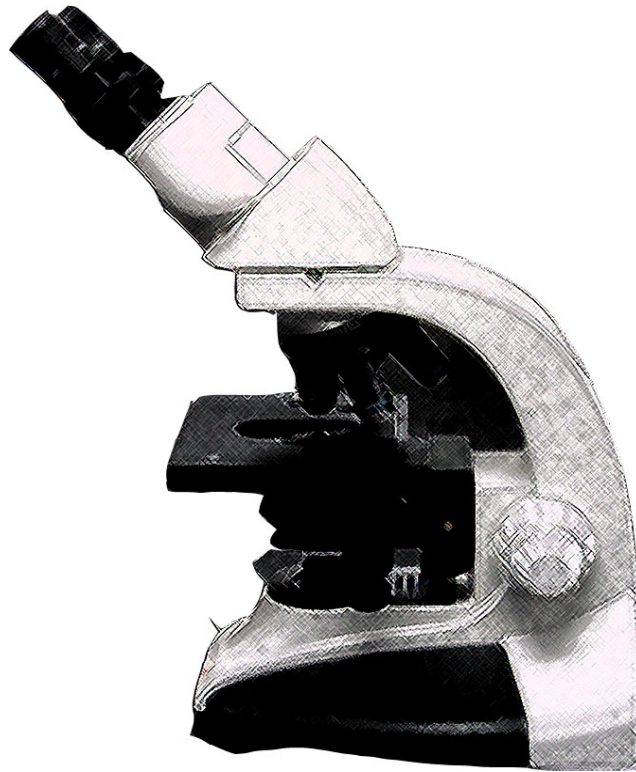


# MICROBIOLOGY LABORATORY MANUAL Biol 2420L



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# CCCCD MICROBIOLOGY



Tutorial Website for  
Microbiology Laboratory

Available at

<http://iws2.collin.edu/dcain/CCCCD%20Micro/index.htm>

## METRIC SYSTEM

This is a scientific laboratory course, and thus a working understanding of the metric system is expected. If you require a review of the metric system (units, conversions, scientific notation etc) please refer yourself to previous textbooks or utilize one of the many online tutorials. We have provided a url below that presents a good review of the metric system. Please talk with your instructor now if you have questions or need additional assistance.

<http://www.swtc.edu:8082/mscenter/mthsci/science/1tools/p02amtrc.pps>

## **BIOSAFETY GUIDELINES**

### **BACKGROUND ON BIOSAFETY**

In addition to following general laboratory safety rules, other protocols must be implemented in the microbiology lab since students are working with living organisms and the risk of student exposure to these organisms must be minimized. The four basic **routes of exposure** to microorganisms are (1) contact with skin and mucous membranes, (2) ingestion, (3) inhalation, and (4) inoculation. Specific lab safety guidelines are designed to address each of these potential routes of exposure. **Contact with skin and mucous membranes** can be minimized by wearing proper personal protective equipment such as lab coats or aprons, gloves, goggles, respirators, and face shields. In addition, students should be prohibited from inserting contact lenses in the lab. **Ingestion** of microorganisms can be minimized by prohibiting eating, drinking, or applying cosmetics in the lab. **Inhalation** of microorganisms can be minimized by adopting measures which decrease the likelihood of generating aerosols. **Inoculation** can be minimized by instituting rigid protocols for the use and disposal of sharps (needles, slides, broken glass, etc.)

Microorganisms are divided into 4 **Biosafety Levels (BSL)** by the Centers for Disease Control and Prevention. The microbes used in our micro lab fall into the BSL-1 and BSL-2 categories.

**Biosafety Level 1** organisms are defined as well-characterized strains of microorganisms not known to cause disease in healthy human adults. Precautions in BSL-1 labs include general lab safety rules such as no eating or drinking, prohibition of mouth pipetting, practicing aseptic technique, and proper disposal of sharps and microbiological waste. Examples of BSL-1 organisms include non-pathogenic laboratory strains of *Escherichia coli*, *Staphylococcus xylosus*, and *Bacillus megaterium*.

**Biosafety Level 2** organisms are defined as moderate-risk microorganisms that are associated with less serious human diseases whose potential for transmission is limited and a proven treatment for the disease exists. Many BSL-2 pathogens are **opportunistic**, meaning they don't ordinarily cause disease in healthy human adults, but may cause disease in children and immunocompromised adults. Additional precautions in BSL-2 labs include using personal protective equipment (PPE) such as disposable gloves and lab coats and limiting lab access to trained individuals. According to guidelines published by the American Society for Microbiology, all environmental samples such as soil or water should be handled using BSL-2 precautions. Examples of BSL-2 organisms include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella*.

**Biosafety Level 3** organisms are defined as high-risk microorganisms with a true potential for infection by aerosols and in which the resulting disease may have serious or lethal consequences. Researchers in BSL-3 labs generally wear double gloves, respirators, and disposable surgical scrubs and gowns, and work in biological safety cabinets in isolated, negative-pressure containment rooms. Examples of BSL-3 organisms include *Mycobacterium tuberculosis* and *Bacillus anthracis*.

**Biosafety Level 4** organisms are defined as easily transmitted, very-high risk microorganisms which cause life-threatening diseases for which there is no vaccine or therapy. Workers in BSL-4 labs work in impermeable positive pressure "space suits" with an external oxygen supply, and precautions such as chemical showers must be taken before exiting the lab. Examples of BSL-4 organisms include Ebola virus, Marburg virus, and Lassa fever virus.



In 2009, the Infectious Diseases Society of America designated six BSL-2 organisms as key hazards in clinical settings because they are responsible for the majority of antibiotic-resistant healthcare-associated infections (HAIs). These have been dubbed the “**ESKAPE**” **pathogens**, and include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and several species of *Enterobacter*. Biosafety experts recommend that ‘safe relatives’ to the ESKAPE pathogens be used whenever feasible, and that proper precautions are exercised whenever any ESKAPE pathogen is used.

### **BIOSAFETY RULES:**

1. Do not eat or drink in the laboratory, chew gum, or place any object on or near your mouth. All books, backpacks, coats, purses, etc. should be cleared from your table before beginning any procedure. **Cell phones** and other electronic devices should also be put away. While actively working with microbial cultures, keep your laboratory manual / instructions at a reasonable distance from your work area. Do not wear loose articles of clothing, such as scarves or bulky jackets.
2. Practice good aseptic technique by performing the following at the **beginning** each class:
  - a) tie back long hair
  - b) wear closed footwear to protect the feet
  - c) wear lab coats to protect clothes
  - d) clean lab table with disinfectant
  - e) wash hands thoroughly before starting any lab exercise
  - f) wear disposable gloves when handling BSL-2 organisms
  - g) wear safety goggles when conducting procedures that pose a splash or aerosol risk, and when dealing with certain chemicals, such as staining reagents.
3. All cultures, whether test tubes or Petri plates, should be labeled with your name or initials, the date, and the name of the organism. Plates can be written on directly; tape labels should be used for test tubes. Permanent markers designated **only** for laboratory use must be used to label cultures.
4. Most microbial cultures will be inoculated using a sterile loop or needle; once the transfer is complete the loop or needle should be sterilized again. Liquid cultures should be transferred using a sterile pipette and mechanical pipetting apparatus. **No mouth pipetting!**
5. All test tubes should be placed in a rack before transporting them from one area of the lab to another. All lids on all test tubes must be secure.
6. All Petri plates should be taped on both sides before carrying to the incubators.
7. All disposable microbiological waste should be discarded in the BIOHAZARD bag. Used test tubes (labels removed) should be placed in the designated pan to be autoclaved and cleaned. Used pipettes should be placed immediately into the provided waste container. **Do not place any hazardous or infectious materials in the regular trash. Do not place any hazardous or infectious materials in the sink.**
8. All used glass slides and coverslips should be placed in SHARPS boxes. **Do not discard slides in the biohazard bags.**

9. Practice good aseptic technique by performing the following at the **end** of each lab exercise:

- a) remove gloves **inside out** and place them in the biohazard bag
- b) remove lab coats, fold them inside out, and place them in the assigned drawer
- c) remove goggles (if used) and clean with alcohol wipes before placing in cabinet for sterilization
- d) decontaminate your work bench by applying an antiseptic wash
- e) wash your hands thoroughly

10. Practice good aseptic technique when making notes or recording results from your experiments in your lab manual -- Do not write in your lab manual or notebook until **AFTER** you have **removed** your gloves and apron and **washed your hands!**

11. If an accident should occur, such as a spill or a broken test tube:

- a) inform your instructor immediately
- b) soak the area with disinfectant and cover with paper towels
- c) dispose of contaminated paper towels in the biohazard bag
- d) dispose of contaminated broken glass in the SHARPS container
- e) wash hands thoroughly

12. If an accident results in bacterial culture coming in contact with eyes, mucous membranes or an open wound:

- a) eyes should be flushed immediately at the eye wash station
- b) affected wound should be washed thoroughly with soap and water
- c) the student should contact their personal physician for advice on further treatment

13. Accident reports must be filed with the lab director no more than 48 hours following the accident.

14. Students who are pregnant or have a medical condition which could compromise their immune system should consult with their attending physician on the advisability of participating in microbiology lab.

## MICROORGANISMS / BIOSAFETY LEVEL:

Below is a list of microorganisms that may be used in Biol2420 lab at Collin College, grouped according to their designated Biosafety Level\*:

### Biosafety Level 1

*Alcaligenes faecalis*  
*Bacillus brevis*  
*Bacillus coagulans*  
*Bacillus licheniformis*  
*Bacillus megaterium*  
*Bacillus polymyxa*  
*Bacillus sphaericus*  
*Bacillus stearothermophilus*  
*Bacillus subtilis*  
*Citrobacter freundii*  
*Clostridium sporogenes*  
*Enterobacter aerogenes*  
*Enterobacter cloacae*  
*Escherichia coli*  
*Flavobacterium capsulatum*  
*Klebsiella oxytoca*  
*Micrococcus luteus*  
*Mycobacterium smegmatis*  
*Providencia alcalifaciens*  
*Pseudomonas fluorescens*  
*Pseudomonas putida*  
*Saccharomyces cerevesiae*  
*Serratia marcescens*  
*Staphylococcus epidermidis*

*Staphylococcus intermedius*  
*Staphylococcus saprophyticus*  
*Staphylococcus xylosus*  
*Streptococcus bovis*  
*Streptococcus mutans*

### Biosafety Level 2

*Acinetobacter lwoffii*  
*Bacillus cereus*  
*Edwardsiella tarda*  
*Enterococcus faecalis*  
*Klebsiella pneumoniae\*\**  
*Morganella morganii*  
*Proteus mirabilis*  
*Proteus vulgaris*  
*Pseudomonas aeruginosa\*\**  
*Salmonella enterica* serotype Choleraesuis  
*Salmonella enterica* serotype Typhimurium  
*Shigella boydii*  
*Staphylococcus aureus\*\**  
*Streptococcus agalactiae*  
*Streptococcus pyogenes*  
*Streptococcus equi*  
*Streptococcus equi* subspecies *zooepidemicus*

\*Note: The Biosafety designation is based on the particular strain of the species that we use in the lab; other strains of a given species may be a different biosafety level.

\*\*ESKAPE pathogen

## ASEPTIC TECHNIQUE

1. Disinfect the tables with antibacterial cleaner and paper towels, which will be disposed of in the trash can.
2. Wear gloves and lab coats. Lab coats should be completely buttoned up and should cover arms.
3. When labeling Petri plates, always write on the bottom of the plate.
4. When using incinerators, give them ample time to warm up before sterilizing loops or needles. **DO NOT** leave loops or needles unattended in the incinerators. They will melt! You will burn your fingers!
5. When inoculating cultures, always sterilize your loop or needle **before** going into a culture and **after** transferring it. Sterilize the loop even if you are going back into the same culture again.
6. Make sure you let your loop cool first – you don't want to kill the bacteria.
7. After removing the lid of a test tube, briefly heat the mouth of the tube before inserting your inoculating loop, and heat again before replacing the cap.
8. Don't put **anything** down on the table – loops, needles, pipettes, test tube lids, etc. Once they touch the table they are **no longer sterile**.
9. Loops/needles can be placed temporarily in slots on the sides of incinerators.
10. Test tube lids can be held with pinky finger.
11. Glass pipettes and plastic pipette tips should remain in canister until just before use.
12. Don't leave media open to the air – bacteria and fungi in the air can contaminate the media.
13. Don't over-inoculate! It doesn't take much inoculum to start a culture. Simply touch the loop or needle to the bacterial growth and obtain a **small amount** on the loop. Don't "scrape" the culture, and don't dig into the agar.
14. When streaking onto an agar plate or slant, make sure the loop doesn't break the surface of the agar. A gentle gliding motion is all that is necessary to distribute the bacteria on the plate.
15. When finished, disinfect the tables again with antibacterial cleaner. Dispose of gloves in the biohazard trash, and place lab coats back in your designated drawers. Wash your hands!

NOTES:

## EXERCISE #0: MICROPIPETTES:

To accurately deliver a precise volume of liquid down to 0.1 ml, scientists have traditionally relied on reusable calibrated glass such as Mohr or serological pipettes. However, to precisely measure and transfer smaller volumes (< 1ml), a micropipette is used. There are several manufacturers and types of micropipettes such as fixed, adjustable, graded, electric, light-guided, multi-channel, and digital.

In this lab we will be using manual adjustable micropipettes to practice measuring and transferring small volumes of colored solution from one microcentrifuge tube to another. These pipettes will be used for several other exercises during the semester, so take time to become familiar with the skill of micropipetting. To understand how to use the micropipette, we will first look at the instrument itself.

### Basic Parts of a Micropipette:

- 1) Plunger
- 2) Tip Eject Button
- 3) Volume Adjustment
- 4) Volume read out
- 5) Tip eject shaft
- 6) Pipette shaft
- 7) Disposable Tip attachment point



### Micropipette Sizes

The maximum number of microliters that a pipette is designed to deliver is found written on the top of the plunger, along with the range of volumes that can be accurately measured by the pipette. Traditional sizes are P20, P200, and P1000. P20 can be used to transfer 0.5 to 20.0  $\mu\text{L}$ ; P200 is used to transfer 20 to 200  $\mu\text{L}$  and P1000 is used to transfer 100 to 1000  $\mu\text{L}$ . (Remember that 1ml = 1000 $\mu\text{L}$ !).

In our lab we will be using P10, P100, and P1000  $\mu\text{L}$  micropipettes.

→ Observe the P10, P100, and P1000 micropipettes on your table. Fill in the chart for the expected ranges of volume that can be administered:

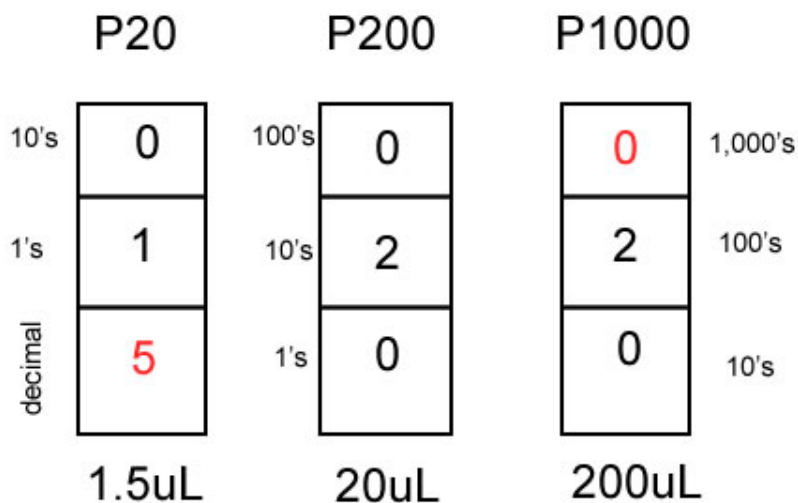
Micropipette	Recommended Range ( $\mu\text{L}$ )	Smallest Increment ( $\mu\text{L}$ )
P10		
P100		
P1000		

There are three numbers on the volume indicator. Set the desired volume by turning the adjustment rings clockwise to increase volume or counterclockwise to decrease volume. The numbers on the volume readout can have both black and red numbers. Depending on the micropipette, the red numbers will indicate a place holder for a decimal if located at the bottom of the indicator or else a larger unit of measurement if located at the top.

**Important: Never turn the indicator dial beyond the upper or lower limits as this will damage the piston!**



Examples below show how to read the micropipette:



→ Write out the volume indicator settings for the following

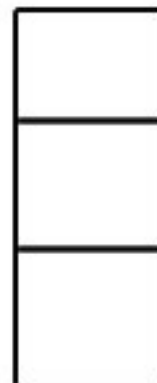
P20 for 17 $\mu$ L



P200 for 163 $\mu$ L



P1000 for 820 $\mu$ L



→ If you wanted to measure 826 $\mu$ L with the p1000, how would you set the dial?

### → PRACTICE:

Using the micropipettes provided in lab, set the indicator to the following volumes and have your instructor check your pipettes. Make sure everyone in your group has a chance to adjust the volume indicator as follows:

P10	to 8.5 $\mu$ L	then reset to	10 $\mu$ L
P100	to 75 $\mu$ L	then reset to	100 $\mu$ L
P1000	to 500 $\mu$ L	then reset to	1000 $\mu$ L

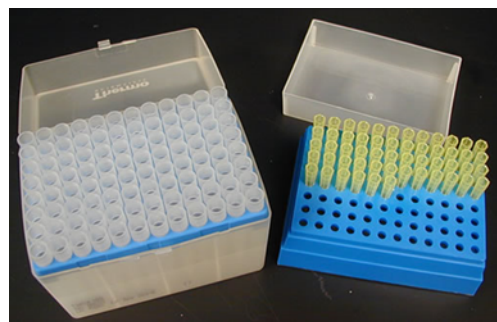
### Disposable tips

Liquids are never drawn into the shaft of the micropipette. Instead, the disposable plastic tips are attached to the shaft. There are three sizes of tips typically used in science labs: Large Blue for 200-1000 $\mu$ L volumes; Smaller Yellow for 2-200 $\mu$ L volumes and Small White tip used for volumes < 2 $\mu$ L.

In our lab, we have available

Small White Tips	for P10
Small Yellow Tips	for P100
Large White Tips	for P1000

The photo shows the large white tips and the small yellow tips in their boxes with the lids removed.



The tips are racked in plastic boxes with covers and are sterilized. Please make sure not to contaminate the tips when touching the box or removing the lid. The box should be closed when not in use in order to prevent air born contamination.

Using the pipette tips:

- 1) Select the correct size tip for the micropipette and open the box without touching the tips.
- 2) Insert the micropipette shaft into the tip and press down firmly to attach the tip to the shaft.
- 3) Remove the pipette with the tip attached and carefully close the box with the lid.
- 4) To remove the tip in the proper biohazard container, the tip ejector button is used.

### →PRACTICE:

Each student should attach the correct tip on the correct micropipette for all three micropipettes, following the steps outlined above.

- Do not lay the micropipette on the table, as this could contaminate the tip.
- Do not hold the pipette upright as this may allow liquid to run down the pipette causing damage.

### Plunger Settings

Micropipettes work on a principle of the movement of an internal piston to create of a partial vacuum by displacing air when the plunger is depressed. The plunger will stop at two different positions when depressed. The first stopping point is used to fill and collect the desired volume to be transferred into the tip. The tip is placed in the solution barely below the surface and the plunger is slowly released allowing the volume to be collected.

When ready to transfer the volume, the plunger is then depressed to the point of initial resistance. After waiting one second, the second stopping point (plunger will be in contact with the body of the instrument) is used to completely discharge and dispense the volume of solution in the tip.



Plunger at rest



Plunger at first stop



Plunger fully depressed



→ **PRACTICE** transferring volume from one microcentrifuge tube to another and proper disposal of tips into the sharps container using the tip eject button following the steps outlined below.

**To Draw up Liquid:**

- 1) Hold the micropipette with the thumb resting on the plunger and the fingers around the upper body
- 2) Push the plunger down to the first stop.
- 3) Place the tip attached to the end of the micropipette **BENEATH** the surface of the liquid (approximately 3mm) but **NOT** at the bottom of the sample tube. The tip should be far enough below the surface as to not draw up any air. If bubbles appear in the tip, return the liquid to the tube and start over with a new tip.
- 4) Release the pressure on the plunger slowly back to its initial resting position.

**To Dispense Liquid:**

- 1) Hold the micropipette so that the end of the tip is inside the new collection tube along the edge at an angle.
- 2) Gently push the plunger to the first stop, waiting 1 second and then depressing all the way to the body of the micropipette to remove any traces of the liquid collected.
- 3) If you were mixing liquids, the plunger would be pushed down to the first stop, repeating several times before final collection.
- 4) Once the liquid is transferred, remove the tip from the liquid before releasing the plunger.
- 5) Release the plunger and allow it to return to its resting position.
- 6) Remove the tip using the tip eject button as explained earlier into the sharps container.

## EXERCISE #1: MICROSCOPY AND MORPHOLOGY/ARRANGEMENT OF BACTERIAL CELLS

### Brightfield Compound Light Microscope

The light microscope is an important tool in the study of microorganisms. The compound light microscope uses visible light to directly illuminate specimens in a two lens system, resulting in the illuminated specimen appearing dark against a bright background. The two lenses present in a compound microscope are the **ocular lens** in the eyepiece and the **objective lens** located in the revolving nosepiece. Compound light microscopes typically have the following components:

**Illuminator:** the light source in the base of the microscope.

**Abbe Condenser:** a two lens system that collects and concentrates light from the illuminator and directs it to the iris diaphragm.

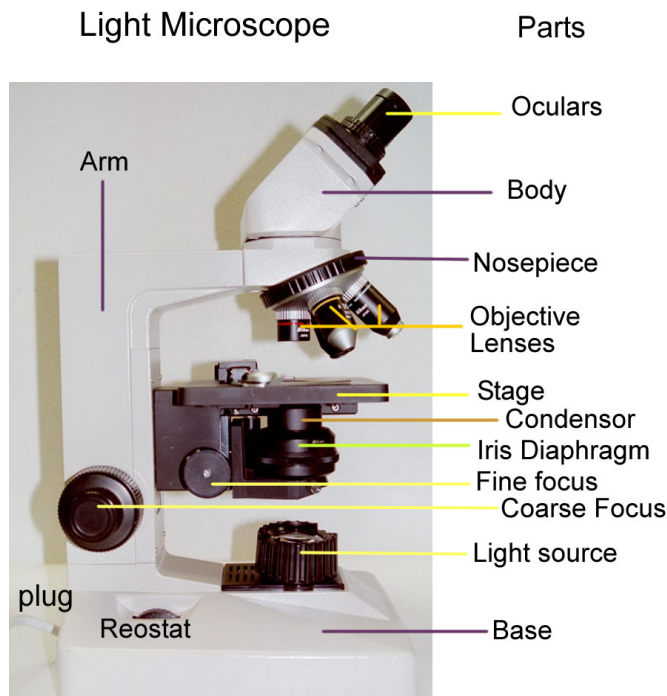
**Iris Diaphragm:** regulates the amount of light entering the lens system.

**Mechanical Stage:** a platform used to place the slide on which has a hole in the center to let light from the illuminator pass through. Often contains **stage clips** to hold the slide in place.

**Body tube:** Houses the lens system that magnifies the specimens

Upper end of body tube -- **Oculars/Eye pieces:** what you view through

Lower end of body tube -- **Nose-piece:** revolves and contains the objectives



### Principles of Microscopy

Basically, a light microscope magnifies small objects and makes them visible. The science of microscopy is based on the following concepts and principles:

**Magnification** is simply the enlargement of the specimen. In a compound lens system, each lens sequentially enlarges or magnifies the specimen. The **objective lens** magnifies the specimen, producing a **real image** that is then magnified by the **ocular lens** resulting in the final image. The **total magnification** can be calculated by multiplying the objective lens value by the ocular lens value.

**Resolving power** is the ability of a lens to show two adjacent objects as discrete entities. In general, the shorter the wavelength of light, the better the resolution, which is why a **blue filter**

is usually connected to the condenser to produce short light waves for optimum resolution. Resolving power is also dependent on the **refractive index** or the bending power of light. Because air has a lower refractive index than glass, light waves have a tendency to bend and scatter as they pass through the air from the glass slide to the objective lens. Addition of **immersion oil**, which has the same refractive index as glass, diminishes the loss of refracted light and improves resolution.

**Contrast** is the ability to distinguish an object from its background. Since most microbes are relatively transparent when viewed under a standard light microscope they are difficult to identify. Using a stain (labs 2-5) that will bind to the microorganism and not the glass slide, dramatically enhances their contrast enabling them to be observed more clearly.

**Depth-of-focus** is the “thickness” of the sample that appears in focus at a particular magnification. As the magnification increases the depth-of-focus decreases, or the “slice” of the sample that appears in focus gets thinner. Many of the newer compound microscopes are **par focal**, which means that if one objective lens has the object in focus, and you go to the next objective lens, only minor adjustment (fine focus) is needed to bring the image back into focus. This is due to the fact that as you increase the magnification, and thus the slice of the sample that appears in focus becomes “thinner”, the correct plane-of-focus will always be within the depth of focus of the previous objective. After you get the sample into focus at scanning or low power using the course adjustment knob, you should only have to use the fine focus knob at the higher magnifications.

**Field-of-View** is the area of the slide that you are observing through the microscope. As you increase the magnification the actual area of the slide that you are looking at is getting smaller. You can think of the field-of-view as a dartboard. At low magnification you are able to see the entire dartboard, but as you increase the magnification you are only observing the bulls-eye, a much smaller portion of the dartboard.

These microscopes are also **par central**, which refers to the ability to keep an object in the middle of your field-of-view when changing from one objective to another. It is useful to remember this as you are increasing magnification. Always keeping your sample in the center of your field-of-view will avoid unnecessary “searching” of the slide for your sample.

**Working distance** is the distance between the objective and the slide. As you increase magnification (by using more powerful objective lenses) the working distance decreases. So much so that by the time you are using the oil-immersion objective (100X) the objective is almost touching the slide, allowing the immersion oil to “connect” the slide and objective. It is important to consider working distance in a number of applications, but practically there are two reasons you should be aware of your working distance. The first is so that you do not inadvertently push the objective through the slide, causing damage to the objective and your sample slide. The second is to estimate whether you are in the correct plane-of-focus.

### Care of Microscopes

Microscopes are very expensive pieces of scientific equipment and must be treated with care. Each pair of students will be assigned a microscope to use throughout the semester, and will be required to sign a microscope agreement form acknowledging responsibility for that microscope. Some basic rules of microscope care include the following:

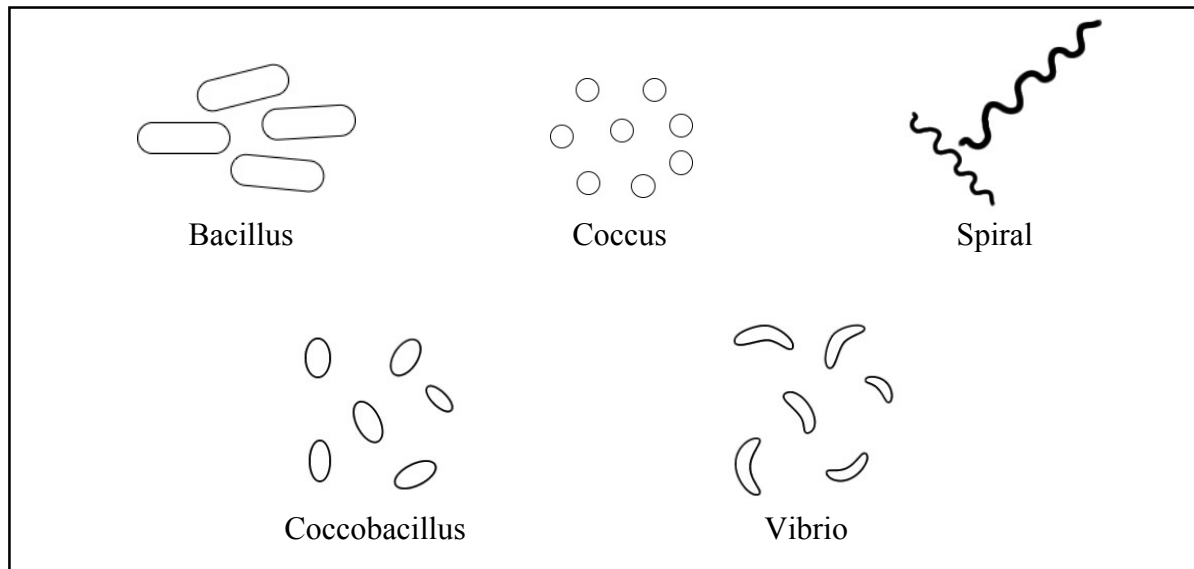
- (1) Always carry a microscope with two hands, one on the base and one on the arm.
- (2) Use the coarse focus knob on the lowest objective only. NEVER use coarse focus on high power or oil immersion, or you may damage the objective lenses.
- (3) Always clean all lenses thoroughly with lens paper and lens cleaner before putting away. Immersion oil which is not removed immediately can dry on the lenses, making it

difficult to view any specimen. Dried immersion oil is also quite difficult to remove from the lenses.

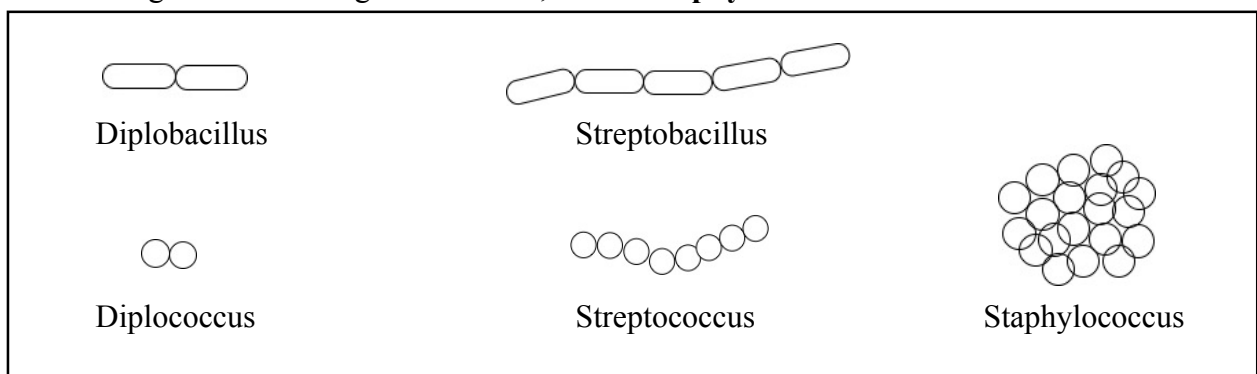
- (4) Always store the microscope with the lowest objective (4X) in place. NEVER store the microscope with the oil immersion objective in place, as it can damage the lens.

### Morphology and Arrangement of Bacterial Cells:

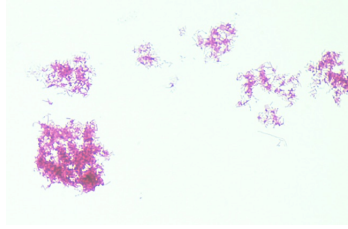
One of the primary ways that bacteria are identified is by their morphology and arrangement. The term **morphology** simply means shape. The three primary shapes of bacterial cells are bacillus, coccus, and spiral. A bacterial cell with a **bacillus** (plural: bacilli) morphology is rod-shaped, while a **coccus** (plural: cocci) has a spherical shape. Spiral-shaped bacteria are divided into two categories depending on whether the spirals are stiff or flexible. A stiff spiral shape is called a **spirillum**, whereas a flexible spiral shape is known as a **spirochete**. Other common shapes you may encounter include coccobacillus (short rods) and vibrio (curved rods).



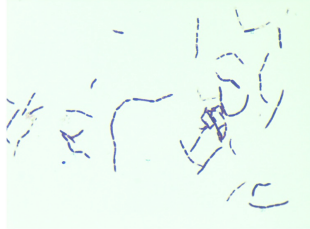
The term **arrangement** refers to the way that multiple bacterial cells associate with one another, and is dependent on (1) the number of planes of cell division and (2) the extent to which the daughter cells separate. With a bacillus, there is a single plane of division, resulting in daughter cells arranged singly, in pairs (**diplobacillus**) or in chains (**streptobacillus**). With a coccus there are infinite potential planes of division. A coccus with a single plane of cell division will reproduce to form daughter cells arranged singly, in pairs (**diplococcus**) or in chains (**streptococcus**). A coccus with two planes of cell division will reproduce to form daughter cells arranged in cubes called **sarcinae**. A coccus with multiple planes of cell division will reproduce to form daughter cells arranged in clusters, termed **staphylococcus**.



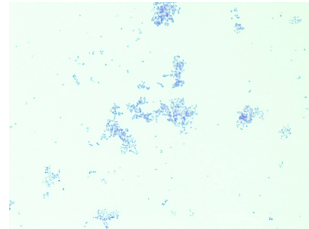
Pictured below are actual microscopic images of several different bacterial morphologies:



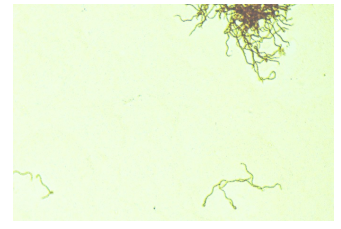
Bacillus  
(single)



Bacillus  
(diplo- and strepto-)



Coccus  
(staphylo-)



Spiral  
(spirillum)

**MATERIALS NEEDED:**

Commercially prepared slides of:

*Staphylococcus*

*Streptococcus*

*Bacillus*

*Escherichia coli*

*Borrelia burgdorferi*

Microscope

Immersion Oil

Lens Paper

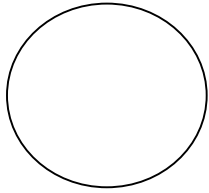
Lens Cleaner

**PROCEDURE:**

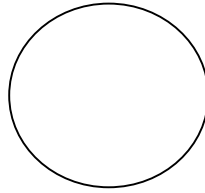
1. Place the slide on the stage and use the coarse and fine focus knobs to bring the specimen into focus under 4X magnification. **Tip:** Bring the stage all the way to the highest position with the coarse focus knob. While looking through the ocular, gradually turn the knob to lower the stage until the specimen comes into focus. Once you have it in focus with the coarse focus knob, fine-tune with the fine focus.
2. Turn the revolving nose piece to bring the 10X objective into place and observe the specimen. Repeat with the 40X objective.
3. Turn the revolving nose piece to bring the 100X objective into place and observe the specimen. Move the nosepiece slightly so you can add a drop of immersion oil to the slide, then bring the oil immersion objective back into place and fine-tune the focus. Observe the specimen. You may need to turn down the amount of light in order to observe the specimen under oil immersion.
4. Draw several cells observed under 100X in the spaces provided below.
5. Repeat steps 1-4 for the remaining prepared slides.
6. When finished, clean all lenses thoroughly with lens paper and lens cleaner.
7. Store the microscope according to the instructions written on the inside of your microscope cabinet.

**RESULTS:**

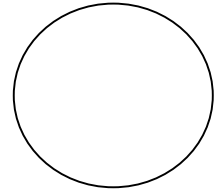
**A. Drawings:**



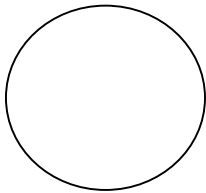
*Staphylococcus*



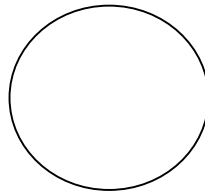
*Streptococcus*



*Bacillus*



*Escherichia coli*



*Borrelia burgdorferi*

**B. Observations:**

For each of the specimens you viewed with the microscope, indicate the cell morphology and arrangement that you observed.

Specimen	Morphology	Arrangement
<i>Staphylococcus</i>		
<i>Streptococcus</i>		
<i>Bacillus</i>		
<i>Escherichia coli</i>		
<i>Borrelia burgdorferi</i>		

**STUDY QUESTIONS:**

1. State the purpose of each of the following microscope components:
  - a. Condenser
  
  - b. Fine-adjustment knob
  
  - c. Coarse-adjustment knob
  
  - d. Iris Diaphragm
  
  - e. Mechanical stage control
  
2. What is the purpose of adding immersion oil when using the 100X objective?
  
  
  
  
  
  
  
  
  
  
3. If the ocular lens has a magnification of 10X and the objective lens has a magnification of 40X, what is the total magnification?
  
  
  
  
  
  
  
  
  
  
4. Are all of the different cell arrangements possible with both cocci and bacillus shaped bacteria? Are there any cell arrangements that could NOT happen with cocci? Are there any cell arrangements that could NOT happen with bacilli? Explain.

## STAINING OF BACTERIAL SPECIMENS

Bacterial specimens are often stained prior to microscopy studies, which allows better visualization of specimens using microscopy. Simply speaking, a stain is a substance that adheres to a cell, giving the cell color. Different stains have different affinities for different organisms, or different parts of organisms. They may be used to differentiate different types of organisms or to view specific parts of organisms.

There are several types of stains which are commonly used in microbiology. The first is a **simple stain**, which uses only one reagent which provides contrast between the background and the heat-fixed bacterium itself. The bacterium takes up stain and becomes colored, while the background remains unstained. Simple stains are typically used on bacterial smears which have been heat-fixed and thus contain non-living microbes.

A second type of stain is a **negative stain**, which uses a single reagent to provide contrast between the background and the living bacterium. Thus, the background is “stained”, while bacterium does not take up any stain. Negative stains are typically used when observing live bacteria is desired.

A **differential stain** is a type of staining that allows you to distinguish between types of bacteria or between specific structures in a bacterium. A differential stain typically uses two or more reagents – a primary stain and a counter stain.

Chemically, there are two main types of stains: **basic stains**, which have a positive charge (cationic) and **acidic stains**, which have a negative charge (anionic). Basic stains have an affinity for negative components of cells, and include dyes such as methylene blue, crystal violet, and carbol fuchsin. Acidic stains have an affinity for positive components of cells, and include dyes such as nigrosin, India ink, and picric acid. Since cell walls are negatively charged, a positive dye will be attracted to and stain the cell wall, whereas a negative dye will be repulsed by the cell wall and not directly stain the cell.

## PREPARATION OF BACTERIAL SPECIMENS

In order to stain bacterial specimens, the bacteria must be placed on a glass slide and prepared for the specific staining process. This is typically done by preparing a **bacterial smear**. A smear is a small volume (a loopful) of specimen-containing medium that is spread (smeared) onto a microscope slide. In preparing smears, the “Goldilocks principle” applies – everything has to be done just right! If smears are too thick, you will have trouble seeing individual cells. If smears are too thin, you may not find the organism. If you stir the drop of medium too much as you spread it on the slide, you will disrupt cell arrangements such as chains or clusters.

Following preparation of a bacterial smear, most smears need to be heat fixed before they are stained. Heat fixation accomplishes three things: (1) it kills the organisms; (2) it causes the organisms to adhere to the slide; and (3) it alters the organisms so that they more readily accept stains (dyes). Again, the Goldilocks principle applies. Slides must be completely dry before heat-fixing, or the organism will be boiled and destroyed. If you heat-fix too little, the organism may wash off the slide. If you heat-fix too much, the organisms may be damaged and the cells won't stain correctly. Specific instructions for preparing a bacterial smear and heat fixing the smear are outlined on the following page.



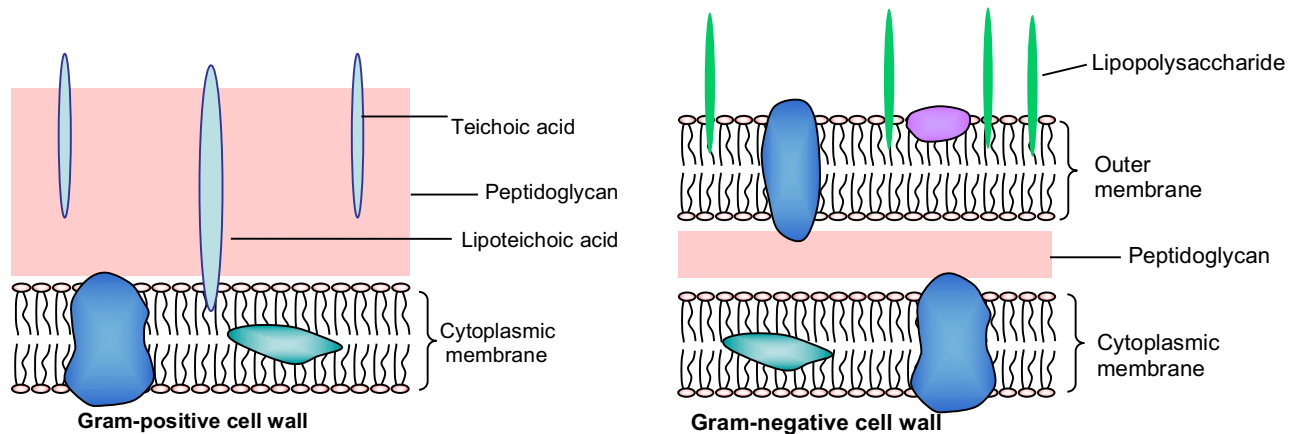
## **PROCEDURE FOR PREPARING A BACTERIAL SMEAR**

1. Obtain a glass slide and clean if necessary.
2. Using a **broth culture**:
  - a. Gently agitate your culture broth tube to disperse the bacteria.
  - b. Sterilize your inoculating loop using an incinerator or a Bunsen burner, and let cool for 20-30 seconds.
  - c. Place loop in the bacterial broth and put the loopful of the broth onto the glass slide. Rub the drop into a nickel-sized smear. Sterilize the loop again to kill any remaining bacteria. Let the smear **air dry completely**. Alternatively, you may use a **slide warmer** (low heat) to dry your smear if one is available. Do **not** use high heat to dry your smear!
3. Using an **agar plate**:
  - a. Using a plastic dropper, place a **small** drop of water in the center of the slide.
  - b. Sterilize your inoculating needle using an incinerator or a Bunsen burner, and let it cool for 20-30 seconds.
  - c. Use the sterile needle to pick up a *small* amount of bacterial growth from the surface of the plate. Do not dig into the agar. Put the bacteria into the drop of water on the glass slide, and rub the drop into a nickel-sized smear. Sterilize the needle again to kill any remaining bacteria. Let the smear **air dry completely**. Alternatively, you may use a **slide warmer** (low heat) to dry your smear if one is available. Do **not** use high heat to dry your smear!
4. **Heat fix** the slide.
  - a. **Incinerator method:** Hold the slide with a wooden clothes pin approximately 1 cm over the barrel of a hot incinerator for 20–30 seconds. Let the slide cool.
  - b. **Bunsen burner method:** Hold the slide with a wooden clothes pin, and pass 10-12 times through the flame. Let the slide cool.

**Hint:** Before you begin the staining process, mark the corner of the slide with a Sharpie so you can distinguish which side of the slide contains the specimen. (Many times when students cannot locate or focus on their specimen under the microscope, it's because their slide is upside down!)

## EXERCISE #2: GRAM STAIN

The **Gram stain** is a differential stain which distinguishes bacteria based on cell wall properties. Bacterial cell walls are composed primarily of **peptidoglycan** and bacteria can be classified into two main groups dependent on the amount of peptidoglycan present in their cell wall. **Gram-positive** organisms have a thick layer of peptidoglycan, whereas **Gram-negative** organisms have a thin layer of peptidoglycan, plus an additional **outer membrane** that is absent in Gram-positive organisms.



In the Gram staining procedure, the **primary stain** is crystal violet, and all cells take up the purple crystal violet stain. Following the primary stain, Gram's Iodine is applied to the bacterial smears. The iodine acts as a **mordant**, enhancing the ability of the stain to enter and bind to the bacteria. Specifically, the iodine binds with crystal violet and locks it into peptidoglycan of bacteria. It also intensifies the purple color. The **decolorizing agent** used in the Gram staining procedure is 95% ethanol, which is a lipid solvent that melts the Gram-negative outer membrane and leads to decolorization of Gram-negative cells. It also dehydrates proteins, helping the primary stain to remain in Gram-positive cell walls. The **counter stain** then used is Safranin, which stains the decolorized Gram-negative cells pink. Thus, at the end of the staining procedure, Gram-positive cells are purple and Gram-negative cells are pink. Note: It is preferable to use fresh cultures for the Gram stain. Old cultures may stain "Gram-variable" (a mix of purple and pink) because they decolorize easily.

### Overview of Gram Stain:

Primary Stain	Crystal Violet
Mordant	Gram's Iodine
Decolorizer	95% Ethanol
Counter Stain	Safranin

### CULTURES NEEDED:

Nutrient broth tubes and/or plates of the following:

*Escherichia coli*

*Staphylococcus xylosum*

*Bacillus megaterium* or *Bacillus subtilis*

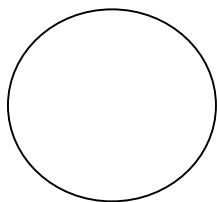
**PROCEDURE:**

Note: This is an individual lab exercise – each student will perform their own Gram stain(s). Your instructor may have you prepare your smears from agar plates, broth tubes, or both.

1. Prepare a bacterial smear with a mixture of all 3 organisms listed above and **heat fix** as described on page 21.
2. Place the slide on a staining tray, and cover the smear with crystal violet. Allow to stain for 60 seconds. (Practice good aseptic technique -- **Do not use your smart phone as a timer!**)
3. Tilt the slide and gently rinse with distilled water for approximately 5 seconds to remove excess stain.
4. Cover the smear with Gram’s Iodine, and allow to sit for 60 seconds.
5. Tilt the slide and gently rinse with distilled water.
6. **IMPORTANT STEP:** Tilt the slide and let 2-3 drops of Decolorizer run over the slide. If the last drop is still purple, continue decolorizing, 2-3 drops at a time, until the decolorizer runs clear. Rinse with distilled water. Tip: thicker smears will require more decolorizer, thinner smears will require less.
7. Cover the smear with Safranin, and stain for 45 seconds.
8. Tilt the slide and rinse with distilled water.
9. Place the slide in a book of Bibulous paper and blot to dry. You do not need a cover slip! Observe the slide under oil immersion, and draw what you see in the results section below. You should see: Small purple cocci (spheres) which are the Gram-positive *S. xylosum*, large purple rods, which are the Gram-positive *B. megaterium* or *B. subtilis*, and small pink rods, which are the Gram-negative *E. coli*. **Label** these in your drawing.
10. Clean your microscope with lens cleaner, paying extra attention to the 40X and 100X objectives
11. Dispose of staining waste in designated waste receptacle. Dispose of slides in a SHARPS container.

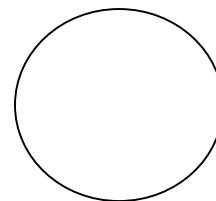
**RESULTS:**

Using colored pencils, draw and label *S. xylosum*, *B. megaterium* (or *B. subtilis*), and *E. coli* in the circle below.



Smear from agar plates

(and/or)



Smear from broth tubes



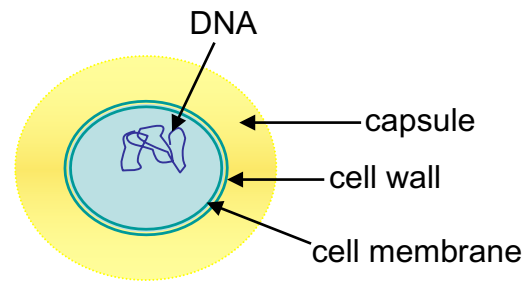
## TROUBLESHOOTING THE GRAM STAIN:

Like many scientific protocols, performing a **Gram stain** is as much art as it is science, and it often takes practice to achieve consistent results. When results do not come out as expected, it is helpful to troubleshoot potential problems so that future issues can be avoided. The table below lists some common Gram stain problems and potential explanations.

<b>Observation</b>	<b>Potential Explanations</b>
Gram-positive cells stain light purple or pink	<ul style="list-style-type: none"><li>• Smears were heat-fixed before drying completely</li><li>• Smears were heat-fixed too long</li><li>• Insufficient iodine used</li><li>• Smears were washed excessively with water</li><li>• Old bacterial cultures were used</li></ul>
Gram-negative cells stain purple or a mix of purple and pink	<ul style="list-style-type: none"><li>• Smears are too thick, and the decolorizer was unable to penetrate well</li><li>• Smears were not decolorized enough to remove crystal violet from Gram-negative cells</li></ul>
Large purple crystals visible on slide	<ul style="list-style-type: none"><li>• Slide was still hot when primary stain was applied, causing crystal violet dye to precipitate onto slide</li></ul>
Nothing visible on slide	<ul style="list-style-type: none"><li>• Slide wasn't heat fixed sufficiently, and specimen was washed off during procedure.</li><li>• Smear wasn't dense enough</li></ul>

### EXERCISE #3: CAPSULE STAIN

The **capsule stain** is a type of **differential stain** which selectively stains bacterial capsules. A capsule is a substance that is synthesized in the cytoplasm and secreted to the outside of the cell where it surrounds the bacterium. Capsules can be polysaccharide, polypeptide, or glycoprotein. Capsules are associated with virulence in several microorganisms, including *Streptococcus pneumoniae* and *Neisseria meningitidis*, because capsules provide a mechanism for these pathogens to evade the host immune system. Because of their structure and composition, heat and water will dislodge capsules from bacteria during laboratory procedures.



**Bacterial capsule**

In the capsule staining procedure, the **primary stain** is crystal violet, and all parts of the cell take up the purple crystal violet stain. There is no mordant in the capsule staining procedure. A 20% copper sulfate solution serves a dual role as both the **decolorizing agent** and **counter stain**. It decolorizes the capsule by washing out the crystal violet, but will not decolorize the cell. As the copper sulfate decolorizes the capsule, it also counter stains the capsule. Thus, the capsule appears as a faint blue halo around a purple cell.

#### **CULTURES NEEDED:**

Milk broth tubes of *Flavobacterium capsulatum*

(Note: your instructor may provide additional organisms for staining)

#### **PROCEDURE:**

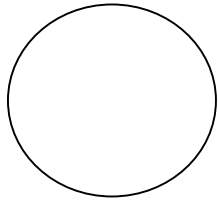
Note: Students will work in pairs to perform the capsule stain.

1. Obtain a clean glass slide. Choose one of the above broth cultures, and agitate your broth culture to disperse the bacteria.
2. Prepare a smear using 2-3 loopfuls of the broth culture. Allow the smear to air-dry at room temperature, but **DO NOT HEAT FIX THIS SLIDE!** Heat will cause the capsule to dislodge.
3. Cover the smear with crystal violet, and stain the smear for 2 minutes.
4. Tilt the slide and rinse with 20% copper sulfate solution. **DO NOT RINSE WITH WATER!** Water will remove the capsule from the cell.
5. Let the slide air dry for a few minutes. **DO NOT blot the slide!** Blotting will remove the bacteria from the slide and/or distort the capsule.
6. Observe the slide under oil immersion, and draw what you see in the results section below. Look for purple cells surrounded by a clear or faint blue halo on a purple background. (The halo is the capsule.) You may need to decrease the amount of light in order to make the capsule easier to see.
7. Clean your microscope with lens cleaner, removing all oil from lenses.

8. Dispose of staining waste in designated waste receptacle. Dispose of slides in SHARPS container.

**RESULTS:**

Using colored pencils, draw the results from the capsule stain:



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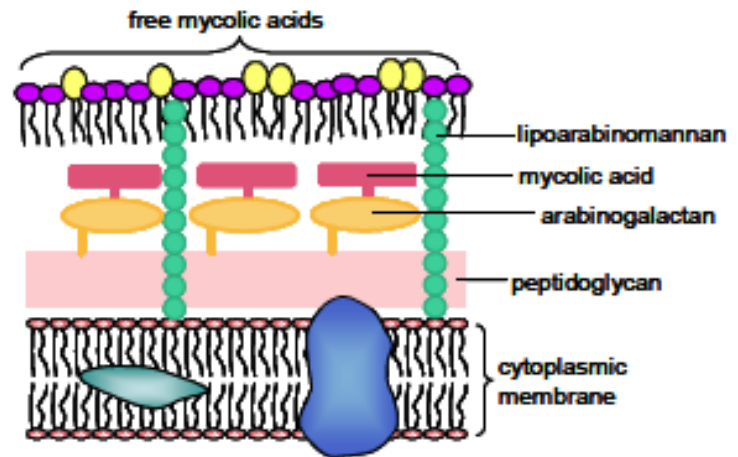
*(name of organism)*

**STUDY QUESTIONS:**

1. Why are capsule stains NOT rinsed with water? (Hint: think about what capsules are made of.)
2. Explain the clinical significance of a bacterial capsule.

## EXERCISE #4: ACID-FAST STAIN

The **acid-fast stain** is a differential stain which distinguishes bacteria based on cell wall properties. Bacteria which are acid-fast are Gram-positive microorganisms that are resistant to the Gram stain, so implementing the acid-fast stain allows us to visualize these bacteria. Acid-fast bacteria have a waxy substance called **mycolic acid** in their cell walls, which comprises up to 60% of the cell wall components. They also contain polymers called arabinogalactan and lipoarabinomannan. Genera which are acid-fast include *Mycobacterium* and *Nocardia*, which include pathogenic organisms such as *M. leprae* and *M. tuberculosis*.



**Acid-Fast Cell Wall**

The **primary stain** in the acid-fast staining procedure is **carbol fuchsin**, which contains **phenol** to solubilize the cell wall and allow the stain to enter the cell. The procedure requires the application of **heat** in order for the stain to penetrate the waxy cell wall. All cells will take up the reddish-pink primary stain. Once the cells have been allowed to cool after the primary stain, the cells are decolorized. The **decolorizer** used in the acid-fast staining procedure is **acid alcohol**, which decolorizes all cells except acid-fast cells. The acid alcohol is unable to penetrate the waxy cell wall of acid-fast microorganisms. The **counterstain** then used is **methylene blue**, which stains the decolorized non acid-fast cells blue. Thus, at the end of the staining procedure, acid-fast cells are reddish-pink and all other cells are blue.

### **CULTURES NEEDED:**

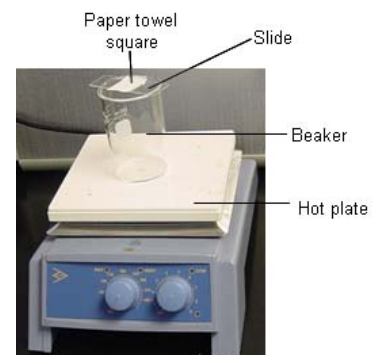
Nutrient broth tubes or plates of *Mycobacterium smegmatis*

Nutrient broth tubes or plates of *Staphylococcus xylosum*

### **PROCEDURE:**

Note: Students will work in pairs to perform the acid-fast stain.

1. Fill a beaker about 1/3 to 1/2 full of **tap water** and place on a hot plate. Turn hot plate on “high” and allow water to boil. Once the water comes to a boil, reduce the heat to low.
2. Prepare a bacterial smear with a mixture of *M. smegmatis* and *S. xylosum* (i.e. 2-3 loopfuls of *M. smegmatis* + 1 loopful *S. xylosum*) and **heat fix** as described on page 21.
3. Place the slide on top of the beaker of water and let steam for 5 minutes.
4. Place a paper towel square on top of the smear area. This will hold the stain in place and keep it from running off the slide.

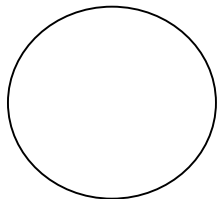




5. Apply enough Carbol Fuchsin to soak the paper towel square and allow to sit for 5 minutes, keeping the paper towel moist with stain. Do not let it dry!
6. Carefully remove the slide with a clothes pin (slide will be hot!) and place on the staining tray to cool. Remove paper towel square.
7. Tilt the slide and rinse with distilled water.
8. Tilt the slide and rinse with Acid Alcohol, drop by drop, until the acid alcohol runs clear.
9. Tilt the slide and rinse with distilled water.
10. Cover the slide with Methylene Blue and allow it to sit for 2 minutes.
11. Tilt the slide and rinse with distilled water.
12. Blot your slide dry with Bibulous paper and observe your slide under oil immersion using proper microscope techniques. Record your results below. You should observe acid fast (reddish-pink) rods, as well as non-acid fast (blue) cocci.
13. Clean your microscope with lens paper and lens cleaner, paying extra attention to the 40X and 100X objectives.
14. Dispose of staining waste in designated waste receptacle. Dispose of slides in SHARPS container.

**RESULTS:**

1. Using colored pencils, draw the results of the acid-fast stain, labeling the *M. smegmatis* and *S. xylosum*:

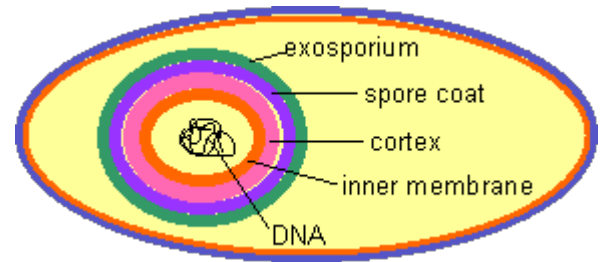


**STUDY QUESTIONS:**

1. Why must heat be used with the application of the primary stain during acid-fast staining?
2. Critical Thinking Question: When and why would the acid-fast stain be used in a hospital or clinical lab?

## EXERCISE #5: ENDOSPORE STAIN

The **endospore stain** is a differential stain which stains bacterial endospores or spores. Some bacteria, including those belonging to the genera *Clostridium* and *Bacillus*, have the capacity to produce metabolically inactive cells called spores. Spores are highly resistant to hostile chemical and physical conditions and are produced by a process called **sporulation** if environmental conditions become unfavorable for normal vegetative growth. The spore structures have a tough outer covering composed primarily of keratin which makes them resistant to heat, radiation, disinfectants, and desiccation. The bacteria will remain in this suspended state until conditions become favorable again and they can germinate and return to their **vegetative** state. The term **endospore** refers to the spore structure contained within a mother cell. The term **spore** refers to the spore structure that exists free of the mother cell. Endospores may be located in the middle of the cells (central), at the end (terminal), or between the end and the middle of the cells (subterminal). The endospores themselves may be round or oval.



The **primary stain** in the endospore staining procedure is **malachite green**, which stains both vegetative cells and endospores. As with the acid-fast stain, **heat** is required to penetrate the endospore coat. Once the cells have cooled, the cells are **decolorized** with **water**, which selectively removes the malachite green from all vegetative cells but not from endospores. The **counterstain** then applied is **safranin**, which stains the decolorized vegetative cells pink. Thus, at the end of the staining procedure, the endospores are dark green, and vegetative cells are pink. Note: Sometimes the endospores don't take up the malachite green very well. In those cases, the endospores will appear as clear ovals or circles within a pink vegetative cell.

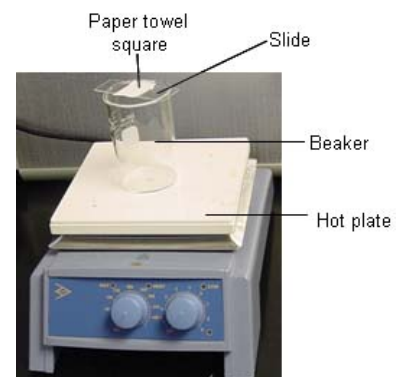
### **CULTURES NEEDED:**

*Bacillus megaterium* or *Bacillus subtilis*

### **PROCEDURE:**

Note: Students will work in pairs to perform the endospore stain.

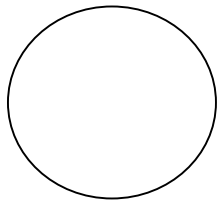
1. Fill a beaker about 1/4 to 1/3 full of **tap water** and place on a hot plate. Turn hot plate on "high" and allow water to boil. Once the water comes to a boil, reduce the heat.
2. Obtain a clean glass slide and prepare a bacterial smear as described on page 13. Air dry and heat fix the slide. Be sure to label your slide!
3. Place the slide on top of the beaker of water and let steam for 5 minutes.
4. Place a paper towel square on top of the smear area. This will hold the stain in place and keep it from running off the slide.
5. Apply enough Malachite Green to soak the paper towel square and allow to sit for 2-3 minutes, keeping the paper towel moist with stain. Do not let it dry!



- Carefully remove the slide with a clothes pin (slide will be hot!) and place on the staining tray to cool. Remove paper towel square.
- Tilt the slide and rinse with distilled water.
- Cover the slide with Safranin and allow to sit for 30 seconds.
- Tilt the slide and rinse with distilled water.
- Blot your slide dry with Bibulous paper and observe your slide under oil immersion using proper microscope techniques. Record your results below. Endospores will appear as dark bluish-green or clear circles or ovals, whereas vegetative cells will be pink rods.
- Clean your microscope with lens paper and lens cleaner, paying extra attention to the 40X and 100X objectives.
- Dispose of staining waste in designated waste receptacle. Dispose of slides in SHARPS container.

### RESULTS:

- Using colored pencils, draw the results of the endospore stain. Label an endospore and a free spore in the drawing. Are the endospores central, terminal, or subterminal? (*circle one*)



*B. megaterium* (or *B. subtilis*)

### STUDY QUESTIONS:

- Why must heat be used with the application of the primary stain during endospore staining?
- Critical Thinking Question: What advantage does the ability to sporulate give to pathogenic bacteria such as *Clostridium tetani*, *Clostridium botulinum* and *Bacillus anthracis*?

## EXERCISE #6– CULTURE TRANSFER TECHNIQUES

The purpose of this lab exercise is to learn how to subculture microorganisms in various types of microbiological media. Simply stated, **subculturing** is transferring microorganisms from one media type to another. Various media types used in microbiology labs include agar slants, agar deeps, agar plates, and broths. An **agar slant** is a solid media in a test tube with a slanted surface on which to culture the microorganism. These are typically inoculated by streaking the surface of the slant with a sterile loop. An **agar deep** is a solid media in a test tube which does not have a slanted surface. These are typically inoculated by stabbing the media with a sterile needle. An **agar plate** is a solid media which is contained in a Petri plate, providing an optimal surface on which to culture microorganisms. Like the agar slants, these are inoculated by streaking the surface with a sterile loop. **Broth tubes** are a liquid medium which can be inoculated by a sterile loop, needle, or pipette. Remember that when inoculating any cultures, it is essential to practice sterile technique at all times!

It is important to know what **sterile media** looks like. Take a minute to observe your **uninoculated** cultures. You will be responsible for making sure there are no contaminants present in the media we provide for you.

### **MEDIA NEEDED:**

- 1 Nutrient agar slant
- 1 Nutrient agar deep
- 1 Nutrient broth tube

### **CULTURES NEEDED:**

*Serratia marcescens* broth cultures and slants

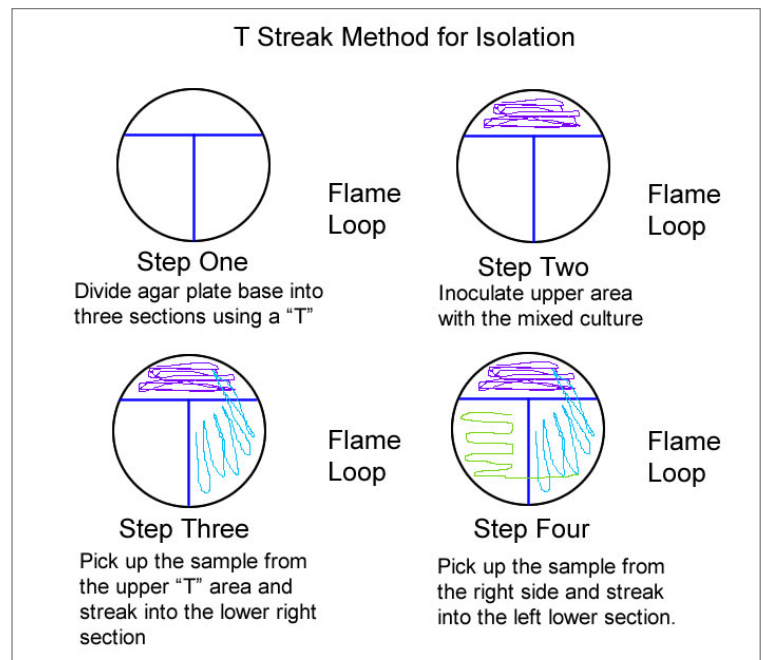
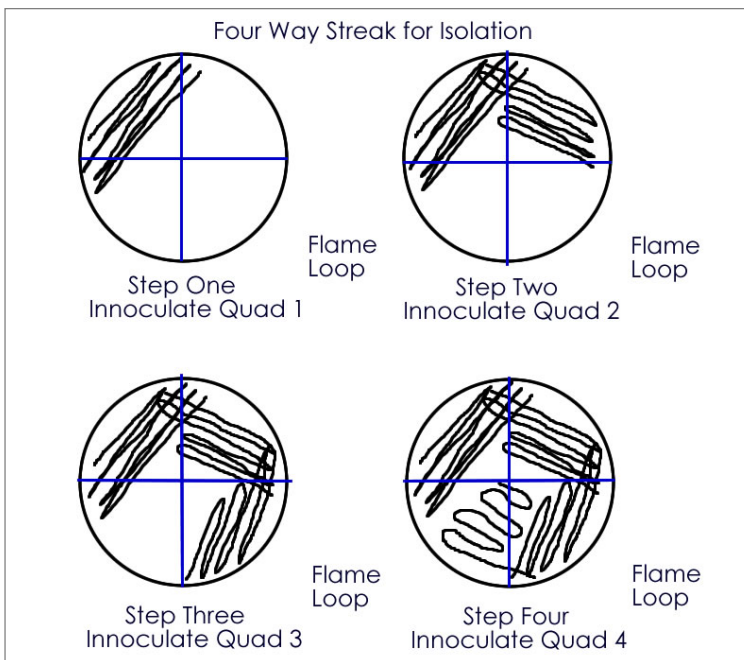
### **PROCEDURE:**

1. **Label** each tube appropriately.
2. Using a sterile **needle**, obtain a small amount of culture from the **broth** tube containing *Serratia marcescens*. Use the needle to inoculate a nutrient agar deep tube by stabbing the needle into the agar deep.
3. Using a sterile **loop**, obtain a loop full of culture from the **broth** tube containing *Serratia marcescens*. Use the loop to inoculate a nutrient agar slant tube by streaking the agar slant with a zig-zag motion.
4. Using a sterile **loop**, obtain a loop full of culture from the **slant** of *Serratia marcescens*. Use the loop to inoculate a nutrient broth tube by gently swishing the loop around in the liquid broth.
5. Make sure all caps are loose, but secure.
6. Incubate at 30 °C for 48 hours.



## EXERCISE #7 – ISOLATION OF PURE CULTURES

The purpose of this exercise is to separate a mixed culture of *Serratia marcescens* and *Escherichia coli* and obtain single, isolated colonies of each bacterial species. When working with microorganisms, it is desirable to start with single, isolated colonies to ensure you are working with a pure culture. Cultures that are visible on the surface of solid media are called colonies. A **colony** forms on a plate when a single microbe is inoculated onto the surface of the plate and reproduces until there are enough cells to form a visible colony. Since a colony theoretically forms from a single cell, a colony should then represent a pure culture. Two of the most common ways to obtain single, isolated colonies are the quadrant streak method and the T-streak method. Both methods, pictured below, allow sequential dilution of the original microbial material over the entire surface of a fresh plate. As the original sample is diluted by streaking it over successive sectors on the plate, the number of organisms decreases until only a few organisms are transferred, and these produce single, discrete colonies.



**MEDIA:** (*per student*)

Nutrient agar plate

**CULTURES:**

Mixed broth culture of *Serratia marcescens* and *Escherichia coli*.

**PROCEDURES:**

Note: Both the quadrant streak method and T-streak method are described below. Your instructor will provide further instructions on which method you will be using.

**QUADRANT STREAK METHOD:**

1. Divide the agar plate into 4 quadrants, and label them clockwise (or counter-clockwise, depending on your preference) 1 through 4.
2. Vortex the culture in the broth tube to ensure it is evenly suspended. Place a loopful of culture onto the plate in Quadrant 1 with a sterile loop and streak the loop very gently using a back and forth motion.
3. Sterilize loop. Go back to the edge of Quadrant 1 and extend the streaks into Quadrant 2, going back into Quadrant 1 twice.
4. Sterilize loop. Go back to the edge of Quadrant 2 and extend the streaks into Quadrant 3, going back into Quadrant 2 twice.
5. Sterilize loop. Go back to the edge of Quadrant 3 and extend the streaks into Quadrant 4, going back into Quadrant 3 twice. Be careful NOT to go back into Quadrant 1!
6. Tape plate closed on both sides. Make sure the plate is labeled with your name, date, and the organism(s), and incubate upside down (to prevent condensation from getting on to agar) at 30 °C.

**T-STREAK METHOD:**

1. Divide the agar plate into 3 sections by drawing a capital “T” on your plate. Label the section above the T sector 1, the section to the right sector 2, and the remaining section on the left sector 3.
2. Vortex the culture in the broth tube to ensure it is evenly suspended. Place a loopful of culture onto the plate in sector 1 with a sterile loop and streak the loop very gently using a back and forth motion.
3. Sterilize loop. Go back to the edge of sector 1 and extend the streaks into sector 2, going back into sector 1 twice. Continue zig-zag streaking on the remaining portion of sector 2.
4. Sterilize loop. Go back to the edge of sector 2 and extend the streaks into sector 3, going back into sector 2 twice. Continue zig-zag streaking on the remaining portion of sector 2. Be careful NOT to go back into sector 1!
5. Tape plate closed on both sides. Make sure the plate is labeled with your name, date, and the organism(s), and incubate upside down (to prevent condensation from getting on to agar) at 30 °C.

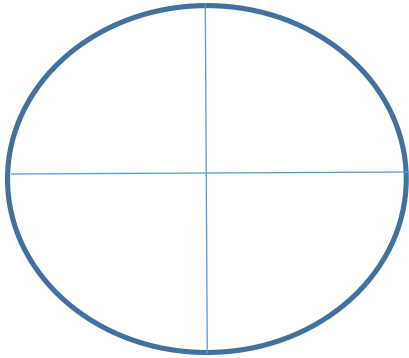
**RESULTS:**

Examine your isolation streak plate. Did you obtain single, isolated colonies of *Serratia marcescens* and *Escherichia coli*?

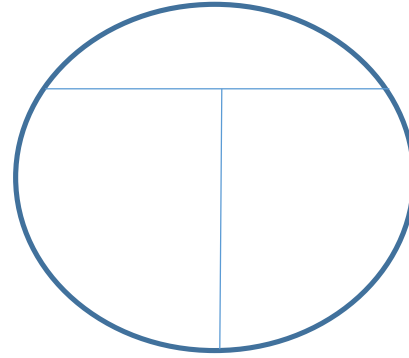
Do you see two distinct colony types on the agar plate? Hint: *S. marcescens* should be pinkish-red and *E. coli* should be cream-colored.

Draw the distribution of the growth and colonies on your plate.

Quadrant streak:



T-streak:



**STUDY QUESTIONS:**

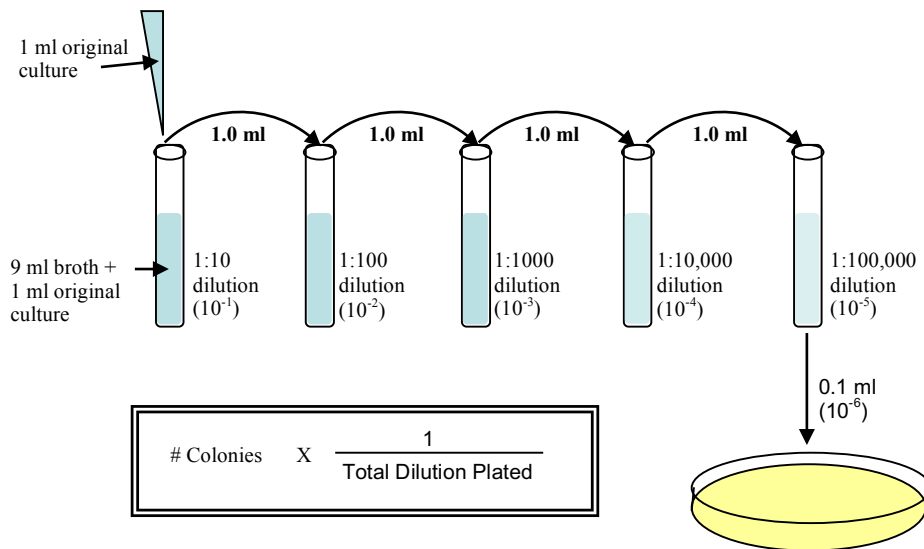
1. Why is it important to start a bacterial culture with a single, isolated colony?
  
  
  
  
  
  
  
  
  
  
2. Suppose you suspect that a supposedly pure bacterial culture is contaminated with another bacterium. Could you use this technique to determine if a contaminant is present? Explain.



## EXERCISE #8: VIABLE PLATE COUNTS

The purpose of this exercise is to quantify the number of bacteria in a broth culture of *E. coli*. In microbiological research, it is often necessary to be able to **quantify** the number of living bacteria in a particular sample. One of the major ways to do this is using **viable plate counts**, in which bacterial cells from a liquid culture are spread onto an agar plate. The plate is incubated, the number of colonies that grow on the plate are counted, and the number of original bacterial cells in the culture is determined. In most cases, however, the liquid culture being quantified contains too many cells to be directly plated onto agar plates – there would be so much growth that it would be impossible to count individual colonies! Therefore, the liquid culture needs to be diluted, often 1-million-fold, before it can be plated.

When such a large dilution is required, an accurate dilution cannot be made in a single dilution step and it is necessary to make **serial dilutions**. Serial dilutions are a step-wise set of dilutions which sequentially dilute the bacterial culture. One or more of the dilutions are then plated on the agar plates to determine the number of colonies present in the original culture. Only plates containing between 30 and 300 colonies are counted to ensure statistically significant data. To estimate the number of bacterial in the original culture, the # of colonies on the plate is multiplied by the total dilution plated. For example, suppose 0.1 ml of a  $10^{-6}$  dilution was plated, and 123 colonies were counted following incubation. The **total dilution plated** would be  $10^{-7}$  (since only 0.1 ml was plated), and the number of bacteria/ml of the original culture would be:  $(123) \times 1/10^{-7} = 1.23 \times 10^9$  CFU/ml. Note that the results are expressed as “colony forming units (CFU)” per ml.



In this exercise, you will be using the viable plate count method to enumerate the bacteria in a provided sample: either an overnight broth culture of *Escherichia coli* or an isolated colony of *Escherichia coli*. Your instructor will provide direction on which sample you will be using.

**MEDIA NEEDED:** (per group of four)

- 3 Nutrient agar plates
- 7 Dilution blanks containing 9 ml water

**CULTURES NEEDED:**

- Overnight broth culture of *Escherichia coli*
- OR** Streak plate containing isolated colonies of *Escherichia coli*

**PROCEDURE:**

1. Label the dilution blanks as follows:  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ .
2. Label the agar plates as follows:  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ .
3. If you are using an overnight broth culture of as your sample, proceed to step 4. If you are quantifying the number of bacteria in an isolated colony, then you will first need to thoroughly resuspend the colony in 1 ml of sterile water first.
  - a. Using a sterile loop, pick up an entire isolated colony and transfer to a small tube containing 1 ml of sterile water.
  - b. Vortex thoroughly to obtain a uniform suspension.
  - c. Proceed to step 4, using the 1 ml suspension you just created to transfer to the first dilution blank.
4. Using a sterile pipette, transfer 1 ml of the *E. coli* broth culture into the tube labeled  $10^{-1}$ . Mix thoroughly.
5. Using a new sterile pipette, transfer 1 ml of the  $10^{-1}$  tube into the tube labeled  $10^{-2}$ . Mix thoroughly.
6. Using a new sterile pipette, transfer 1 ml of the  $10^{-2}$  tube into the tube labeled  $10^{-3}$ . Mix thoroughly.
7. Using a new sterile pipette, transfer 1 ml of the  $10^{-3}$  tube into the tube labeled  $10^{-4}$ . Mix thoroughly.
8. Using a new sterile pipette, transfer 1 ml of the  $10^{-4}$  tube into the tube labeled  $10^{-5}$ . Mix thoroughly.
9. Using a new sterile pipette, transfer 1 ml of the  $10^{-5}$  tube into the tube labeled  $10^{-6}$ . Mix thoroughly.
10. Using a new sterile pipette, transfer 1 ml of the  $10^{-6}$  tube into the tube labeled  $10^{-7}$ . Mix thoroughly.
11. Using a new sterile pipette, transfer 0.1 ml of the  $10^{-5}$  tube to the nutrient agar plate labeled  $10^{-6}$ , and spread the liquid thoroughly and evenly over the surface of the plate using a sterile disposable spreader. Be careful not to let the spreader dig into the agar! [Note: Since we're only plating 0.1 ml of the  $10^{-5}$  dilution, the total dilution plated is  $10^{-6}$ .]

12. Using a new sterile pipette, transfer 0.1 ml of the  $10^{-6}$  tube to the nutrient agar plate labeled  $10^{-7}$ , and spread the liquid thoroughly and evenly over the surface of the plate using a sterile disposable spreader.
13. Using a new sterile pipette, transfer 0.1 ml of the  $10^{-7}$  tube to the nutrient agar plate labeled  $10^{-8}$ , and spread the liquid thoroughly and evenly over the surface of the plate using a sterile disposable spreader.
14. After the liquid has absorbed into the plates, tape them closed on both sides. Make sure your plates are labeled with your name, date, and the organism, and incubate upside down at  $30\text{ }^{\circ}\text{C}$ .

**RESULTS:**

Examine the nutrient agar plates for growth, and count the number of colonies on each plate. Remember, the number has to be between 30 and 300 in order to be statistically accurate. If your plate has fewer than 30 colonies, record the number as “TFTC” for “too few to count”. If your plate has more than 300 colonies, record the number as “TNTC” for “too numerous to count”. Then, use the formula on the previous page to determine the number of CFU/ml of the original broth culture.

Dilution	Number of colonies	Number of CFU/ml of original sample <i>(Remember to use scientific notation!)</i>
$10^{-6}$		
$10^{-7}$		
$10^{-8}$		

**STUDY QUESTIONS:**

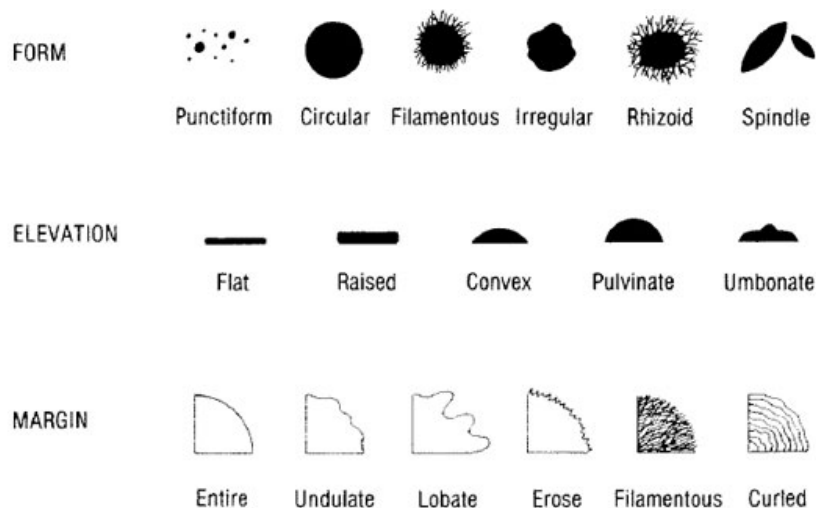
1. Why do you think it is important to be able to quantify the number of viable bacteria in a sample?
  
2. Critical Thinking Question: Give an example of a commercial or industrial setting where quantifying viable bacteria would be a useful tool.
  
3. (Optional) If you used an isolated colony as your sample: How many individual bacterial cells make up a single bacterial colony of *Escherichia coli*?

## EXERCISE #9 – GROWTH CHARACTERISTICS OF COLONIES AND BROTH CULTURES

When properly isolated, bacteria grow on solid media in the form of a colony. A colony is formed when a single, microscopic cell (i.e. colony forming unit) reproduces to form a visible mound of cells on the surface of solid media. To the untrained eye, all colonies may look alike, but upon careful observation, many variations can be noted when comparing the colonies of different species of bacteria. Conversely, colonies produced by a single species of bacteria will typically be uniform in appearance.

The most common characteristics for differentiating bacterial colonies on nutrient (TSA) agar plates are size, pigmentation, form, elevation, and margin. The term **size** simply refers to how large or small the colonies are and include terminology such as pinpoint, small, medium and large. The size is usually determined by measuring the diameter of the isolated bacterial colony. **Pigmentation** refers to the chemicals produced by the organism to create color. Intracellular pigments are produced within the organism and give color to the surface of the colonies. Extracellular pigments are released external to the organism and if soluble, will produce a color on the agar medium. The **form** describes the overall shape of the colony with the general growth distribution delineating the edge. **Elevation** depicts how the colony sits on the surface of the agar and includes terms that can describe the overall raised growth or what is happening within the center of the colony. The **margin** refers to the outer edge of the colony and indicates differences between a sharp boarder verses one that is indented, wavy, tooth like, or spreading out.

See below for examples of the different types of form, elevation, and margin.

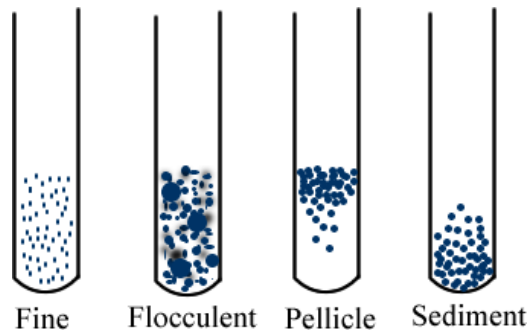


Source: [www.asm.org](http://www.asm.org)

Additional differences in media growth can be visualized using broth cultures. Patterns of growth can be identified by the following descriptive terms:

- Fine turbidity: finely dispersed small uniform growth
- Flocculent: large flaky clumps
- Sediment: growth primarily at the bottom
- Pellicle: growth primarily at the surface

Examples of broth media growth are illustrated in the picture below.



In this exercise, you will be characterizing the agar plate colonies of seven different species of bacteria based on size, pigmentation, form, elevation, and margin, as well as describing the growth of an organism in a liquid broth medium.

**MEDIA NEEDED:**

None

**Equipment Needed:**

Dissecting Microscope  
Magnifying lens

**CULTURES NEEDED:**

Streak plates of various bacteria  
Broth cultures of various bacteria and yeast

**PROCEDURE:**

1. Select a broth specimen, observe the growth pattern and record your results. Repeat for the specimens provided.
2. Select an agar plate specimen and observe the isolated colonies as follows, recording your results in the table provided in the Results section. Don't forget to record the name of the specimen as well!
  - a. Measure the diameter of the colony in millimeters.
  - b. Determine the pigmentation (i.e. color) of the colony.
  - c. Determine the form of the colony, using the diagram above as a guideline.
  - d. Determine the elevation of the colony, using the diagram above as a guideline.
  - e. Determine the type of margin, using the diagram above as a guideline.
3. Repeat with the remaining 6 specimens.

**RESULTS:**

Name of Specimen in Broth	Growth Pattern Description

Name of Organism on Agar Plate	Size (mm)	Pigmentation/Color	Form	Elevation	Margin



## EXERCISE #10 – EFFECT OF TEMPERATURE ON GROWTH OF MICROORGANISMS

Bacterial growth is dependent on the ability of bacterial enzymes to carry out metabolic processes, and is therefore dependent on temperature. In general, enzymes are less active at low temperatures due to low kinetic energy, and are increasingly active as temperatures increase. However, when temperatures become too high, enzymes will become denatured and will cease to function properly. Another important factor in bacterial growth is membrane fluidity. At low temperatures, the lipids in a cell membrane can pack too tightly and solidify, prohibiting the membrane from functioning properly. When temperatures become excessively high, the lipid bilayer can become too fluid and lose its integrity. As a group, bacteria are capable of growth within a range from -5 °C to 80 °C, with some archaea able to withstand even higher temperatures. However, each individual bacterial species will have a more narrow range in which it can grow. For every organism there are three cardinal temperatures: the minimum growth temperature, the optimum growth temperature, and the maximum growth temperature. The **minimum growth temperature** is the **lowest** temperature at which a specific organism will grow and reproduce. The **optimum growth temperature** is the temperature at which a specific organism exhibits maximum growth and reproduction. The **maximum growth temperature** is the **highest** temperature at which a specific organism will grow and reproduce. Although the optimum growth temperature is different for each organism, it is generally closer to the maximum growth temperature than the minimum.

All prokaryotes can be classified into four general groups depending on their temperature requirements. If an organism grows at temperatures ranging from -5 to 20 °C, it's classified as a **psychrophile**. An organism that grows in a range from 20 to 45 °C is classified as a **mesophile**. (The term **psychrotroph** is often used to describe organisms which are able to grow at temperatures below 10 °C, but also grow in the mesophile range.) **Thermophiles** are able to grow in a range from 45 to 80 °C. Some archaea are classified as **hyperthermophiles**, which can grow at temperatures greater than 80 °C, some up to 105 °C.

It is important to note that the specific growth range will vary for each species in a given temperature class. For example, while *Staphylococcus aureus* and *Staphylococcus epidermidis* are both classified as mesophiles, the maximum growth temperature for *S. epidermidis* is only 40 °C, whereas *S. aureus* will grow at 45 °C.

For this lab exercise, each group will design and conduct an experiment to determine the temperature classification of five different species of bacteria, using the provided media and equipment.

**MEDIA AVAILABLE:** (*per group of four*)

4 nutrient agar plates

**EQUIPMENT AVAILABLE:**

Incubators set at various temperatures (4 °C, 25 °C, 37 °C, 55 °C)

(Note: the 4 °C “incubator” is the refrigerator, and 25 °C is approximately room temperature)

**CULTURES NEEDED:**

*Escherichia coli*, *Bacillus stearothermophilus*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Micrococcus luteus*



**PROCEDURE:**

(Outline your experimental design in the space provided below.)

**DATA:**

Temperature	<i>Serratia marcescens</i>	<i>Pseudomonas fluorescens</i>	<i>Escherichia coli</i>	<i>Bacillus stearothermophilus</i>	<i>Micrococcus luteus</i>

**CONCLUSIONS:**

Organism	Temperature Class*
<i>Serratia marcescens</i>	
<i>Pseudomonas fluorescens</i>	
<i>Escherichia coli</i>	
<i>Bacillus stearothermophilus</i>	
<i>Micrococcus luteus</i>	

(\*psychrophile, psychrotroph, mesophile, or thermophile)

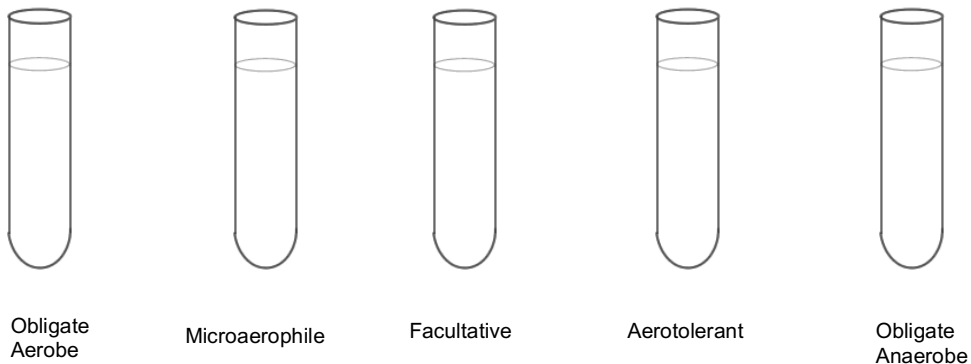


## EXERCISE #11 -- ATMOSPHERIC OXYGEN REQUIREMENT FACTORS

Bacterial growth is also dependent on the presence of oxygen in the environment, as different bacteria have different oxygen requirements depending on the types of enzymes they possess. The major bacterial oxygen classes are aerobes, microaerophiles, obligate anaerobes, aerotolerant anaerobes, and facultative anaerobes. **Aerobes** require atmospheric O<sub>2</sub> (20%), and use O<sub>2</sub> as the final electron acceptor in the electron transport system. **Microaerophiles** require O<sub>2</sub> at below atmospheric concentrations, typically 2-10%. Microaerophiles have a limited ability to neutralize toxic oxygen, so excess O<sub>2</sub> will kill the bacteria. However, microaerophiles do use O<sub>2</sub> as final electron acceptor in the electron transport system. **Obligate Anaerobes** cannot survive in the presence of any oxygen. Obligate anaerobes lack the enzymes necessary to break down the toxic by-products of oxygen. In these bacteria, the final electron acceptor in the electron transport system is a molecule other than O<sub>2</sub>. **Aerotolerant Anaerobes** grow equally well in the presence or absence of oxygen. They do possess enzymes necessary to neutralize toxic oxygen by-products, but they never use O<sub>2</sub> as a final electron acceptor. **Facultative Anaerobes** are also able to live either in the presence or absence of oxygen, but they prefer oxygen so they can carry out aerobic respiration with O<sub>2</sub> as final electron acceptor to maximize ATP yields. These organisms can use other electron acceptors if O<sub>2</sub> is not available, such as fumarate and nitrate. They can also utilize fermentative metabolism in the absence of oxygen.

One simple way to determine the oxygen requirements of a bacterium is to inoculate the bacterial culture into a melted agar deep tube of brain heart infusion (BHI) media, mixing well to evenly distribute the bacteria throughout the liquefied agar. Once the agar solidifies, an oxygen gradient is created, with atmospheric oxygen concentrations at the top of the tube and little to no oxygen present at the bottom of the tube. The bacteria present in the agar deep tube will only be able to grow where their oxygen requirements are met, and will localize to the area(s) of their oxygen requirements in the tube.

Based on your knowledge of oxygen requirements, sketch where you think growth would occur in the BHI tube for each oxygen class:



**MEDIA NEEDED:** (per group of 4)  
4 Brain Heart Infusion (BHI) deeps

**CULTURES NEEDED:**  
*Staphylococcus xylosum*, *Mycobacterium smegmatis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Saccharomyces cerevisiae*, *Clostridium sporogenes* *Bacillus megaterium*, and *Serratia marcescens*

- PROCEDURE:**
1. Obtain 4 melted BHI tubes from the water bath and label with 4 of the above bacteria. Each person in the group should be responsible for one BHI tube. Coordinate with another group so that all microorganisms are tested!!
  2. While the BHI tube is still liquefied, add 2 drops of the appropriate inoculum into the BHI tube using a sterile Pasteur pipette. Alternatively, you may gently inoculate the liquefied BHI tube using a sterile loop or needle. Place cap loosely (but securely) on the tube. You need to work quickly so the agar doesn't solidify!
  3. Roll the BHI tube between the palms of your hands to disperse the bacteria throughout the tube, taking care not to create bubbles or aerate the agar.
  4. Place the BHI tube in ice bath until solidified.
  5. Remove from ice bath, and place in incubator. Incubate at 37 °C for 48 hours.

**RESULTS:**  
Examine the BHI deep tubes for presence/absence of microbial growth and location of growth. Record results in the table below.

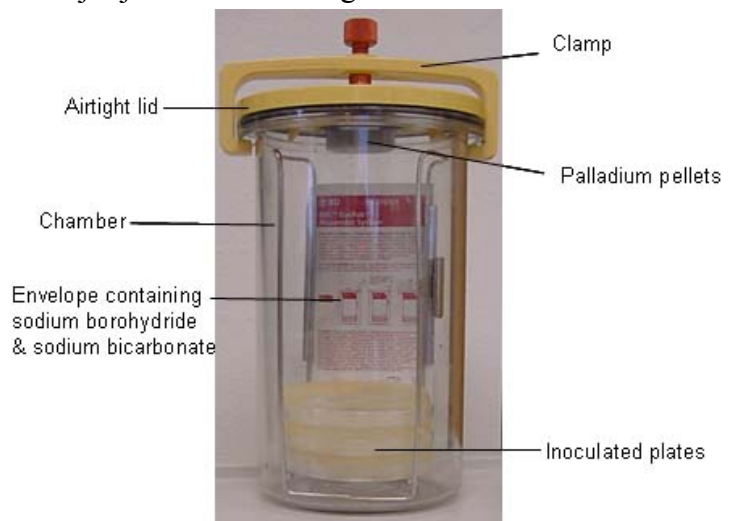
Species	Distribution of growth	Oxygen Requirement Classification
<i>Staphylococcus xylosum</i>		
<i>Mycobacterium smegmatis</i>		
<i>Enterococcus faecalis</i>		
<i>Micrococcus luteus</i>		
<i>Saccharomyces cerevisiae</i>		
<i>Clostridium sporogenes</i>		
<i>Bacillus megaterium</i>		
<i>Serratia marcescens</i>		



## EXERCISE #12 – CULTIVATION OF ANAEROBIC ORGANISMS

Specialized methods are necessary to culture organisms **anaerobically**. One such method is the use of **fluid thioglycollate broth**, which is a reducing medium. It contains **sodium thioglycollate**, which reacts with molecular oxygen keeping free oxygen levels low. The sodium thioglycollate in the broth creates a **redox potential** in the tube, with higher levels of oxygen at the top of the tube, and a complete absence of oxygen at the bottom of the tube. Fluid thioglycollate broth also typically contains a redox potential indicator such as resazurin, which produces a pink coloration in an oxidized environment. As with the BHI media described in Exercise #10, organisms will only be able to grow where their oxygen requirements are met, and will localize to the area(s) of their oxygen requirements in the fluid thioglycollate broth

A second method used to culture organisms anaerobically is the use of a **GasPak Jar**. This is a specialized culture vessel in which an anaerobic environment is generated after inoculated media are sealed into the chamber. Anaerobic conditions are created by adding water to a gas generator envelope that is placed in the jar just before sealing. There are two chemical tablets in the envelope, **sodium borohydride** and **sodium bicarbonate**. Water reacts with these chemicals, producing **hydrogen** gas from the sodium borohydride and **carbon dioxide** from the sodium bicarbonate. The hydrogen gas combines with free oxygen in the chamber to produce water, thus removing all free oxygen from the chamber. This reaction is catalyzed by the element **palladium** (#46 on the periodic table), which is attached to the underside of the lid of the jar. The carbon dioxide replaces the removed oxygen, creating a completely anaerobic environment.



For this lab exercise, each group will design and conduct an experiment to determine the oxygen classification of four different species of bacteria, using the provided media and equipment.

### **MEDIA AVAILABLE:** (*per group of four*)

- 4 thioglycollate broth tubes
- 2 nutrient agar plates

### **EQUIPMENT AVAILABLE:**

- Gas Pak Jar
- Incubators set at 37 °C

### **CULTURES NEEDED:**

*Bacillus megaterium*, *Mycobacterium smegmatis*, *Escherichia coli*, *Clostridium sporogenes*

**PROCEDURE:**

(Outline your experimental design in the space provided below.)





### EXERCISE #13: USE OF SELECTIVE, DIFFERENTIAL, AND ENRICHED MEDIA

Microbiologists often used multiple types of media to cultivate microorganisms in order to either specifically grow a particular organism, or to obtain information about the biochemical properties of the organisms that grow. Three commonly used types of media are selective media, differential media, and enriched media.

**Selective media** is designed to suppress the growth of some microorganisms while allowing the growth of others (i.e., it *selects* for certain microbes). Solid media is usually employed with selective media so that individual colonies may be isolated. Examples of selective media include mannitol salt agar, MacConkey agar, eosin-methylene blue agar, and Columbia C-CNA agar.

**Differential media** allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies based on specific biochemical properties of the organisms. Most differential media contain a substrate and a chemical indicator, often a pH indicator. As with selective media, solid media is usually employed so individual colonies may be observed. Examples of differential media include mannitol salt agar, DNase agar, blood agar, MacConkey agar and eosin-methylene blue agar.

**Enriched media** contains specific growth factors needed by fastidious bacteria to support their growth. Examples of enriched media include blood agar and chocolate agar.

Described below are the specific properties of some of the more commonly used selective, differential, and enriched media. Note that a particular type of media can have multiple properties – e.g. it can be both selective and differential!

#### Mannitol Salt Agar:

Mannitol salt agar (MSA) is both selective and differential. MSA contains 7.5% NaCl, which selects for halotolerant organisms such as members of the *Staphylococcus* genus. The media also contains the carbohydrate substrate mannitol, and a pH indicator, phenol red. Organisms which are able to ferment the mannitol will produce acid fermentation products which lower the pH, causing the phenol red indicator to turn yellow.

#### Blood Agar:

Blood agar is a differential medium. It is also commonly used as an enriched medium for growing fastidious bacteria. Some bacteria produce exotoxins called hemolysins that cause lysis of red blood cells. The degree of the hemolysis is an especially useful tool for differentiation among Gram-positive cocci, although it may also be used in differentiating some Gram-negative organisms as well. The three types of hemolysis are:

1. **Beta hemolysis**, which is the **complete lysis** of red blood cells and hemoglobin. This results in complete clearing of the blood around colonies.
2. **Alpha hemolysis** refers to the **partial lysis** of red blood cells and hemoglobin. This results in a greenish-grey or greenish-yellow discoloration of the blood around the colonies.
3. No hemolysis, sometimes called **gamma hemolysis** results in no change in the medium.

#### MacConkey Agar:

MacConkey Agar is both selective and differential. This media contains crystal violet and bile salts, which inhibit most Gram-positive organisms and select for Gram-negative organisms. It also contains the substrate lactose and the pH indicator neutral red, which allow differentiation among Gram-negative bacteria based on their ability to ferment lactose. When lactose is

fermented by coliforms such as *Escherichia coli*, acid end-products lower the pH of the media below 6.8, with the resulting colonial growth turning pinkish-red. If an organism is unable to ferment lactose, the colonies will be colorless, taking on the color of the medium.

#### Eosin Methylene Blue Agar:

Eosin Methylene Blue (EMB) agar is both selective and differential. This media contains the dyes eosin and methylene blue, which inhibit Gram-positive growth and select for Gram-negative organisms. It also contains lactose, allowing differentiation between organisms which ferment lactose and produce acid end-products, and organisms that do not ferment lactose. Small amounts of acid production result in a pink colored growth (e.g. *Enterobacter aerogenes*) while large amounts of acid cause the acid to precipitate on the colony, resulting in a characteristic greenish, metallic sheen (e.g. *E. coli*). Organisms which do not ferment lactose will be colorless, taking on the color of the medium.

#### Columbia C-NA Agar:

Columbia C-NA (CCNA) agar is both selective and differential. This media contains two antibiotics, colistin and nalidixic acid, which prohibit the growth of most Gram-negative organisms. Gram-positive organisms such as members of the *Staphylococcus* and *Streptococcus* genera are allowed to grow. Addition of blood to the agar allows differentiation by hemolytic patterns.

#### **MEDIA NEEDED:** (per group of 4)

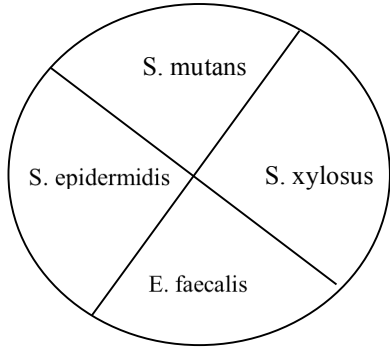
- 1 Mannitol Salt agar plate
- 1 Blood agar plate
- 1 MacConkey agar plate
- 1 Eosin-Methylene Blue (EMB) agar plate
- 1 Columbia C-NA (CCNA) agar plate

#### **CULTURES NEEDED:**

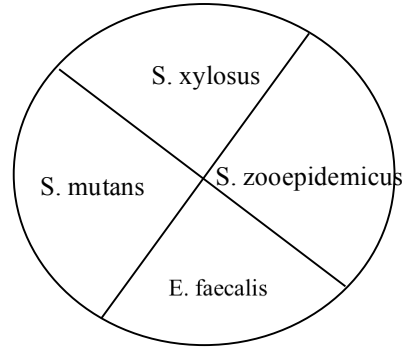
*Enterobacter aerogenes*, *Escherichia coli*, *Streptococcus zooepidemicus*, *Streptococcus mutans*, *Enterococcus faecalis*, *Morganella morganii*, *Staphylococcus xylosum*, *Staphylococcus epidermidis*

#### **PROCEDURE:**

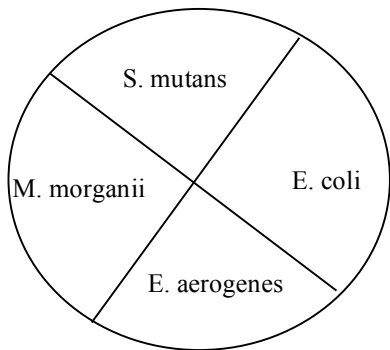
1. Label each plate according to the diagram on the following page. Don't forget to also put your name/initials and the date.
2. Using a sterile loop, inoculate each one of the plates using a straight line streak according to the diagram on the following page.
3. Tape all plates on both sides.
4. Place the blood agar plates and CCNA plates into an Anaeropak box so they can be incubated in an environment enriched with carbon dioxide to encourage the growth of capnophiles.
5. Incubate all plates at 37 °C for 48 hours.



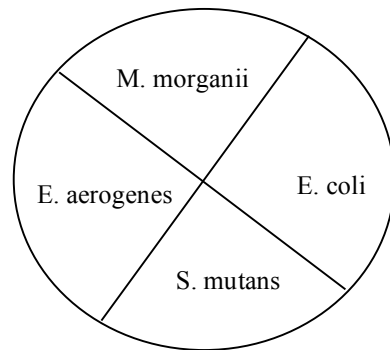
MSA



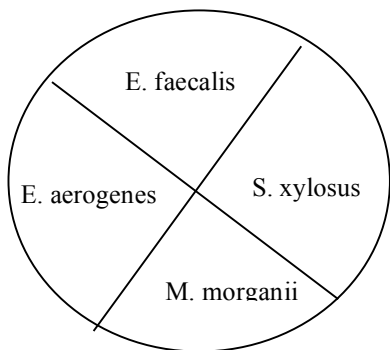
Blood



MacConkey plate



EMB plate



C-CNA

## RESULTS:

Examine plates for the following:

1. Mannitol Salt Agar Plates: Growth on mannitol salt agar indicates the organism is halotolerant. A change in the color of the agar from pink to yellow indicates the organism has the ability to ferment mannitol. Note: this yellow coloration can usually be seen around the sides of the bacterial growth.
2. Blood Plates: All organisms should grow on the blood plates – they are NOT selective. Growth surrounded by a complete clearing of the blood indicates beta hemolysis. Growth surrounded by a partial clearing of the blood (often a greenish-grey color) indicates alpha hemolysis. Growth which produces no changes in the blood indicates the bacteria are non-hemolytic. (This lack of hemolysis is sometimes called gamma hemolysis.)
3. MacConkey Agar Plates: Growth on MacConkey agar indicates the organism is resistant to crystal violet and bile salts, and is likely to be Gram-negative. Growth which is a pinkish-red color indicates the organism has the ability to ferment lactose, and is probably a coliform.
4. Eosin Methylene Blue Agar: Growth on Eosin Methylene Blue agar indicates the organism can grow in the presence of the dyes eosin and methylene blue and is likely a Gram-negative. Growth which is a pink color indicates the organism can ferment lactose to form weak acid end-products, and growth which exhibits a green metallic sheen indicates the organism can ferment lactose to form strong acid end-products.
5. C-CNA Agar: Growth on Columbia C-NA agar indicates the organism is resistant to the antibiotics colistin and naladixic acid, and is likely a Gram-positive. Hemolysis patterns can allow differentiation (see above).

Record your results on the data charts below.

### Mannitol Salt Agar

Organism	Growth	Color	Interpretation of Results
<i>Streptococcus mutans</i>			
<i>Staphylococcus epidermidis</i>			
<i>Staphylococcus xylosum</i>			
<i>Enterococcus faecalis</i>			

Blood

Organism	Growth	Hemolytic pattern	Interpretation of Results
<i>Streptococcus mutans</i>			
<i>Streptococcus zooepidemicus</i>			
<i>Staphylococcus xylosum</i>			
<i>Enterococcus faecalis</i>			

MacConkey

Organism	Growth	Color	Interpretation of Results
<i>Escherichia coli</i>			
<i>Streptococcus mutans</i>			
<i>Morganella morganii</i>			
<i>Enterobacter aerogenes</i>			

EMB

Organism	Growth	Color	Interpretation of Results
<i>Escherichia coli</i>			
<i>Morganella morganii</i>			
<i>Streptococcus mutans</i>			
<i>Enterobacter aerogenes</i>			

C-CNA

Organism	Growth	Hemolysis	Interpretation of Results
<i>Morganella morganii</i>			
<i>Enterococcus faecalis</i>			
<i>Staphylococcus xylosus</i>			
<i>Enterobacter aerogenes</i>			

**STUDY QUESTIONS:**

1. What property or properties of Gram-negative organisms would likely make them more resistant to crystal violet and bile salts than Gram-positive organisms?

2. An unknown bacterium produces colorless colonies when inoculated onto an EMB plate. Predict what you would see if you inoculated that same unknown onto the following media, and explain why:

MacConkey agar:

C-CNA Agar:

## EXERCISE #14: CHEMICAL CONTROL OF MICROORGANISMS

Antimicrobial chemicals, disinfectants, and antiseptic solutions are routinely used to decontaminate surfaces, objects, and even skin and tissues. Examples include household cleaners such as Lysol and Clorox, first aid treatments such as isopropanol and hydrogen peroxide, and hospital cleaners such as Amphyll and Vesphene. Chemical agents work through two main mechanisms: (1) Alteration of cell walls or cytoplasmic membranes and (2) Interference with protein and nucleic acid structure. Like antibiotics, chemical agents may be “static” or “cidal” in action, although most of the newer chemical agents are microbicidal.

There are many methods to test the effectiveness of antimicrobial chemicals, including the disk diffusion assay. In this assay, small filter disks are impregnated with the chemical to be tested, and are placed on a plate inoculated to form a bacterial lawn (even, confluent bacterial growth). The plates are incubated to allow growth of the bacteria and time for the chemicals to diffuse into the agar. As a chemical diffuses into the agar, it becomes less concentrated. If an organism is susceptible to a chemical, a clear **zone of inhibition** will appear around the disk where the growth has been inhibited. The size of this zone of inhibition depends on the sensitivity of the bacteria to the specific chemical and the chemical's ability to diffuse through the agar.

We will be testing four different chemicals today using the disk diffusion assay. Students may test the chemicals provided in the laboratory, or they are welcome to bring in their own disinfectants for testing.

### **MATERIALS NEEDED:** (*per group of 4*)

1 Mueller-Hinton plate

### **CULTURES NEEDED:**

*Escherichia coli*, *Staphylococcus aureus*

### **PROCEDURE:**

1. With a marker, divide the bottom of the plate into quadrants. Label A, B, C, and D. (Key: A = isopropanol; B= hydrogen peroxide; C= Lysol antibacterial disinfectant; D= Micro-90 dish detergent) Note: If you brought your own disinfectants to test, record those here: A = \_\_\_\_\_ B = \_\_\_\_\_  
C = \_\_\_\_\_ D = \_\_\_\_\_
2. Select a broth culture of either *E. coli* or *S. aureus*. Coordinate with another lab group in your class so that each group chooses a different one. Write the name of the bacteria on the plate, as well as your initials and the date.
3. Gently agitate your broth tube to resuspend any bacteria that have settled to the bottom of the tube. With a sterile swab, dip into the broth tube and completely swab the surface of the plate. Rotate the plate 90° and repeat, dipping a fresh sterile swab into the broth tube and completely swabbing the surface of the plate. There should be a blanket of uniform growth following incubation.
4. Using forceps, dip a sterile filter disk in Solution A, allow the excess solution to drip off, and then place the disk in the center of Quadrant A. Repeat with Solutions B, C, and D.



5. GENTLY press each disk onto the agar to make sure it stays, but do not puncture the agar.
6. Tape the plate, and incubate at 37 °C for 48 hours.

**RESULTS:**

Using a plastic ruler, measure the diameter in millimeters (mm) of the zone of inhibition for each chemical tested. Record your results in the chart below.

<b>Chemical</b>	<b><i>E. coli</i> Zone of Inhibition</b>	<b><i>S. aureus</i> Zone of Inhibition</b>
Isopropanol		
Hydrogen peroxide		
Clorox cleaner		
Lab cleaner		

Based on your results, which chemical is most effective against *E. coli*? Which is least effective against *E. coli*?

Which is most effective against *S. aureus*? Which is least effective against *S. aureus*?

Which chemical would you choose if you wanted maximum effectiveness against both *E. coli* and *S. aureus*?

**STUDY QUESTIONS:**

1. Using your textbook as a reference, describe the mechanisms of action of isopropanol, hydrogen peroxide, and Clorox bleach against bacteria.

## EXERCISE #15: CHEMOTHERAPEUTIC AGENTS

Antimicrobial chemotherapy is the use of chemicals to inhibit or kill microorganisms in or on the host. Antimicrobial therapy is based on **selective toxicity**. This means that the agent used must inhibit or kill the microorganism in question without seriously harming the host. In order to be selectively toxic, a chemotherapeutic agent must interact with some microbial function or microbial structure that is either not present in the host, or is substantially different from that of the host. For example, in treating infections caused by prokaryotic bacteria, the agent may prevent peptidoglycan synthesis or inhibit bacterial enzymes such as RNA polymerase or DNA gyrase. Human cells do not contain peptidoglycan or DNA gyrase, and have structurally different RNA polymerases. Therefore, drugs with these targets will have little, if any, effect on the host.

Based on their origin, there are 2 general classes of antimicrobial chemotherapeutic agents: (1) Antibiotics, which are substances produced as metabolic products of one microorganism which inhibit or kill other microorganisms; and (2) Antimicrobial chemotherapeutic chemicals, which are chemicals synthesized in the laboratory which can be used therapeutically on microorganisms. Today the distinction between the two classes is not as clear, since many antibiotics are extensively modified in the laboratory (semisynthetic) or even synthesized without the help of microorganisms.

Some antimicrobial agents are “cidal” in action: they kill microorganisms (e.g., penicillins, cephalosporins, neomycin). Others are “static” in action: they inhibit microbial growth long enough for the body's own defenses to remove the organisms (e.g., tetracyclines, erythromycin, sulfonamides).

Antimicrobial agents also vary in their spectrum. Drugs that are effective against a variety of both Gram-positive and Gram-negative bacteria are said to be **broad spectrum** (e.g., tetracycline, streptomycin, cephalosporins, ampicillin, sulfonamides). Those effective against just Gram-positive bacteria, just Gram-negative bacteria, or only a few species are generally termed **narrow spectrum** (e.g., penicillin G, erythromycin, clindamycin, gentamicin). The major classes of antibiotics and their mechanisms of action have been covered in the text used in lecture.

Antibiotic sensitivity testing is used to determine the susceptibility of bacteria to various antibiotics. In a clinical setting, these tests are used to measure the effectiveness of a variety of antibiotics on a specific organism in order to prescribe the most suitable antibiotic therapy.

One commonly used test is the **Disk Diffusion Assay**, also known as the **Kirby-Bauer** test. In this qualitative test, a series of antibiotic-impregnated paper disks are placed on a plate inoculated to form a bacterial lawn (even, confluent growth). The plates are incubated to allow growth of the bacteria and time for the antibiotics to diffuse into the agar. As the drug diffuses into the agar, its strength decreases. If an organism is susceptible to an antibiotic, a clear circular **zone of inhibition** will appear around the disk where the growth has been inhibited. The size of this zone of inhibition depends on the sensitivity of the bacteria to the specific antibiotic and the antibiotic's ability to diffuse through the agar. After incubation, the zones of inhibition are measured and compared with tables giving the interpretation of measurement for each antibiotic.

Another commonly used test is the **Epsilon meter** or **E test**, which quantitatively measures the minimal inhibitory concentration (MIC) of the antibiotic for the microorganism being tested. The test employs a rectangular strip that contains a defined concentration gradient of the antimicrobial agent. As with the Kirby-Bauer test, the strip is placed on a plate inoculated to form a bacterial lawn, and the plates are incubated to allow the bacteria to grow and the antibiotic to diffuse into the agar. The resulting zone of inhibition has an elliptical shape, with

the smallest part of the ellipse intersecting with the strip at lowest concentration of antibiotic that effectively inhibits the growth of the bacteria (the MIC).

**MEDIA NEEDED:** (*per group*)

2 Mueller Hinton Agar plates

**CULTURES NEEDED:**

*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus vulgaris*

**PROCEDURE:**

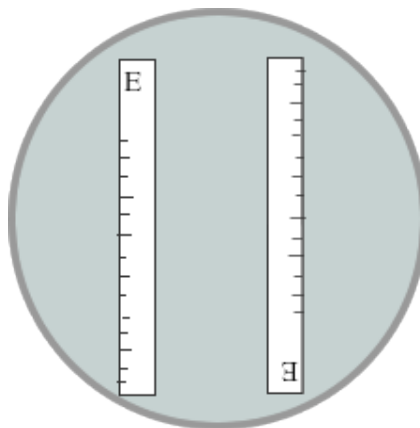
Each **group** will choose one of the five cultures listed above. Make sure to label your plates with your names or initials and the name of the organism.

Disk Diffusion Assay / Kirby-Bauer test:

1. Gently agitate your broth tube to resuspend any bacteria that have settled to the bottom of the tube. With a sterile swab, dip into the broth tube and completely swab the surface of the M-H plate. Rotate the plate 90° and repeat, dipping a fresh sterile swab into the broth tube and completely swabbing the surface of the plate. There should be a blanket of uniform growth following incubation.
2. Carefully place the provided antibiotic discs onto the plate using the disc dispenser provided. (Your lab instructor will demonstrate how to properly use the disc dispenser.) You will need to take sterile forceps and lightly touch each disc to make sure it will stay in place.

E-test: (Optional – check with your instructor)

1. Gently agitate your broth tube to resuspend any bacteria that have settled to the bottom of the tube. With a sterile swab, dip into the broth tube and completely swab the surface of the M-H plate. Rotate the plate 90° and repeat, dipping a fresh sterile swab into the broth tube and completely swabbing the surface of the plate. There should be a blanket of uniform growth following incubation.
3. Using a pair of sterile forceps, carefully place the provided E-strips onto the plate, tapping each one lightly to make sure it will stay in place. (See diagram below)



4. Incubate both plates inverted at 37 °C for 48 hours.

**RESULTS:**Disk Diffusion Assay:

Using a plastic ruler, measure the diameter in millimeters (mm) of the zone of inhibition for each antibiotic tested. Record your results in the chart below. Refer to the table provided to determine sensitivity and resistance.

Antibiotic	Inhibition Zone Diameter (mm)	Susceptible/ Intermediate/ Resistant
Nalidixic Acid (NA30)		
Trimethoprim/Sulfamethoxazole (SXT25)		
Gentamycin (CN10)		
Vancomycin (VA30)		
Penicillin (P10)		
Cefoxitin (FOX30)		
Tetracycline (TE30)		
Azithromycin (AZM15)		

## Antibiotic Zone Diameters:

Disc Code	Resistant (<mm)	Intermediate (mm range)	Susceptible (>mm)
AZM15	13	14-17	18
CN10	12	13-14	15
VA30 -- <i>Enterococcus</i>	14	15-16	17
VA30 -- <i>other</i>	9	10-11	12
P10 -- <i>Staphylococcus</i>	28	-	29
P10 -- <i>Enterococcus</i>	14	-	15
P10 -- <i>Streptococcus</i>	19	20-27	28
P10 -- <i>other</i>	19	-	20
TE30	14	15-18	19
FOX30	14	15-17	18
SXT25	10	11-15	16
NA30	13	14-18	19
W5	10	11-15	16

E-test:

Observe each zone of inhibition on the plate and determine where the smallest part of the ellipse intersects with the E-strip, as illustrated below. Use the measurements marked on the strip to determine the minimal inhibitory concentration (MIC) of the antibiotic for the microorganism tested and use the chart below to determine if the MIC indicates susceptibility or resistance.

	MIC Breakpoint	
	MIC $\mu\text{g}/\text{ml}$	ml
	S $\leq$	R $>$
Ampicillin (AM)	8	8
Ciprofloxacin (CI)	0.5	1
Daptomycin (DPC)	1	1
Imipenem (IP)	2	8
Tetracycline (TC)	1	2
Tigecycline (TGC)	1	2
Trimethoprim & Sulfa (TS)	2	4



MICs:

Antibiotic	MIC (mcg/ml)	Susceptible/ Intermediate/ Resistant

**STUDY QUESTIONS:**

1. Compare and contrast the Disk Diffusion and Epsilon tests for determining antibiotic susceptibility. Is one better than the other? Why or why not?

2. Experimental results indicate that antibiotics such as streptomycin, tetracycline, and chloramphenicol effectively inhibit the growth of prokaryotic cells, but have little effect on eukaryotic cells such as fungi and protozoa. Based on your knowledge of the mechanism of action of these particular antibiotics, why do you think this is the case? (*Hint: Consult your textbook to review mechanisms of action of the different classes of antibiotics.*)

3. Distinguish between broad spectrum and narrow spectrum antibiotics, and give an example of a clinical situation where each type of antibiotic might be prescribed.

4. Discuss some of the common side effects of antibiotics.

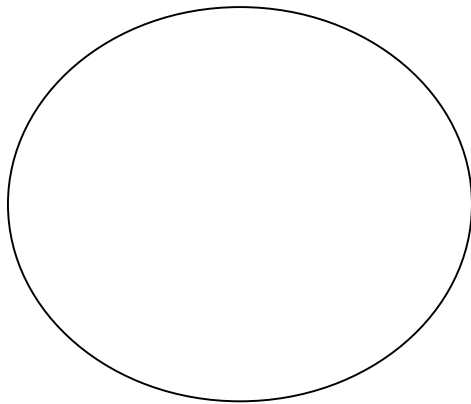
**EXERCISE #16: ADDITIVE AND SYNERGISTIC EFFECTS OF ANTIBIOTICS (DEMO)**

Antibiotics are often used in combination with each other in treatment of bacterial diseases. For example, treatment of tuberculosis often involves a cocktail of antibiotics including rifampin, isoniazid, and/or ethambutol. The goal of antibiotic combination therapy is to (a) reduce the incidence of antibiotic-resistance (b) allow lower doses of antibiotics to be used and/or (c) enhance the agents' antibacterial activity. Antibiotics used in combination can have either an additive effect or a synergistic effect. An **additive effect** means that the overall effect of the antibiotic combination is no greater than the sum of the individual antibiotics. A **synergistic effect** means that the overall effect of the antibiotic combination is greater than the sum of the individual antibiotics. In synergism, the antibiotics enhance the effect of each other.

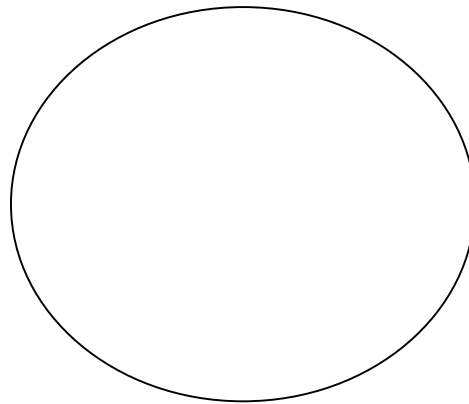
Additive and synergistic effects of drug combinations can be determined in the lab by placing the two antibiotic discs on a lawn of cells plated on Mueller-Hinton agar. If each disk has its own uniform zone of inhibition, the effect of the two antibiotics is additive. If the zones of inhibition for the antibiotics merge in the middle, the effect is synergistic. Demonstrations of additive and synergistic effects will be available on the front table during the results lab session.

**RESULTS:**

Draw the results of the additive and synergistic combinations of drugs:



Additive



Synergistic

## EXERCISE #17: MICROBIOTA OF THE MOUTH

That “fuzzy” feeling that can be felt on unbrushed teeth is an accumulation of a biofilm of bacteria. There are several bacteria within a person’s mouth which make up this biofilm and are **cariogenic**, meaning they have a tendency to cause **dental caries**. These include *Lactobacillus acidophilus*, *Streptococcus mutans* (and *S. gordonii*, *S. salivarius*), and *Actinomyces odontolyticus*. These organisms are able to ferment carbohydrates to lactic acid, thus lowering the pH at the surface of the enamel which can **decalcify** the enamel and lead to cavities. Enamel decalcification will begin with a pH of 5.5 and accelerates as the pH reaches 4.4. For example, *S. mutans* produces the enzyme **dextranucrase**, which polymerizes sucrose to form a polysaccharide called **glucan**. *L. acidophilus* produces a similar polymer called **lexan**. Glucan and lexan are both **glycocalyxes** which bind tightly to teeth, allowing the bacteria to form a biofilm. The bacteria then produce acids as a product of carbohydrate fermentation.

The Snyder Test is used to determine a person’s susceptibility to dental caries based on acid production that is assumed to be a result of the growth of lactobacilli on the teeth or in other areas of the mouth. (Note that the Snyder Test does not test for cariogenic streptococci or actinomycetes.) The Snyder Test agar contains **2% glucose** and has a pH of about 4.8. This low pH inhibits the growth of most organisms, but it is ideal for lactobacilli. If growth occurs, and lactic acid is produced by the lactobacilli, the pH will drop below 4.8 to about 4.4. The indicator used to show this change in pH is **bromocresol green**, which will change from green to yellow upon acid production. The rate of change is another factor to be considered, as it is an indication of the number of acid-producing organisms present (i.e. the faster the rate of change, the more organisms present). The extent to which a culture changes color following incubation indicates a person’s susceptibility to the formation of dental caries:

Color of Agar	Green	Light Green	Yellow-Green	Yellow
Susceptibility	Slight	Mild	Moderate	High

Alternatively, the tubes can be read at different time intervals and the amount of time it takes for the tube to turn yellow will correlate with an individual’s susceptibility to dental caries:

24 hours = very high susceptibility

48 hours = high susceptibility

72 hours = moderate susceptibility

### **MEDIA NEEDED:** (*per student*)

1 Snyder deep agar tube

1 Sterile empty tube or beaker

### **CULTURES NEEDED:** Saliva from student’s oral cavity

### **PROCEDURE:**

1. Obtain a sterile test tube or small beaker. Collect approximately  $\frac{1}{4}$  to  $\frac{1}{2}$  inch of saliva in the test tube or beaker.
2. Obtain a melted Snyder Agar deep tube that has been tempered in a 45 °C water bath.
3. While the Snyder Agar deep tube is still liquefied, aseptically pipette 0.2 ml of saliva into the tube.



4. Place the cap *loosely* on the tube and roll the tube gently between the palms of your hands. Let solidify.
5. Incubate the Snyder tube at 37 °C for 48 hours.

**RESULTS:**

Examine Snyder deep agar tubes from the previous lab period for a change in the color of the culture medium. Record your results in the table below for yourself and your lab partners:

Tube Identification (name or initials)	Color of test culture	Caries susceptibility? (Slight, Mild, Moderate, Severe)

Based on **your** results, what is your tendency to form dental caries?

**STUDY QUESTIONS:**

1. Explain how the Snyder agar medium is both a selective and a differential medium. How is the differential nature of the medium used to detect susceptibility to dental caries?

2. Explain how resident mouth microbiota are responsible for the formation of dental caries. Are all members of resident mouth microbiota capable of initiating dental caries?

## EXERCISE #18: NORMAL MICROBIOTA OF THE THROAT AND SKIN

In healthy humans, the internal organs and tissues such as muscles, the brain, and blood do not contain microorganisms. However, surface tissues, such as the skin and mucous membranes, are in continuous contact with environmental microbes and become readily colonized by specific bacteria. The population of microbes regularly found in the body is referred to as the **normal microbiota**. The term **transient microbiota** refers to members of the normal microbiota that are present for only a short time before disappearing. A person's normal microbiota is an important part of the immune system, as the normal microbiota often inhibit pathogenic microbes from colonizing the host, a process called **microbial antagonism**. Different types of bacteria will colonize different niches in a person's body due to variations in moisture level, pH, atmospheric pressure, oxygen levels, and body secretions. Accordingly, different types of media must be used to culture the various human microbiota.

### Streptococcus

Members of the genus *Streptococcus* are often found as normal microbiota of the mouth and throat. *S. mutans*, *S. gordonii*, and *S. salivarius* are examples of resident mouth microbiota. *S. pyogenes* (one of the Group A Strep) is found in approximately 5-15% of normal individuals as part of the normal throat microbiota. **Blood agar** is typically used to cultivate and differentiate among *Streptococcus* species. Since streptococci are **capnophiles**, blood plates containing these microbes should be incubated in the presence of carbon dioxide.

### Neisseria /Moraxella

Members of the genus *Neisseria* are fastidious organisms that require special media and conditions for growth. *Neisseria* are typically cultivated on **chocolate agar**, which contains **lysed (boiled) red blood cells** and provides the nutrients and growth factors needed for these organisms. *Neisseria* are also capnophiles, so chocolate plates containing *Neisseria* should be incubated in the presence of carbon dioxide. Many saprophytic strains of *Neisseria* are normal microbiota of the mouth and throat. In addition, the pathogen *Neisseria meningitidis* is found in approximately 20-40% of young adults as a normal resident of the nasopharynx. Members of the genus *Moraxella*, which are closely related to *Neisseria*, will also grow on chocolate agar. Like *Neisseria*, *Moraxella* are also common microbiota of the nasopharynx, particularly in children and young adults.

Since chocolate agar is not a selective medium, *Neisseria* and *Moraxella* must be identified using the oxidase test, which tests for the presence of *cytochrome c* in the electron transport chain. In this test, a drop of **oxidase reagent** is added to growth on the agar plate, and a purple-black coloration that appears within 30 seconds signifies a positive reaction.

### Diphtheroids

Diphtheroids are species of the genus *Corynebacterium* that are non-pathogenic and are part of normal skin microbiota. Diphtheroids are cultivated on **Mueller-Hinton Tellurite (MHT)** plates. If Diphtheroids are present, they will produce gray to black colonies because the **tellurite** in the media is reduced intracellularly to tellurium, which appears as a gray precipitate.

### Staphylococcus

*Staphylococcus epidermidis* and *Staphylococcus aureus* are normal microbiota of the skin. *S. aureus* tends to be found in moist environments such as the nostrils and armpits, whereas *S. epidermidis* is usually found in drier areas of the skin. *S. epidermidis* is typically non-pathogenic, whereas *S. aureus* is widely known as a troublesome opportunistic pathogen.

Approximately 20% of healthy people are permanently colonized with *S. aureus*, and 60% of healthy people are transient carriers of *S. aureus*. Recent studies have also shown that anywhere from 1-4% of the U.S. population is colonized with methicillin-resistant *S. aureus* (MRSA), with a few studies indicating that the MRSA colonization rate may be as high as 10%. Mannitol salt agar (MSA) is used to select for both of these members of the *Staphylococcus* genus. MSA also allows differentiation between *S. aureus* and *S. epidermidis* because *S. aureus* will ferment mannitol to produce a golden-yellow colony growth, whereas *S. epidermidis* will not. To determine if *S. aureus* strains are methicillin-resistant, specialized selective media such as HardyCHROM™ MRSA agar is used. Due to proprietary selective and differential ingredients in the medium, MRSA will grow as magenta colored colonies on HardyCHROM™ MRSA plates.

### **Environmental Organisms**

Many of the microbes found in the environment are fungi such as yeasts and molds. **Sabouraud** agar is an **acidic** medium (pH 5.5) which is used to cultivate acidophiles such as fungi from environmental specimens. Other environmental specimens which are not acidophiles can be cultured on nutrient agar.

#### **MEDIA NEEDED:** (1 each per *pair*)

Blood agar plate  
Mannitol salt plate  
Chocolate agar plate  
Sabouraud agar plate  
M-H Tellurite plate  
Nutrient agar plate  
0.85% saline tube  
HardyCHROM™ MRSA plates (*optional*)

#### **CULTURES NEEDED:**

Swabs of throat  
Swabs of skin or nose  
Swab of ears or palms  
Swabs of environment

#### **PROCEDURE:**

1. Using a permanent marker, divide the blood plate, chocolate agar plate, Mannitol Salt Agar (MSA) plate and Mueller-Hinton-Tellurite (MHT) plate in half so that each lab partner will use half of each plate. Label the halves with your names or initials.
2. Swab the back of your lab partner's throat with a sterile swab (or you may swab your own throat, if you prefer) and rub onto the appropriate sector of the blood agar plate in a zig-zag pattern. Swab the throat again with a fresh swab, and rub onto the appropriate sector of the chocolate agar plate. Switch roles and swab the other person's throat with fresh sterile swabs and rub onto the other halves of the blood and chocolate agar plates. Tape the plates, and place in an Anaeropak box with an activated CO<sub>2</sub> generator.
3. Using a fresh swab, swab anywhere on your skin or up in your nostril and rub onto your half of the MSA plate. Your lab partner should do the same.
4. Using a fresh swab, swab either your ears or the center of your palms and rub onto your half of the MHT agar plate. Your lab partner should do the same.
5. Swab anywhere in the environment (bathroom door, toilet seat, door handle, water fountain, etc.) and rub onto a Sabouraud agar plate. You may want to moisten your swab with sterile saline prior to taking your environmental sample. (Note: if you

- want to divide this plate in half and obtain two environmental samples, that is fine.)  
**Repeat**, this time inoculate the environmental specimens onto a nutrient agar plate.
6. *Optional*: If your instructor has provided HardyCHROM™ MRSA plates:
    - a.) The larger plate is to test if you carry MRSA as part of your normal microbiota. Use a Sharpie to divide the plate in half, labelling one half with your initials and the other with your lab partner's initials. Using a sterile cotton swab, each student should sample the inside of their nostril and inoculate their portion of the plate in a zigzag motion.
    - b.) The smaller plate is a **contact plate** and is designed to detect MRSA on environmental surfaces. To you use this plate, simply remove the lid and press the agar firmly on the surface that you wish to sample. *S. aureus*, including MRSA, is often spread via indirect contact, so when deciding what to sample, think about surfaces that might be repeatedly touched by a lot of different people!
  7. Tape all plates on both sides, invert, and incubate at 37°C for 48 hours.
  8. **ALL used swabs are to be discarded immediately in the biohazard waste!**

#### RESULTS:

1. DO NOT OPEN THE BLOOD AGAR PLATE! Examine the blood agar plate cultures for zones of hemolysis.
2. Open the lid of the chocolate plate just enough to add a drop **oxidase** reagent to the growth on plate. Close immediately. A purplish color that develops within 30 seconds on the surface of any of the colonies indicates the presence of *Neisseria/Moraxella*.
3. Examine the Mueller-Hinton Tellurite plate for black diphtheroid colonies.
4. DO NOT OPEN THE MANNITOL SALT PLATE! Examine the mannitol salt agar plate for the presence of growth of *Staphylococcus*. What color is the media surrounding the growth? A **yellow** color indicates *S. aureus*, which has fermented the mannitol and lowered the pH of media. No color change indicates *S. epidermidis*.
5. Environmental Samples -- Examine Sabouraud agar plates for growth. Identify mold vs. yeast growth on the plate. Examine the nutrient agar plate for growth. Compare the growth visible on the Sabouraud and nutrient plates.
6. DO NOT OPEN THE HardyCHROM™ MRSA PLATES! Examine the plates for the presence of magenta-colored colonies, which indicate the presence of methicillin-resistant *S. aureus*. (Note: any growth that is not magenta is something other than MRSA.)

Record all results in the table below:

Specimen	Observations and Results
Blood agar: type(s) of hemolysis Types of organisms present?	
Chocolate agar: purple colonies? Types of organisms present?	
Mueller-Hinton-Tellurite agar (+) or (-) black colonies Types of organisms present?	
Sabouraud Agar (Environmental sample)	
Nutrient Agar (Environmental sample)	
Mannitol Salt Agar (+) or (-) growth Color of medium Types of organisms present?	
HardyCHROM MRSA plates (+) or (-) magenta colonies Types of organisms present?	
HardyCHROM MRSA contact plates (Environmental sample) (+) or (-) magenta colonies Types of organisms present?	

**STUDY QUESTIONS:**

1. What is meant by the term “normal microbiota”? Are these organisms harmful or beneficial to their host? Why?

2. Even if all students are healthy, why is it still advisable NOT to open the blood plates on which throat specimens are grown or the MSA plates or HardyCHROM MRSA plates on which skin/environmental specimens are grown?

3. Compare the growth from the environmental samples inoculated onto the Sabouraud and Nutrient Agar plates. Are there any differences? Would you expect there to be a difference? Why or why not?

## **EXERCISE #19: CULTIVATION OF URINE SPECIMENS**

Urine from the urinary bladder should be sterile! The act of voiding or **micturation** helps to rid the lower urethra orifice of any bacteria that may try to ascend into the urinary bladder. Bacteriuria, the presence of bacteria in the urine, can indicate a urinary tract infection, called a UTI. Such infections include urethritis, cystitis, and pyelonephritis. **Urethritis** is an infection involving only the urethra. **Cystitis** is an infection which involves the urinary bladder. **Pyelonephritis** is an infection involving the kidneys, and tends to be a more severe infection, often resulting in the destruction of renal corpuscles. Bacteria that commonly cause UTIs include *Escherichia coli* (primary culprit), *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis* and *P. vulgaris*, as well as *Pseudomonas aeruginosa* and *Salmonella* and *Shigella* species.

The first step usually taken to diagnose a UTI is a dipstick test, which detects the presence of leukocyte esterase and nitrate reductase activity associated with these types of infections. If the dipstick test is positive, the next step in diagnosis of a UTI is often the examination of a urine specimen for the presence of bacteria. Samples may be taken aseptically using a needle for cystocentesis or a clean sample using a sterile catheter. More commonly, a “clean catch” voided sample is obtained. In this procedure, the urine sample is collected **midstream** following adequate cleansing of the external genitalia. It is crucial to process fresh, unrefrigerated urine samples immediately to avoid overgrowth of normal genital microbiota resulting in false positive results.

There are different types of agar plates that may be inoculated from a patient sample depending on what type of information is desired. Non-selective/ non-differential media such as nutrient agar is often used to simply determine the total number of bacteria in a urine sample. In order to tentatively identify specific organisms, a differential media such as HardyCHROM™ UTI agar must be used. The HardyCHROM™ UTI agar is marketed by Hardy Diagnostics as “a non-selective chromogenic medium recommended for the cultivation, differentiation, and enumeration of urinary tract pathogens based on colony color and morphology”. The ingredients include peptones and a proprietary blend of chromogenic substrates and indicators that cause different types of microorganisms to produce different colored colonies on the agar..

### **MEDIA NEEDED:**

- 1 Nutrient Agar plate
- 1 HardyCHROM™ UTI agar plate (*optional – check with your instructor*)

### **CULTURES NEEDED:**

Student urine specimen (voluntary only)

### **PROCEDURE:**

1. Students should collect their own midstream urine sample in a sterile specimen cup.
2. Using a sterile 10 µl plastic calibrated loop, dip the loop into the urine and streak onto the surface of the nutrient agar plate. Incubate at 37 °C for 48 hours.
3. Using a new sterile 10 µl plastic calibrated loop, dip the loop into the urine and streak onto the surface of the HardyCHROM™ UTI agar plate. Incubate at 37 °C for 48 hours.
4. Discard the used loop in the biohazard waste.
5. Follow your instructor’s directions for disposing of remaining urine, and discard the specimen cup in the biohazard waste.

## RESULTS:

### Nutrient Agar Plate:

Examine the urine culture plate for the presence of colonies. Count how many bacterial colonies are present on the nutrient agar plate, and calculate the number of CFU/ml by dividing the number of colonies by the size of the calibrated loop, which is 0.01 ml:

$$\frac{\# \text{ of colonies}}{0.01 \text{ ml}} = \frac{\text{CFU}}{\text{ml}}$$

Does there seem to be only one colony type, or are multiple colony types represented?

**What do the numbers mean?** The critical threshold between bacterial infection and simple bacterial contamination from a clean catch urine sample is usually considered to be  $1 \times 10^5$  bacteria per ml of urine. Traditionally, a UTI is diagnosed when the number of bacteria present in the urine is greater than  $1 \times 10^5$  per ml, **and** the patient presents with symptoms consistent with a UTI. Some recent studies suggest, however, that lower count bacteriuria ranging from  $1 \times 10^2$  to  $1 \times 10^4$  bacteria per ml of urine may indicate an early phase of UTI.

### HardyCHROM™ UTI plate:

Examine the urine culture plate and note the color of each colony type present on the plate. Using the table below, determine the type(s) of organisms tentatively present in the sample.

Colony Color	Colony Size	Types of Organisms(s)	Present on plate? Y/N
Rose/magenta	Medium-large	<i>Escherichia coli</i>	
Dark blue/indigo	Large	<i>Klebsiella, Enterobacter, Serratia</i>	
Teal/turquoise	Small	<i>Enterococcus</i>	
Clear/light yellow with orange halo	n/a	<i>Proteus, Morganella, Providencia</i>	
Blue/green with orange halo	n/a	<i>Proteus vulgaris</i>	
Opaque cream/white	Small-medium	<i>Staphylococcus aureus</i> *	
Opaque pink	Medium	<i>Staphylococcus saprophyticus</i> *	
White	Very small	<i>Staphylococcus epidermidis</i> *	
Light yellow/green/colorless	Medium-large	<i>Pseudomonas</i>	
Opaque white moist colonies	Small	<i>Candida</i> *	
Dark blue with pink halo	Small-medium	<i>Citrobacter</i>	

\*For these organisms, further tests must be used to confirm their identification in a clinical setting.



## **STUDY QUESTIONS:**

1. How accurate is a lab analysis of a 24-hour, unrefrigerated, non-midstream urine culture? Explain.
2. Why do you think that further tests are needed to confirm the identification of some of these organisms?
3. Critical Thinking Question: Why do you think different species of bacteria produce different colored colonies on the HardyCHROM UTI agar?
4. Critical Thinking Question: What do you think are some of the limitations of the HardyCHROM™ UTI agar?

## **EXERCISE #20: IDENTIFICATION OF BACILLUS SPECIES**

*Bacillus* represents a genus of rod-shaped, Gram-positive bacteria which are ubiquitous in nature, and are commonly found in places such as soil, water, and airborne dust. All *Bacillus* species are able to perform aerobic respiration – some are facultative and some are strict aerobes. Most *Bacillus* species produce capsules, and all are capable of motility by peritrichous flagella. A unique characteristic of this genus is its ability to produce endospores when environmental conditions are stressful. Some *Bacillus* species are natural flora in the human intestines, and other *Bacillus* species are ecologically important as insect pathogens. Several species of *Bacillus* are antibiotic producers, including *B. brevis*, *B. cereus*, *B. licheniformis*, *B. polymyxa*, and *B. subtilis*.

Although most species of *Bacillus* are harmless saprophytes, two species are considered medically significant: *Bacillus anthracis* and *Bacillus cereus*. *B. anthracis* is the causative agent of anthrax, which occurs in two main forms in humans: pulmonary anthrax and cutaneous anthrax. *B. cereus* is a cause of food poisoning associated with dried foods such as rice and beans.

In order to differentiate between *Bacillus* species, several microbiological tests are used including an assay for the presence of extracellular enzymes, growth and fermentation on mannitol salt agar, nitrate reduction, citrate utilization, and esculin hydrolysis.

### **EXTRACELLULAR ENZYMES:**

Bacteria secrete extracellular enzymes which are responsible for a variety of enzymatic activities. Among them are enzymes which hydrolyze substrates such as starch, lipids, and proteins. We will be assaying for these enzymatic activities using the following kinds of **differential** media.

#### **Starch media:**

Starch media contains the polysaccharide **starch** as a substrate. Starch is a polymer of many glucose monomers joined together in glycosidic linkages. *Amylase* is the enzyme that breaks the starch down into smaller subunits of maltose (a disaccharide). Another enzyme, *maltase*, then catabolizes the maltose into glucose (a monosaccharide). Organisms which hydrolyze starch will show a clear zone in the starch agar around the growth of the microorganism following the addition of iodine to the media.

#### **Lipid media:**

Lipid media contains the lipid substrate **Tributylin**. Lipids are degraded by an enzyme called *lipase*, which breaks the ester bonds of triglyceride molecules to produce glycerol and fatty acids. Organisms which hydrolyze lipids will show a clear zone in the lipid agar around the growth of the microorganism.

#### **Casein media:**

Casein media contains the substrate **casein**, which is a major milk protein. Proteins are polymers of amino acids joined together by peptide bonds. Hydrolysis of proteins is referred to as proteolysis. Enzymes which degrade casein include *protease* and *casease*. In proteolysis of casein, the protein is broken into peptones, then into polypeptides, then into dipeptides, and finally into amino acids. This is known as the “Peptonization Process”. Organisms which hydrolyze casein will show a clear zone in the casein agar around the growth of the microorganism.

### Gelatin media:

Gelatin media contains the substrate **gelatin**, which is a protein produced by the hydrolysis of collagen. Gelatin is degraded by the enzyme *gelatinase* into its amino acid components. Organisms which hydrolyze gelatin will cause the gelatin to liquefy.

### OTHER TESTS:

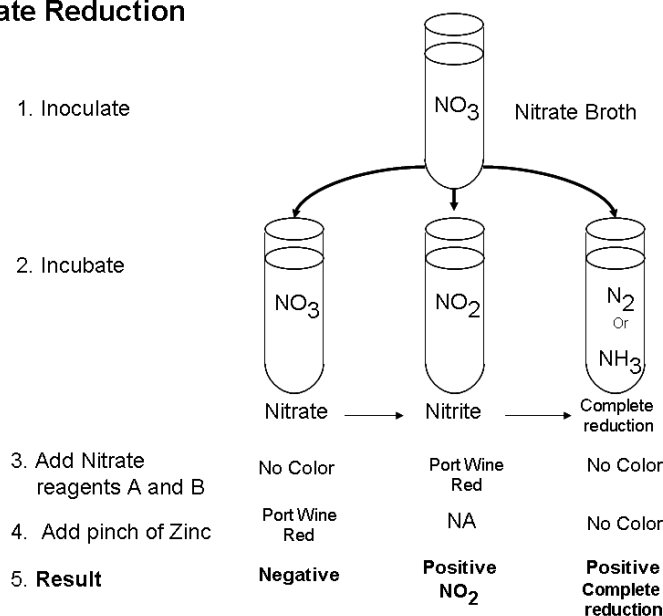
#### Mannitol Salt Agar:

MSA is both selective and differential. It contains 7.5% NaCl, which selects for organisms which are halotolerant. The media also contains mannitol and phenol red, which allows differentiation of organisms based on whether or not they are able to ferment mannitol. If mannitol is fermented, the acidic fermentation products react with the phenol red pH indicator, which changes color from red to yellow.

#### Nitrate Reduction:

Some organisms possess an enzyme called *nitrate reductase* which enables them to anaerobically reduce nitrate into nitrite. Some of these organisms can then further reduce the nitrite to ammonia or completely to molecular nitrogen. Nitrate broth tubes contain beef extract, peptone, and nitrate. After incubation of an organism in a nitrate broth tube, reagents are added to determine if the nitrate has been reduced, and if so, to what extent. Nitrate Reagent A, containing sulfanilic acid, is added to the test tube, followed by addition of Nitrate Reagent B, containing alpha-naphthylamine. Formation of a red color indicates the presence of nitrites, indicating the organism has reduced the nitrate to nitrite and is **positive** for nitrate reduction. If no color appears, a pinch of zinc is added to the tube. If a red color appears, the organism is **negative** for nitrate reduction, because the zinc has reduced the nitrate to nitrite, causing the red color. However, if there is still no color change after the addition of zinc, this indicates the nitrates were reduced beyond nitrites to ammonia or molecular nitrogen, and the organism is **positive** for nitrate reduction.

#### Nitrate Reduction



**Citrate Utilization:**

The purpose of this test is to determine if an organism can use citrate as a carbon source. The citrate agar slant contains sodium citrate, bromthymol blue (pH indicator), sodium, and water. If the organism is able to utilize citrate, an enzyme called *citruse* will break the citrate into oxaloacetic acid and acetic acid. The oxaloacetic acid is then broken down into pyruvate and the acetic acid is converted to CO<sub>2</sub>. The CO<sub>2</sub> reacts with the water and sodium in the media to produce alkaline sodium carbonate. The sodium carbonate reacts with the pH indicator to produce a “Prussian blue” color.

**Bile esculin agar (BEA):**

BEA is both selective and differential. It contains bile salts, esculin, ferric ammonium citrate, and beef and gelatin extracts. The bile salts give BEA its selective property, because only some organisms are able to grow in the presence of bile. The esculin and ferric ammonium citrate give BEA its differential property, because it distinguishes those organisms which are able to hydrolyze the carbohydrate esculin. When esculin is hydrolyzed to glucose and esculetin, the esculetin reacts with ferric ammonium citrate to form a blackish precipitate. The beef and gelatin extracts provide macromolecules for general growth requirements.

**MEDIA NEEDED:** (*per student*)

- 1 Tri-plate made of starch agar, lipid agar, and casein agar
- 1 Mannitol salt agar (MSA) mini-plate
- 1 Gelatin deep tube
- 1 Bile esculin tube
- 1 Citrate agar slant
- 1 Nitrate broth tube

**CULTURES NEEDED:**

Each student will select one of the following:

*Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus sphaericus*, *Bacillus licheniformis*, *Bacillus brevis*, or *Bacillus polymyxa*.

(Your instructor may supply additional *Bacillus* species for you to choose from.)

**PROCEDURE:**

1. Using a straight line streak, inoculate each portion of the tri-plate with your *Bacillus*.
2. Using a straight line streak, inoculate the MSA mini-plate with your *Bacillus*.
3. Using a sterile **needle**, inoculate the gelatin deep tube with your *Bacillus* by stabbing into the gelatin. Make sure to replace the cap *loosely*.
4. Using a sterile loop, inoculate the nitrate broth with your *Bacillus*.
5. Inoculate the citrate agar slant with your *Bacillus* by streaking the surface of the slant with a sterile loop in a zig-zag motion. Do not stab the butt!
6. Using a sterile loop, streak the Bile Esculin slant tube with your *Bacillus*.
7. Make sure all plates are taped on both sides, and all test tube lids are loose but secure. Make sure all plates and tubes are labeled with your name and the name of the organism. Incubate all plates and tubes at 30 °C for 48 hours.

**RESULTS:**

1. Observe your tri-plates for the following reactions:
  - a. Positive starch hydrolysis – clear zone around bacterial growth upon addition of iodine
  - b. Negative starch hydrolysis– blackish blue color upon addition of iodine indicates presence of starch (meaning starch has not been hydrolyzed)
  - c. Positive lipid hydrolysis – clear zone around bacterial growth
  - d. Positive casein – clear zone around bacterial growth
2. Observe your mini MSA plate for growth and mannitol fermentation (yellow).
3. Observe your gelatin deeps for a positive or negative reaction. Positive gelatin hydrolysis is indicated by liquefaction of the gelatin at room temperature.
4. Analyze your nitrate broth tube to determine if nitrate reduction has occurred:
  - a. Add **5 drops** of Nitrate Reagent A to the broth tube and mix thoroughly. Next, add 5 drops of Nitrate Reagent B and mix. If a red color is produced, this indicates a positive result for nitrate reduction. This means the organism has reduced the nitrate to nitrite.
  - b. If no red color is produced, add a pinch of Zinc and mix thoroughly. Allow to sit for several minutes. If the broth turns red, this indicates that the zinc has reduced the nitrates and the organism is negative for nitrate reduction.
  - c. If the addition of zinc does NOT cause a color reaction, the test is positive. This means that the organism has reduced the nitrate to nitrites and then reduced the nitrites to ammonia or molecular nitrogen.
5. Observe your citrate slant for color and bacterial growth. Growth and a blue color indicate a positive result, which means the organism is able to use citrate as a carbon source.
6. Observe your bile esculin tube for esculin hydrolysis (black precipitate throughout agar).
7. Record your results below or on the chart provided by your lab instructor.

Bacillus species	MSA Growth	MSA Fermentation	Nitrate	Citrate	Bile Esculin	Starch	Lipid	Casein	Gelatin

**STUDY QUESTIONS:**

1. Why is it important to be able to determine the identity of species of *Bacillus* in the laboratory?

2. Critical Thinking Question: Why do you think soil-dwelling organisms such as *Bacillus* possess extracellular enzymes such as *lipase*, *proteases*, *casease* and *gelatinase*?

## EXERCISE #21: TRANSFORMATION

Transformation refers to the ability of a microorganism to take up free DNA from the environment, and incorporate that DNA into their genome. Cells that have the capacity to do this are referred to as **competent cells**. Some genera of bacteria are naturally competent, such as *Streptococcus*, *Bacillus*, *Haemophilus*, and *Pseudomonas*. Those genera which are not naturally competent can be manipulated in the laboratory to become **artificially competent**. Artificial competency is typically induced using chemicals such as calcium chloride to increase the permeability of the cells. The most commonly used artificially competent species is the genetic workhorse *Escherichia coli*, which is an indispensable asset to the fields of genetic engineering and biotechnology.

Plasmids are often used as vectors in transformation experiments. A **vector** is a piece of DNA that delivers a gene to a recipient cell. Plasmids are small, covalently closed circular pieces of extrachromosomal DNA that replicate independently of the chromosome in a bacterial cell. Plasmid vectors are typically engineered to contain two things: (1) a **selectable marker** such as an antibiotic-resistance gene to allow selection of transformed cells and (2) a **multiple cloning site** where a gene of interest can be inserted.

The success of a transformation experiment is determined by calculating the **transformation efficiency**, which is simply the number of transformants obtained per microgram of DNA. For example, if 2 ng of plasmid DNA was transformed into competent *E. coli*, and 237 transformants resulted, the transformation efficiency would be calculated as follows:

Step 1: Convert nanograms to micrograms

$$2 \text{ ng} = 0.002 \text{ }\mu\text{g}$$

Step 2: Divide the # of transformants by the amount of DNA

$$237 / 0.002 \text{ }\mu\text{g} = 118,500 \text{ transformants}/\mu\text{g DNA} = 1.19 \times 10^5 \text{ transformants}/\mu\text{g DNA}$$

The purpose of today's exercise is to transform the plasmid **pGREEN** into chemically competent *E. coli* cells. The selectable marker on the pGREEN plasmid is a beta-lactamase gene which confers resistance to the antibiotic ampicillin. The pGREEN plasmid also has a gene encoding a green fluorescent protein inserted into the cloning site. Recipient cells that are successfully transformed with the pGREEN plasmid will be ampicillin-resistant and will produce the green fluorescent protein, giving them a green coloration which is visible under ambient light conditions, and will fluoresce under UV light.

### **MEDIA NEEDED:** (*per group of 4*)

2- LB plates containing Ampicillin (LB-amp)

2- LB plates

LB broth

Ice-cold Calcium chloride ( $\text{CaCl}_2$ )

### **CULTURES NEEDED:**

*Escherichia coli* competent cells

pGREEN plasmid DNA (5 ng/ $\mu\text{l}$ ), on ice

**PROCEDURE:**

1. Label one of the LB-amp plates “Before” and the other LB-amp plate “After”. Label one of the LB plates “Before” and the other LB plate “After”.
2. Pre-warm all 4 plates and the small tube of LB broth at 37 °C for 30 min.
3. Label an empty microfuge tube as “Tube A”. Pipet 250 µl of ice-cold CaCl<sub>2</sub> into Tube A. Place Tube A on ice.
4. Use a sterile loop to transfer an isolated colony of *E. coli* to Tube A. Mix the bacteria with the CaCl<sub>2</sub> until no visible clumps remain in the tube. The CaCl<sub>2</sub> will increase the permeability of the bacterial cells, making them “competent” to take up DNA. Thus, they are now referred to as “competent cells”. Put Tube A back on ice.
5. Using a sterile loop, streak a loopful of the competent cells from Tube A onto the LB plate labeled “Before”.
6. Using a sterile loop, streak a loopful of the competent cells from Tube A onto the LB-amp plate labeled “Before”.
7. Pipet 10 µl of the pGREEN plasmid solution into Tube A, and mix by tapping gently.
8. Incubate Tube A on ice for 15 minutes.
9. Heat-shock the cells by placing Tube A in a 42 °C water bath for 90 seconds. Place the tube immediately back on ice.
10. Add 250 µl of pre-warmed LB broth to Tube A. Incubate the tube at 37 °C for 10 minutes.
11. Using a sterile disposable spreader, spread 100 µl of cells from Tube A on the pre-warmed LB-amp plate labeled “After”.
12. Using a sterile loop, streak a loopful of the cells from Tube A onto the LB plate labeled “After”.
13. Incubate all plates overnight at 37 °C.

**RESULTS:**

1. Examine the LB and LB-amp plates for growth and pigmentation, and record your results in the table below:

	Growth on LB	Growth on LB-amp	Pigmentation
“Before”			
“After”			

2. Count the number of colonies on the LB-amp “After” plate. \_\_\_\_\_
3. Calculate the transformation efficiency:



**STUDY QUESTIONS:**

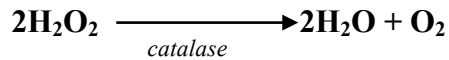
1. Was there any growth on the LB-amp “Before” plate? Was there any growth on the LB-amp “After” plate? Explain these results.

2. Why is it important to streak the cells on non-selective media (i.e. plain nutrient agar) both before and after the transformation procedure?

3. Explain how natural transformation could lead to the spread of antibiotic resistance in pathogenic bacteria.

## EXERCISE #22: CATALASE TEST

*Staphylococcus* species produce an enzyme called *catalase*, which reacts with the toxic oxygen by-product hydrogen peroxide ( $H_2O_2$ ), breaking it down into water ( $H_2O$ ) and molecular oxygen ( $O_2$ ).



*Streptococcus* and *Enterococcus* species do not produce *catalase* (although they produce *superoxide dismutase* and *peroxidase*). Therefore, the *catalase* test is widely used to distinguish *Staphylococcus* from *Streptococcus* and *Enterococcus*.

### CULTURES NEEDED:

Your *Staphylococcus* and *Streptococcus/Enterococcus* cultures from Exercises 22 and 23.

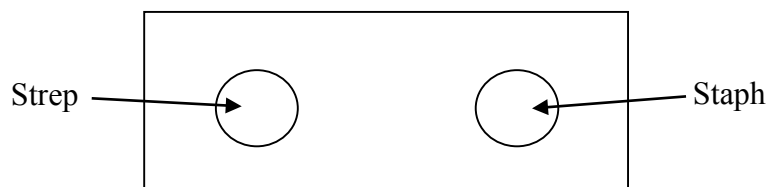
### PROCEDURE:

1. Drop 2 small drops of 3% hydrogen peroxide onto a clean glass slide. Using a sterile loop, put a loopful of the *Staphylococcus* organism into one of the drops. Observe for a bubbling reaction. (The bubbles are the molecular oxygen that is produced as the *catalase* breaks down the hydrogen peroxide.)

Catalase-positive: bubbles observed

Catalase-negative: no bubbles

2. Repeat with *Streptococcus* organism using a sterile loop to put a loopful of the bacteria into the other drop of hydrogen peroxide.



### RESULTS:..

Organism: \_\_\_\_\_ Catalase Reaction: + or - (circle one)

Organism: \_\_\_\_\_ Catalase Reaction: + or - (circle one)



### EXERCISE #23: STREPTOCOCCUS IDENTIFICATION

Members of the genus *Streptococcus* are Gram-positive cocci of significant clinical importance. Due to similarities between the genera *Streptococcus* and *Enterococcus*, members of the *Enterococcus* genus are grouped into the same family as the *Streptococcus* species. Streptococcal species are divided into groups based on their **Lancefield classification**, which categorizes streptococci based on similar surface antigens. Most of the clinically important streptococci fall in the following groups:

Lancefield Group	Species	Significant Human Diseases
A	<i>S. pyogenes</i>	Acute pharyngitis, scarlet fever, impetigo Necrotizing fasciitis Glomerulonephritis, rheumatic fever
B	<i>S. agalactiae</i>	Neonatal meningitis, pneumonia, bacteremia Puerperal fever
C	<i>S. equi</i>	Veterinary pathogen (equine strangles)
D	<i>E. faecalis</i> <i>S. bovis</i>	Urinary tract infections, Biliary tract infections
K	<i>S. salivarius</i>	Dental caries, endocarditis
Viridans (no Lancefield antigens)	<i>S. mutans</i> <i>S. mitis</i>	Dental caries, endocarditis
Pneumococci (no Lancefield antigens)	<i>S. pneumoniae</i>	Pneumonia, meningitis, bacteremia

Acute *S. pyogenes* (Group A Strep or GAS) infections may take the form of pharyngitis, scarlet fever, impetigo, or cellulitis. Invasive infections can result in necrotizing fasciitis, myositis, and streptococcal toxic shock syndrome. Patients infected with Group A Strep may also develop immune-mediated complications such as acute rheumatic fever and acute glomerulonephritis.

Adults infected with *S. agalactiae* (Group B Strep or GBS) may experience vaginitis, puerperal fever, urinary tract infection, skin infection, and endocarditis. In newborn infants, *S. agalactiae* is a common cause of meningitis, neonatal sepsis, and pneumonia.

Members of the Group C Strep are important veterinary pathogens, the most notable being *S. equi* which causes a highly contagious respiratory disease in horses called strangles. Group C Strep are rarely pathogenic to humans.

Members of the Group D Strep, in particular *E. faecalis*, are commonly associated with urinary tract and biliary tract infections.

Both Group K Strep (*S. salivarius*) and the Viridans streptococci (*S. mitis* and *S. mutans*) can cause dental caries and endocarditis.

Members of the *Streptococcus* genus can be distinguished from *Staphylococcus* by a negative **catalase** test and by the inability of most streptococcal species to grow in the presence of high salt (NOTE: *Enterococcus* species can grow in high salt media). Streptococci are **capnophiles**, and grow best in the presence of carbon dioxide. *Streptococcus* species are distinguished from one another based on colony morphology, hemolytic properties, and biochemical reactions. Most *Streptococcus* species produce **hemolysins** that hydrolyze red

blood cells, and their hemolytic patterns are classified as **alpha**, **beta**, or **gamma** based on the degree of hemolysis. In addition to hemolytic patterns, *Streptococcus* species can be distinguished from one another by their susceptibility to the antibiotics bacitracin and SXT, the ability to grow on high-salt media, and the ability to hydrolyze esculin in bile esculin slants. During today's lab we will be inoculating different *Streptococcus* species onto mannitol salt agar, blood agar, and bile esculin agar to examine the different characteristics of each species.

### **Mannitol Salt Agar:**

MSA is both selective and differential. However, it is used solely as a **selective** medium in the identification of streptococci. MSA contains 7.5% NaCl, which selects for organisms which are halotolerant. The only members of the *Streptococcus* family that are able to grow on MSA are the *Enterococcus* species.

### **Blood Agar:**

Blood agar is a differential medium. It is also commonly used as an enriched medium for growing fastidious bacteria. Some bacteria produce exotoxins called hemolysins that cause lysis of red blood cells. The degree of the hemolysis is an especially useful tool for differentiation among *Streptococcus* species. The three types of hemolysis are:

1. **Beta** hemolysis, which is the complete lysis of red blood cells and hemoglobin. This results in complete clearing of the blood around colonies.
2. **Alpha** hemolysis refers to the partial lysis of red blood cells and hemoglobin. This results in a greenish-grey discoloration of the blood around the colonies.
3. No hemolysis, sometimes called **gamma** hemolysis, results in no change in the medium. The blood agar will also be used to test the susceptibility of the *Streptococcus* species to the antibiotics bacitracin and SXT.

### **Bile esculin agar:**

BEA is both a selective and differential medium. It contains bile salts, esculin, ferric ammonium citrate, and beef and gelatin extracts. The bile salts give BEA its selective property, because only enteric organisms are able to grow in the presence of bile. The esculin and ferric ammonium citrate give BEA its differential property, because it distinguishes those organisms which are able to hydrolyze the carbohydrate esculin. When esculin is hydrolyzed to glucose and esculetin, the esculetin reacts with ferric ammonium citrate to form a blackish precipitate. The beef and gelatin extracts provide macromolecules for general growth requirements.

### **MEDIA NEEDED:** (*per student*)

- 1 Blood agar plate
- 1 Mannitol salt agar (MSA) mini-plate
- 1 Bile Esculin agar slant
- 1 0.85% saline tube

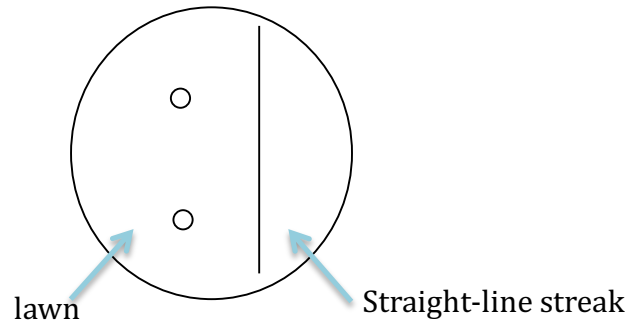
### **CULTURES NEEDED:**

- Streptococcus pyogenes*
- Streptococcus bovis*
- Streptococcus mutans*
- Streptococcus equi*
- Enterococcus faecalis*
- Streptococcus agalactiae*
- Streptococcus zooepidemicus*

(Note: Your lab instructor may not use all the bacteria on this list, and/or may add additional Strep species to the above list.)

**PROCEDURE:**

1. Each student will choose **one** of the *Streptococcus* (or *Enterococcus*) cultures listed above. Try to ensure that each member in your lab group chooses a different culture. Make sure to label your plates and slants with your name and the name of the organism.
2. Using a sterile loop, inoculate your MSA mini-plate with your *Streptococcus* (or *Enterococcus*) organism using a **straight-line streak**.
3. With a sterile loop, streak a bile esculin slant tube with your *Streptococcus* (or *Enterococcus*) organism using a **zig-zag** motion. Replace the cap so that it is loose, but secure.
4. With a sterile loop, inoculate  $\frac{1}{4}$  of the blood plate with a **straight-line streak**.
5. Suspend several loopfuls of the organism into an 0.85% saline tube. The inoculated saline should be slightly to moderately turbid. Using a sterile swab, dip into the inoculated saline and completely swab the other  $\frac{3}{4}$  of the blood agar plate, **creating a lawn**.
6. Place and seat a Bacitracin disc and an SXT (trimethoprim-sulfamethoxazole) disc equidistant within the designated area on the swabbed area of the blood plate.



7. Place the blood agar plate and mannitol salt agar plate into an Anaeropak box (for an aerobic environment enriched with carbon dioxide). Incubate at 37 °C for 48 hours.
8. Incubate the bile esculin slant tube at 37 °C for 48 hours.

**RESULTS:**

Examine *Streptococcus* cultures for the following:

1. Susceptibility to Bacitracin and SXT (trimethoprim-sulfamethoxazole) on blood plate: Susceptible organisms will not grow adjacent to the antibiotic discs. Measure the diameter of the zone of inhibition of the SXT disc in millimeters. If the zone of inhibition is greater than 20 mm, the organism is susceptible to SXT. If there is ANY zone of inhibition with the Bacitracin disk, the organism is susceptible.
2. Hemolysis pattern on blood plate – is it alpha, beta, or gamma?
3. Growth on Mannitol Salt agar. (Note: The **color** of the agar does not matter. We are exclusively looking for growth!)
4. Ability to hydrolyze esculin in Bile Esculin slants, which is indicated by a black precipitate. Note: Greater than half the media must turn black for the bile esculin test to be considered positive.
5. Record your results on the data chart below.

Organism	Hemolysis	Bile Esculin	MSA Growth	SXT Diameter /S or R	Bacitracin Diameter /S or R

**STUDY QUESTIONS:**

1. Explain the medical significance of *Staphylococcus* and *Streptococcus* species. Why is it important to be able to distinguish between these two genera?

## **EXPERIMENT #24: RAPID STREP TEST**

The rapid strep test allows for a quick diagnosis of Group A *Streptococcus* directly from throat swabs, eliminating the need for 1 or 2 day incubations on blood plates to positively identify Group A Strep. The rapid test uses a sandwich immunoassay to detect group A streptococcal antigens. The test cassette contains a membrane strip that has been coated with anti-Strep A antibodies. If the specimen being tested contains Group A Strep bacteria, the Strep A antigens extracted from the throat swab will bind to the antibodies. The antigen-antibody complex then binds with a second antibody that is conjugated with a chromogen, producing a colored line. Thus, a colored line at the test line region of the cassette indicates the sample is positive for Group A Strep. However, rapid Strep tests are not foolproof, giving false negative results approximately 15% of the time. For this reason, most clinical microbiologists recommended that a traditional throat culture also be performed when rapid Group A Strep test gives a negative result.

### **CULTURES NEEDED:** *(per group of 4)*

Polyester swab containing throat specimen

### **MATERIALS NEEDED:** *(per group of 4)*

Test Device Cassette in sealed foil pouch

Reagent A

Reagent B

Extraction tube and dropper tip

### **PROCEDURE:**

**Note:** *Depending on the brand of test kit being used, your instructor may provide you with a different protocol than the one below.*

1. Add 4 drops of Reagent A to the Extraction tube. The solution should be light red.
2. Add 4 drops of Reagent B to the Extraction tube. The solution should turn pale yellow.
3. Place the throat swab in the Extraction tube. Agitate the swab 10 times in the tube. Leave the swab in the tube for 1 minute.
4. Squeeze the swab firmly against the tube to expel as much liquid as possible from the swab. Discard the swab in the Biohazard trash.
5. Fit the dropper tip on top of the extraction test tube.
6. Remove BD Directigen EZ Group A test device from its pouch and place on a level surface. Add 3 full drops of solution into the specimen well.
7. Read results in 5 minutes. Do not read results after 10 minutes. A positive result should have a red line in both the test and control line regions on the device. A negative result will only have a red line in the control region. Note: Any line, regardless of intensity, should be interpreted as a line.



**RESULTS:**

Sketch the results displayed on the test device:

Was the sample positive or negative for Group A Strep? \_\_\_\_\_

**STUDY QUESTIONS:**

1. What autoimmune-related complications can result from this infection?

## **EXERCISE #25: IDENTIFICATION OF *STAPHYLOCOCCUS* SPECIES**

Members of the genus *Staphylococcus* are Gram-positive cocci of significant clinical importance. The *Staphylococcus* genus is classified into two major groups: *S. aureus* and non-*S. aureus*. *S. aureus* is a leading cause of skin and soft tissue infections (SSTIs), as well as toxic shock syndrome (TSS) and scalded skin syndrome. *S. aureus* has also been found to be the causative agent in such ailments as pneumonia, meningitis, arthritis, and osteomyelitis. It can be distinguished from other pathogenic species of *Staphylococcus* by its ability to ferment mannitol and by a positive result in a *coagulase* test.

An important subclass of *S. aureus* are the methicillin-resistant *S. aureus* (MRSA), which are resistant not only to the penicillin-derived methicillin, but to other antibiotics as well. MRSA emerged in hospitals and hospital-like settings in the early 1960's, and has become a significant threat to public health. Much attention is currently being given to an emerging type of MRSA called community-acquired MRSA (CA-MRSA), which is not hospital associated, and is causing serious infections in otherwise healthy people.

Of the non-*S. aureus* species, *S. epidermidis* is the most clinically significant. This bacterium is an opportunistic pathogen which is a normal resident of human skin, and often infects IV drug users, newborns, elderly, and those using catheters or other artificial appliances.

Members of the *Staphylococcus* genus can be distinguished from other Gram-positive cocci by two main properties: (1) the ability to grow on high salt media, and (2) the production of the enzyme *catalase*. *Staphylococcus* species are distinguished from one another based on their ability to ferment mannitol, their susceptibility to the antibiotic Novobiocin, and whether or not they produce a hydrolytic enzyme called *DNase*, which degrades DNA. During today's lab we will be inoculating different *Staphylococcus* species onto mannitol salt agar, *DNase* agar, and Mueller-Hinton agar to examine the different characteristics of each species.

### **Mannitol Salt Agar:**

MSA is both selective and differential. It contains 7.5% NaCl, which selects for organisms which are halotolerant. The media also contains mannitol and phenol red, which allows differentiation of organisms based on whether or not they are able to ferment mannitol. If mannitol is fermented, the acidic fermentation products react with the phenol red pH indicator, which changes color from red to yellow.

### **DNase agar:**

*DNase* agar contains an emulsion of DNA, peptides, and methyl green dye. The dye and polymerized DNA form a complex that gives the agar a blue-green color at pH 7.5. Bacterial colonies that secrete *DNase* will hydrolyze the DNA in the medium into smaller fragments unbound from the methyl green dye. This results in clearing around the bacterial growth.

### **Mueller-Hinton agar:**

M-H agar is a type of nutrient agar standardized for use in antimicrobial testing that allows for antibiotic diffusion into the agar. In addition, the levels of thymine, thymidine, calcium ions and magnesium ions are controlled in this medium so as not to interfere with susceptibility testing. The M-H agar will be used to test susceptibility to the antibiotic Novobiocin.

**MEDIA NEEDED** (*per student*):

- 1- *Staphylococcus* tri-plate
  - Mannitol Salt agar (pink)
  - DNase* agar (bluish-green)
  - Mueller-Hinton agar (yellowish)
- 1- 0.85% Saline tube

**CULTURES NEEDED:**

- Staphylococcus aureus*
  - Staphylococcus epidermidis*
  - Staphylococcus xylosum*
  - Staphylococcus intermedius*
  - Staphylococcus saprophyticus*
- (Your instructor may supply additional *Staphylococcus* species)

**PROCEDURE:**

1. Each student will choose **one** of the *Staphylococcus* cultures listed above. Try to ensure that each member in your lab group chooses a different culture. Make sure to label your plate with your name and the name of the organism.
2. With a sterile loop, inoculate the plate with a **straight line streak** on the MSA portion and the *DNase* agar portion of the tri-plate.
3. Create a liquid suspension of your *Staphylococcus* species by diluting 2-3 loopfuls of the organism into a saline tube using a sterile loop. Using a sterile swab, completely cover the Mueller-Hinton portion of the plate with the suspension to create a bacterial lawn. Place and seat a Novobiocin disc in the center with sterile forceps.
4. Incubate your tri-plates at 37 °C for 48 hours.



## **EXERCISE #26: COAGULASE TEST (DEMO)**

The **coagulase test** allows differentiation of *Staphylococcus aureus* from other *Staphylococcus* strains by the fact that most *S. aureus* strains produce an enzyme called **coagulase**, which acts within host tissue to convert fibrinogen to fibrin. Two versions of the coagulase test may be used: the tube test or the slide test. To perform the **tube test**, *S. aureus* is inoculated into a tube of citrated plasma and incubated at 37 °C for four hours and periodically examined for fibrin formation or coagulation. If a clot forms within four hours, the test is positive for the presence of **coagulase**, and signifies virulent *S. aureus*. Alternatively, several *S. aureus* colonies can be emulsified in a drop of citrated plasma on a glass slide in the **slide test** procedure. If **coagulase**-positive, visible white clumps will appear almost instantly. Although the slide test is much quicker and more economical, it is considered to be less sensitive than the tube test. Therefore, isolates which test negative using the slide test are usually re-tested with the tube test.

A demonstration of a positive **coagulase** test will be available on the front table during the results lab session.

### **NOTES / OBSERVATIONS:**

### **STUDY QUESTIONS:**

1. How does coagulase contribute to the virulence of pathogenic *Staphylococcus aureus*?

## **EXERCISE #27: RAPID STAPH TEST**

The rapid *Staphylococcus aureus* test is based on the fact that most pathogenic strains of *S. aureus* produce a bound coagulase and contain Protein A on their cell surface. [Remember from lecture that coagulase and Protein A are two of the many virulence factors that *S. aureus* has. The coagulase converts fibrinogen to fibrin, producing clots that allow the bacteria to “hide” from the immune system. Protein A binds to the Fc region of IgG antibodies, inhibiting IgGs which have opsonized the bacteria from binding to receptors on phagocytes.] The rapid Staph test kit contains latex particles coated with human fibrinogen and IgG. These latex particles will bind with any Staph bacteria that contain bound coagulase and/or Protein A, resulting in visible clumping or agglutination of the latex particles. A positive result from the rapid test is conclusive for *Staphylococcus aureus*, and replaces the need to grow the bacteria on selective and differential media such as mannitol salt agar and *DNase* agar.

### **CULTURES NEEDED:** (per group of 4)

Nutrient agar plate containing suspected *S. aureus* colonies

### **MATERIALS NEEDED:** (per group of 4)

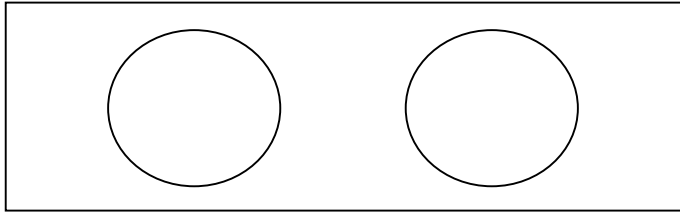
BBL Staphyloslide Test Latex  
BBL Staphyloslide Control Latex  
BBL Staphyloslide Latex Reaction Card

### **PROCEDURE:**

1. Make sure card is flat.
2. Dispense 1 drop of Test Latex onto one of the circles on the reaction card.
3. Dispense 1 drop of Control Latex onto the other circle on the reaction card.
4. Using a sterile loop, pick up and smear 5-10 colonies onto the Test Latex-containing circle, and spread to cover the circle.
5. Repeat step 4 for the Control Latex.
6. Pick up and hand-rock the card for 20 seconds and observe for agglutination. The Control Latex should NOT agglutinate.
7. Dispose of the reaction card in the Biohazard waste.

**RESULTS:**

Sketch the results displayed on the reaction card:



Was the patient sample positive or negative for *Staphylococcus aureus*? \_\_\_\_\_

**STUDY QUESTIONS:**

1. Based on the results, the attending physician wants a blood sample taken from the patient to test for bacteremia. Why do you think this additional test has been ordered?
  
2. Critical Thinking Question: What further tests do you think should be performed on the specimen to ensure proper treatment? Why?

## **EXERCISE #28: IDENTIFICATION OF GRAM-NEGATIVE SPECIES**

Gram-negative bacteria are distinguished from Gram-positive bacteria by their cell wall properties. Gram-negative organisms possess a thin layer of peptidoglycan in their cell wall, plus an outer phospholipid bilayer membrane containing lipopolysaccharide (LPS). Gram-negative bacteria can be found in diverse environments, including soil, fresh water, and salt water. Gram-negative bacteria are found in abundance as normal flora in the intestinal tracts of mammals. Gram-negative bacteria also constitute the largest group of human bacterial pathogens. Almost every Gram-negative bacterium has the capacity to cause morbidity and mortality in humans under the right conditions. The pathogenesis of Gram-negative bacteria is due in part to the lipid A component of the LPS in the outer membrane. Lipid A, also known as endotoxin, can trigger symptoms such as fever, vasodilation, inflammation, shock, and blood clots within blood vessels. Many pathogenic G-negative bacteria also produce additional toxins and virulence factors.

In order to distinguish among the Gram-negative bacteria, the following tests will be utilized. Each student will choose ONE of the Gram-negative organisms listed below and use that organism in each of the following tests. Be sure to label all tubes with the name of the organism, as well as your name and date.

*Acinetobacter lwoffii*

*Alcaligenes faecalis*

*Citrobacter freundii*

*Edwardsiella tarda*

*Enterobacter aerogenes*

*Enterobacter cloacae*

*Escherichia coli*

*Klebsiella oxytoca*

*Klebsiella pneumoniae*

*Morganella morganii*

*Proteus mirabilis*

*Proteus vulgaris*

*Pseudomonas aeruginosa*

*Pseudomonas putida*

*Providencia alcalifaciens*

*Salmonella enterica* serotype Choleraesuis

*Salmonella enterica* serotype Typhmuri

*Shigella boydii*

(Note: Your lab instructor may not use all the bacteria on this list, and/or may add additional Gram-negative bacteria to the above list.)

### **MACCONKEY AGAR**

MacConkey Agar is both selective and differential. This media contains crystal violet and bile salts, which inhibit most Gram-positive organisms and select for Gram-negative organisms. It also contains the carbohydrate substrate lactose and the pH indicator neutral red, which allow differentiation among Gram-negative bacteria based on their ability to ferment lactose. When lactose is fermented by coliforms such as *Escherichia coli*, acid end-products lower the pH of the media below 6.8, with the resulting colonial growth turning pinkish-red. If an organism is unable to ferment lactose, the colonies will be colorless, taking on the color of the medium.

**MEDIA NEEDED:** (per student)

1 MacConkey plate



**PROCEDURE:**

1. Obtain a MacConkey plate and label it with your name and the name of your microorganism.
2. Perform an isolation streak using either the quadrant streak method or T-streak method. Refer back to Exercise 7 for directions.
3. Invert the plate, and incubate overnight at 37 °C.  
*(Note to instructors: After incubating overnight at 37 °C, the plates may be refrigerated at 4 °C until the next lab period. Plates that are incubated at 37 °C longer than 24 hours may give aberrant results.)*

**RESULTS:**

1. Observe your MacConkey plate for growth. Because the agar is selective for Gram-negative bacteria, all Gram-negatives should grow on this agar.
2. Observe the color of the growth on the agar. Growth that is pinkish-red indicates the organism is able to ferment lactose. Growth that is colorless indicates the organism is unable to ferment lactose.
3. Record your results.

**TRIPLE-SUGAR IRON (TSI) AGAR TEST**

TSI agar slants are used to differentiate between various Gram-negative bacilli. There are seven ingredients in a TSI slant: (1) 0.1% glucose (2) 1.0% sucrose (3) 1.0% lactose (4) peptones (5) phenol red – pH indicator (6) sodium thiosulfate and (7) ferrous sulfate. Glucose, sucrose, and lactose are carbohydrate substrates, and fermentation of these carbohydrates into acid end-products will result in a yellow color on the slant and/or butt as the acids react with the phenol red. Results are reported as “A” for acid or “Alk” for alkaline. Note: all organisms will preferentially ferment glucose before fermenting lactose and/or sucrose. Gas production can also be determined with this test by observing the butt of the tube for gas bubbles or cracks in the agar. If the microorganism produces hydrogen sulfide, it will utilize the sodium thiosulfate as a substrate, producing H<sub>2</sub>S which reacts with the ferrous sulfate (the H<sub>2</sub>S indicator) to cause a black precipitate.

**MEDIA NEEDED: (per student)**

1 TSI slant

**PROCEDURE:**

1. Obtain a TSI slant and label it with your name and the name of your microorganism.
2. Using a sterile loop, inoculate the slant of the tube using a zig-zag streak. Then, use a sterile needle to stab the butt of the tube 5-10 times to push the organism down into the butt of the tube.
3. Make sure the cap is loose but secure. Incubate overnight at 37 °C.

**RESULTS:**

1. Observe your TSI slant for the following reactions:
  - a. Slant: acid (yellow) or alkaline (red, orange, or pink)

- b. Butt: acid (yellow) or alkaline (red, orange, or pink)
- c. Gas production: bubbles or cracks throughout the media
- d. Hydrogen sulfide production: indicated by black coloration in the tube

Note: Hydrogen sulfide production is always acidic, even though black coloration masks yellow color of pH indicator.

2. Interpretation:

- a. Red (alk) slant and yellow (acid) butt: only glucose fermentation has occurred. Since glucose is present in a 0.1% concentration, the small amount of acid produced by glucose fermentation is rapidly oxidized on the slant, resulting in an alkaline reaction. In the butt, the acid reaction is maintained because of reduced oxygen tension and slower growth of the organisms.
- b. Yellow (acid) slant and yellow (acid) butt: lactose and/or sucrose fermentation has occurred.
- c. Red (alk) slant and red or orange-red (alk) butt: no carbohydrate fermentation has occurred. Instead, peptones are catabolized resulting in an alkaline pH due to the production of ammonia.

3. Record your results.

### **UREASE TEST**

Some microorganisms are able to degrade urea using a hydrolytic enzyme called *urease*. *Urease* breaks the covalent carbon-nitrogen bonds in the compound, hydrolyzing the urea into ammonia and carbon dioxide. (Water is also produced). Urea broth tubes are used in the lab to determine if a microorganism is *urease*-positive or -negative. The tube contains urea as a substrate and phenol red as a pH indicator. If the bacterium is *urease*-positive, the ammonia produced by urea hydrolysis will raise the pH and react with the pH indicator, creating a “hot pink” color.

### **MEDIA NEEDED: (per student)**

1 Urea broth tube

### **PROCEDURE:**

1. Obtain a urea broth tube and label the tube with your name and the name of your microorganism.
2. Using a sterile loop, inoculate your tube.
3. Make sure the cap is loose but secure and incubate at 37 °C.

### **RESULTS:**

1. Observe your tube for a positive or negative result.
  - a. Positive is indicated by a hot pink coloration.
  - b. Negative is indicated by no color change in the media.
2. Record your results.

### **SIM AGAR TEST**

A SIM agar deep contains the amino acid tryptophan, as well as peptones, sodium thiosulfate, and ferrous sulfate. SIM agar deep tubes test for three things: (1) indole production, (2) hydrogen sulfide production, and (3) motility.

**Indole production:** If an organism produces an enzyme called tryptophanase, it will degrade tryptophan into indole, pyruvate, and ammonia. To test for the presence of tryptophanase, the agar deep is inoculated with a sterile needle and incubated for 24-48 hours. After incubation, a solution called Kovac's Reagent is added to the tube. Kovac's Reagent contains HCl, Butanol, and para-dimethyl-amino-benzaldehyde (pdaba). The acidified butanol extracts any indole that is produced and brings it to the surface of the agar, where it reacts with the pdaba, turning red.

**Hydrogen sulfide production:** Organisms which produce the enzyme thiosulfate reductase can reduce sulfur to hydrogen sulfide gas. In the SIM deep tubes, the source of sulfur is sodium thiosulfate. If hydrogen sulfide gas is produced, it reacts with ferrous sulfate, giving a black precipitate.

**Motility:** Organisms which are motile will exhibit diffuse growth that spreads out from the initial stab line, whereas organisms which are not motile will only grow where the agar was initially stabbed.

#### **MEDIA NEEDED (*per student*):**

1 SIM agar deep

#### **PROCEDURE:**

1. Obtain a SIM deep tube and label the tube with your name and the name of your microorganism.
2. Inoculate SIM deep tube with a straightened sterile needle, stabbing the butt all the way to the bottom a single time.
3. Make sure the cap is loose but secure and incubate at 37 °C.

#### **RESULTS:**

1. Retrieve your SIM deep tube inoculated during the previous lab period.
2. Add 10 drops of Kovac's Reagent slowly down the side of the SIM deep tube. If a cherry red color develops, the result is positive for indole production.
3. Observe your SIM tube for a black precipitate, which indicates the production of hydrogen sulfide gas.
4. Observe the pattern of growth along the stab line. If the growth spreads out from the stab line, your organism is positive for motility.
5. Record your results.

### **MRVP TEST**

The MRVP test consists of two separate components, the **Methyl Red (MR)** test and the **Voges Proskauer (VP)** test. The purpose of these tests is to determine if the organism is able to ferment glucose, and if so, what types of fermentation acids are produced. To perform both tests,

the bacteria are initially grown in MRVP broth, which contains peptone, glucose, and phosphate buffer.

### **Methyl Red (MR) test:**

This test was initially designed to distinguish between *Escherichia coli* and *Enterobacter aerogenes*. During growth in the MRVP broth, *E. coli* will ferment the glucose in the media to produce a mixture of fermentation acids – lactic, acetic, and formic acids. *E. aerogenes* will produce only acetic acid. The acids produced by *E. coli* are strong enough to overcome the buffering capacity of the phosphate buffer in the media, thus lowering the pH. The acetic acid produced by *E. aerogenes* is not strong enough to overcome the buffering capacity of the phosphate buffer. After incubation of the organism in the MRVP tube, methyl red is added to the tube to determine the pH. A red color indicates a positive MR reaction, indicating a pH of 4.4 or lower. Note: Although the test was originally designed to distinguish *E. coli* from *E. aerogenes*, there are other organisms besides *E. coli* which produce a mixture of fermentation acids and give a positive methyl red test!

### **Voges-Proskauer (VP) test:**

The purpose of this test is to detect bacteria which ferment glucose, but only produce one acid end-product, usually acetic acid. The acetic acid produced initially lowers the pH of the media, but is quickly converted to acetylmethylcarbinol, which leads to a pH of approximately 6.2. The VP test uses an MRVP broth tube (described above). After incubation of the organism in the MRVP tube, Barritt's Reagent A ( $\alpha$ -naphthol) and B (40% KOH) are added to the media. The chemicals in the reagents will react with acetylmethylcarbinol, and a positive reaction will show a dark red band at the top of the broth in the tube which will diffuse over time into the rest of the media. NOTE: An organism will NEVER be positive for both the MR and VP tests!!

### **MEDIA NEEDED (per student):**

1 MRVP broth tube

### **PROCEDURE:**

1. Obtain an MRVP broth tube and label the tube with your name and the name of your microorganism.
2. Inoculate the MRVP broth tube with a sterile loop.
3. Make sure the cap is loose but secure and incubate at 37 °C.

### **RESULTS:**

1. Retrieve your MRVP broth tube inoculated during the previous lab period.
2. Transfer 1 ml of your MRVP broth to an empty tube. Add 10 drops of **methyl red** color indicator. Mix well and observe for a continuing red color, which indicates a positive result for the methyl red test. A positive MR result means that the organism has fermented glucose to produce a mixture of fermentation acids that were able to lower the pH below 4.4.
3. Transfer another 1 ml of your MRVP broth to an empty tube. Add 10 drops of Barritt's Reagent A (VPA), but do not mix. Then add 5 drops of Barritt's Reagent B (VPB) to the same tube, and mix thoroughly. Incubate at room temperature for a minimum of 25 minutes to allow the reaction to occur. If a dark red band begins to appear at the top of the liquid, this indicates a positive result for the Voges-Proskauer test. A positive VP

results means that the organism has fermented glucose to produce acetic acid only, which has been converted to acetylmethylcarbinol.

4. Record your results.

### **CITRATE UTILIZATION TEST**

The purpose of this test is to determine if an organism can use citrate as a carbon source. The citrate agar slant contains sodium citrate, bromthymol blue (pH indicator), sodium, and water. If the organism is able to utilize citrate, an enzyme called *citruse* will break the citrate into oxaloacetic acid and acetic acid. The oxaloacetic acid is then broken down into pyruvate and the acetic acid is converted to CO<sub>2</sub>. The CO<sub>2</sub> reacts with the water and sodium in the media to produce alkaline sodium carbonate. The sodium carbonate reacts with the pH indicator to produce a “Prussian blue” color.

#### **MEDIA NEEDED (per student):**

- 1 Citrate agar slant

#### **PROCEDURE:**

1. Obtain a citrate agar slant and label the tube with your name and the name of your microorganism.
2. Inoculate the citrate agar slant by streaking the surface of the slant with a sterile loop in a zig-zag motion. Do not stab the butt!
3. Make sure the cap is loose but secure and incubate at 37 °C.

#### **RESULTS:**

1. Retrieve your citrate agar slant inoculated during the previous lab period.
2. Observe your citrate slant for color and bacterial growth. A “Prussian blue” color indicates a positive result, which means the organism is able to use citrate as a carbon source.
3. Record your results.

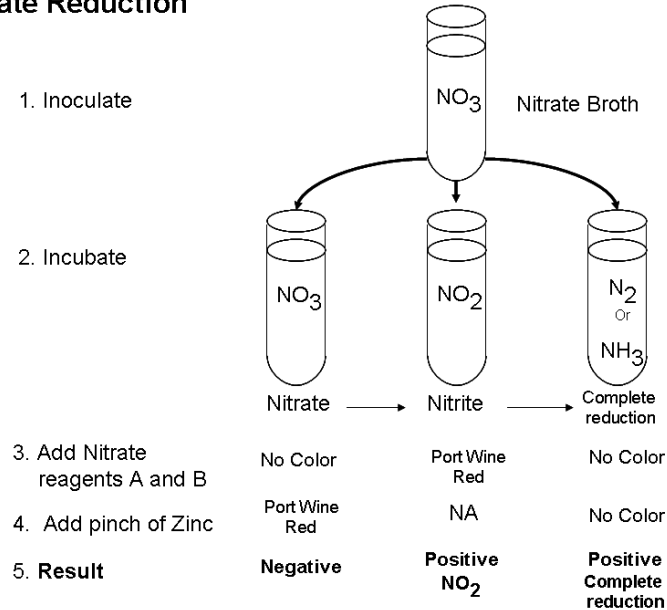
NOTE: Historically, the previous three types of media (SIM, MRVP, and Citrate) have been grouped together in a test called the IMViC test, which stands for Indole, Methyl Red, Vogues-Proskauer, and Citrate.

### **NITRATE REDUCTION TEST**

Some organisms possess an enzyme called nitrate reductase which enables them to anaerobically reduce nitrate into nitrite. Some of these organisms can then further reduce the nitrite to ammonia or completely to molecular nitrogen. Nitrate broth tubes contain beef extract, peptone, and nitrate. After incubation of an organism in a nitrate broth tube, reagents are added to determine if the nitrate has been reduced, and if so, to what extent. Nitrate Reagent A, containing sulfanilic acid, is added to the test tube, followed by addition of Nitrate Reagent B, containing alpha-naphthylamine. Formation of a red color indicates the presence of nitrites, indicating the organism has reduced the nitrate to nitrite and is positive for nitrate reduction. If

no color appears, a pinch of zinc is added to the tube. If a red color appears, the organism is negative for nitrite reduction, because the zinc has reduced the nitrate to nitrite, causing the red color. However, if there is still no color change after the addition of zinc, this indicates the nitrates were reduced beyond nitrites to ammonia or molecular nitrogen, and the organism is positive for nitrate reduction.

### Nitrate Reduction



#### MEDIA NEEDED: (per student)

1 Nitrate broth tube

#### PROCEDURE:

1. Obtain a nitrate broth tube and label the tube with your name and the name of your microorganism.
2. Inoculate the nitrate broth with a sterile loop.
3. Make sure the cap is on loose but secure and incubate at 37 °C.

#### RESULTS:

1. Retrieve your nitrate broth tube inoculated during the previous lab period.
2. Add 5 drops of Nitrate Reagent A to the broth tube and mix thoroughly. Next, add 5 drops of Nitrate Reagent B and mix. If a red color is produced, this indicates a positive result for nitrate reduction. This means the organism has reduced the nitrate to nitrite.
3. If no red color is produced, add a pinch of Zinc and mix thoroughly. Allow to sit for several minutes. If the broth turns red, this indicates that the zinc has reduced the nitrates and the organism is negative for nitrate reduction.
4. If the addition of zinc does NOT cause a color reaction, the test is positive. This means that the organism has reduced the nitrate to nitrites and then reduced the nitrites to ammonia or molecular nitrogen.
5. Record your results.

## LYSINE IRON AGAR

The purpose of this test is to determine if the organism is able to carry out **lysine decarboxylation**, which is an anaerobic process, or **lysine deamination**, which is an aerobic reaction. The LIA slant contains lysine, glucose, peptones, bromcresol purple (pH indicator), sodium thiosulfate and ferric ammonium citrate. Bromcresol purple is a dark purple color at pH 6.8 or higher, and turns yellow when the pH drops below 5.2.

Upon fermentation of glucose, some organisms will synthesize an enzyme called *lysine decarboxylase*, which cleaves a carboxyl group from the lysine. This lysine decarboxylation creates a diamine end-product called cadaverine, which raises the pH above 6.8 and reacts with the pH indicator to give a dark purple color in the butt of the tube. Other organisms may ferment glucose but are unable to synthesize *lysine decarboxylase*. These bacteria will produce acid end-products that lower the pH, resulting in a yellow color in the butt of the tube.

If the organism has the ability to deaminate lysine, the ammonia produced will react with the ferric ammonium citrate to produce  $\alpha$ -ketocarboxylic acid, which produces a dark red color on the slant of the tube. Deamination requires oxygen, so it never occurs in the butt of the tube.

Lysine iron agar also tests for hydrogen sulfide production. Organisms which produce the enzyme thiosulfate reductase can reduce sulfur to hydrogen sulfide gas. In LIA tubes, the source of sulfur is sodium thiosulfate. If hydrogen sulfide gas is produced, it reacts with ferric ammonium citrate, giving a black precipitate.

### **MEDIA NEEDED:** (*per student*)

1 Lysine Iron Agar (LIA) slant

### **PROCEDURE:**

1. Obtain a LIA slant and label the tube with your name and the name of your microorganism.
2. Using a sterile loop, inoculate the slant of the tube using a zig-zag streak. Then, use a sterile needle to stab the butt of the tube 5-10 times to push the organism down into the butt of the tube. Do not stab into the slant, only into the butt!
3. Make sure the caps are loose but secure, and incubate at 37 °C.

### **RESULTS:**

1. Retrieve your Lysine Iron Agar slant inoculated during the previous lab period.
2. Observe the color of the slant of the media. A PURPLE color indicates a **negative deamination** reaction and a RED color indicates a **positive deamination** reaction. If unsure of red vs. purple, use a light source such as an overhead projector.
3. Observe the color of the butt of the media. A PURPLE color indicates a **positive decarboxylation** reaction, and a YELLOW color indicates a **negative decarboxylation** reaction.
4. Note: If you have a red slant, you will nearly always have a yellow butt!
5. Observe your LIA slant for a black precipitate, which indicates the organism is positive for hydrogen sulfide production.
6. Record your results.

**RESULTS TABLE:**

Gram Negative Organism	MacConkey Agar	Triple Sugar Iron Agar				Urease	Citrate	SIM		MR	VP	Nitrate	Lysine Iron Agar	
		Slant	Butt	Gas	H <sub>2</sub> S			Indole	H <sub>2</sub> S				Slant Deamination	Butt Decarb-oxylation
	Lactose Fermentation													

**SIM:** Motility \_\_\_\_\_

**LIA:** H<sub>2</sub>S \_\_\_\_\_

**STUDY QUESTIONS:**

1. Why are the TSI and LIA slants inoculated with both a streak and a stab?
  
2. The nitrate test requires the addition of several reagents to the culture after incubating the culture for 48 hours. Explain why the **absence** of a red color after the addition of Reagents A and B and zinc signifies a **positive** result.
  
3. Name the three different types of media used to test for **hydrogen sulfide** production during this week's lab exercises. What is the substrate available for H<sub>2</sub>S production in each type of media?





## **EXERCISE #29: EPIDEMIOLOGY INVESTIGATION**

One of the jobs of an epidemiologist is to investigate outbreaks of infectious diseases. When a suspected outbreak occurs in a community, epidemiologists gather data to first determine the cause of the disease and its the route of transmission, and also to trace the pattern of transmission with the goal of identifying the original carrier, often termed “patient 0”. Once the patients involved in the outbreak have been identified, steps can be taken to prevent the outbreak from spreading further.

This laboratory exercise simulates an outbreak of a disease transmitted via body fluids, and students will assume the role of an epidemiologist to trace the pattern of transmission and identify the original “carrier” of the disease.

### **CULTURES NEEDED:**

None

### **MATERIALS NEEDED:**

Test tube containing “Body fluids” – 1 per student

Phenol red indicator solution

Plastic pipettes

### **PROCEDURE:**

1. Select numbered “Body fluid” test tube from the rack and record your number. \_\_\_\_\_  
*Note: one of the test tubes contains the “diseased body fluid”. The remainder of the tubes contain “uninfected body fluids”.*
2. Using your plastic pipette, draw up about **half** of the solution from your test tube. Then do the following:
  - a. Choose someone at random in the class. Note: it should NOT be someone who sits at your lab table!
  - b. Dispense the contents of your pipette into your contact’s test tube as he/she dispenses the contents of their pipette into your test tube.
  - c. Using your pipette, mix the solution in your tube.
  - d. Record your contact’s name and tube number: \_\_\_\_\_

**IMPORTANT: Wait until everyone in the class completes this step before proceeding to the next step!**

3. Repeat Step 2 twice more (Rounds 2 and 3), each time with a different contact. Again, it is imperative that you wait until everyone in the class completes Round 2 before moving on to Round 3.

Round 2 contact’s name and tube number: \_\_\_\_\_

Round 3 contact’s name and tube number: \_\_\_\_\_

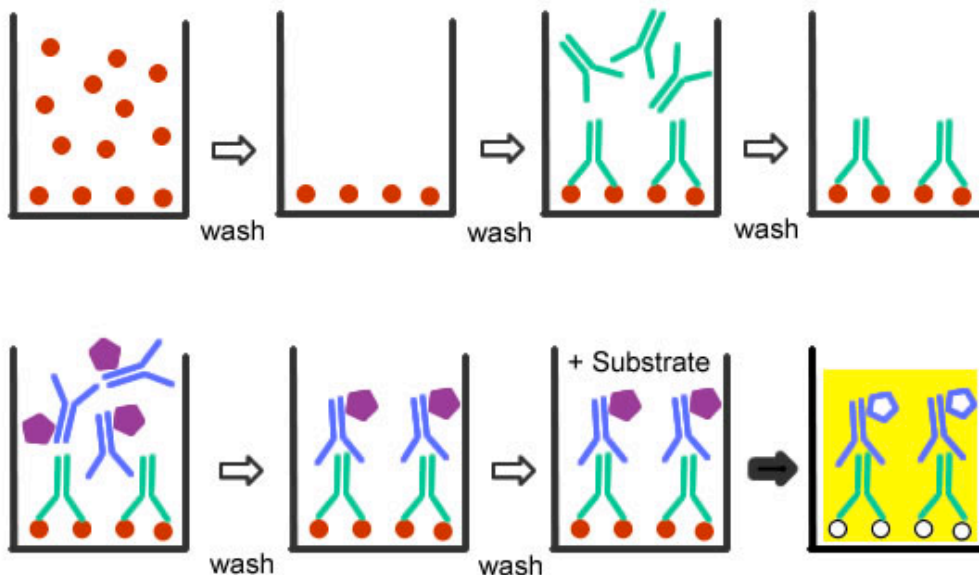
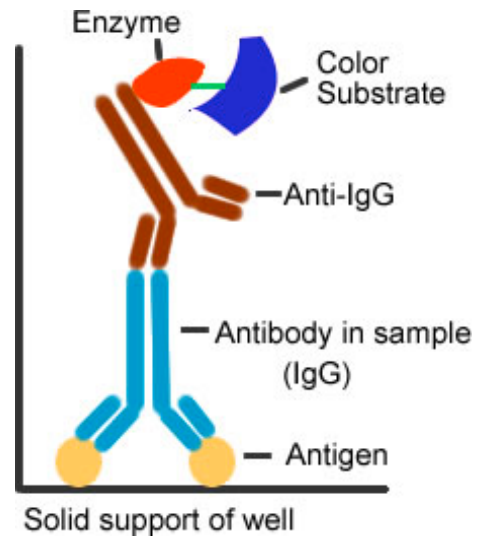
4. Once you have completed Rounds 1-3, add 1 drop of phenol red to your test tube. If the solution turns red, that indicates your tube is positive for the “disease”. If the solution turns yellow, your tube is negative for the “disease”.
5. Using the results from the entire class, complete the class data table.



### EXERCISE #30: ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TESTING

The enzyme-linked immunosorbent assay, or ELISA (sometimes called EIA, or enzyme immunoassay), is a commonly used tool in diagnostic medicine. It is used to test for many diseases and conditions, including HIV infection, Lyme disease, pregnancy, thyroid deficiencies, and lupus. The test is a type of immunoassay based on specific antigen-antibody binding. ELISA can directly test for a particular antigen, such as in a pregnancy or Lyme disease test, or, it can test for specific antibodies, such as in an HIV or lupus test.

For this lab exercise, we will be using the BioRad ELISA Immuno Explorer Kit to conduct an HIV test on simulated patient samples. In this kit, the test strips contain wells that will be coated with HIV antigens. Samples containing patient serum are then added to the wells. If the patient's serum contains antibodies to HIV, those antibodies will bind to the antigen in the wells. At this point, the wells are washed with a buffer to remove any unbound antibody. In order to detect the antibody-antigen complexes in the wells, a second antibody is then added, which will bind to the first antibody. Again, the wells are washed with a buffer to remove any unbound antibody. The secondary antibody is conjugated with a chromogenic enzyme, which will elicit a color change in the sample when a detection reagent containing the substrate is added. The more intense the color, the more antibody is present in that sample. The overall procedure is outlined in the diagram below.



**MATERIALS NEEDED:** (*per table*)

- Test Samples A and B (*Yellow tubes*)
- (+) Positive control (*Violet tube*)
- (-) Negative control (*Blue tube*)
- (AG) Purified antigen (*Green tube*)
- (SA) Secondary antibody (*Orange tube*)
- (SUB) Enzyme substrate (*Brown tube*)
- 12-well microplate strip
- Micropipette
- Yellow tips
- Disposable plastic transfer pipette
- Wash buffer
- Paper towels

**PROCEDURE:**

1. Identify the yellow tubes labeled “Sample A” and “Sample B” that contain the samples being tested.
2. Label your 12-well strip. On each strip label the first 3 wells with a “+” for the positive controls and the next 3 wells with a “-” for the negative controls. Label the remaining wells to identify the samples being tested (3 wells each).



3. Use a fresh pipette tip to transfer 50  $\mu$ l of purified antigen (AG) into all 12 wells of the microplate strip.
4. Wait 5 minutes for the antigen to bind to the plastic wells.
5. WASH:
  - a. Tip the microplate strip upside down onto the paper towels, and gently tap the strip a few times upside down. Make sure to avoid splashing sample back into wells.
  - b. Discard the top paper towel.
  - c. Use your transfer pipette to fill each well with wash buffer, taking care not to spill over into neighboring wells. Note: the same transfer pipette is used for all washing steps.
  - d. Tip the microplate strip upside down onto the paper towels and tap.
  - e. Discard the top 2–3 paper towels.
6. Repeat wash step 5.
7. Use a fresh pipette tip to transfer 50  $\mu$ l of the positive control (+) into the three “+” wells.
8. Use a fresh pipette tip to transfer 50  $\mu$ l of the negative control (-) into the three “-” wells.

9. Use a fresh pipette tip to transfer 50  $\mu$ l of Sample A into the appropriately labeled three wells.
10. Use a fresh pipette tip to transfer 50  $\mu$ l of Sample B into the appropriately labeled three wells.
11. Wait 5 minutes for the antibodies to bind to their targets.
12. Wash the unbound primary antibody out of the wells by repeating all of wash step 5 two times.
13. Use a fresh pipet tip to transfer 50  $\mu$ l of secondary antibody (SA) into all 12 wells of the microplate strip.
14. Wait 5 minutes for the antibodies to bind to their targets.
15. Wash the unbound secondary antibody out of the wells by repeating wash step 5 three times.
16. Use a fresh pipet tip to transfer 50  $\mu$ l of enzyme substrate (SUB) into all 12 wells of the microplate strip.
17. Wait 5 minutes. Observe and record the results.

**RESULTS:**

Record your results in the table below:

	+ Control	-- Control	Sample A	Sample B
Color				
Positive/Negative				

Do all of the wells that show a color change have the same color intensity?

Explain the significance of some wells having a darker color than others.

## STUDY QUESTIONS:

1. In a clinical setting, a positive ELISA is typically repeated to confirm the results, and additional samples are sent to a certified laboratory to re-confirm the results via other methods. Why is this important? What types of tests would be used to reconfirm the results?
2. The ELISA test is the most commonly used tool for diagnosing HIV infections. However, it can give a false negative result if a patient was only recently infected (i.e. less than 3 months prior). Explain how a patient can be infected with HIV but still have a negative result in this test.
3. The assay used in this lab exercise employed an **indirect** ELISA because we were testing patient samples for the presence of specific antibodies to a particular antigen, which is what is illustrated in the diagram on page 112. Other diagnostic tests use a **direct** ELISA, which tests for the presence of the antigen itself, using wells that are coated with antibodies to capture the targeted antigen. A secondary antibody is subsequently used to detect any bound antigen. In the space below, draw the “sandwich” that would result in a direct ELISA test.

### **EXERCISE #31: YOGURT PRODUCTION**

Yogurt is one of many types of fermented milk products that is consumed around the world. Commercial yogurt is made in North America from cow's milk and prepared under precisely controlled conditions in order to favor the metabolism and growth of different types of lactic acid bacteria. These bacteria ferment the milk to produce the distinct taste of yogurt. To produce yogurt, milk is first brought almost to the point of boiling to kill any bacteria present in the milk. It is then cooled so that when the lactic acid bacteria are added, they are not destroyed. When the milk has cooled sufficiently, two of the "active cultures" are added: *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

The first step of yogurt making is to incubate the mixture at 45°C, which encourages the growth of *Streptococcus thermophilus*, allowing the bacteria to rapidly ferment the lactose in the milk to lactic acid. This causes the pH of the milk to decrease. Cow's milk has a natural pH of about 6.6. At this pH, the casein (milk protein) exists as a colloidal suspension of a calcium salt called calcium caseinate which gives milk its white color and turbid appearance. When lactic acid is produced during lactose fermentation, the calcium caseinate is converted to calcium lactate and free (soluble) casein. As the pH drops to 4.6, the casein will denature to form smooth, semi-solid curds. This first step is continued only until the coagulated milk has the proper texture. If the step continues too long, too much lactic acid will be produced and the yogurt will be too sour.

After the proper texture has been achieved, the second step involves cooling the yogurt. The lower temperature prevents further growth of *S. thermophilus* and promotes growth of *Lactobacillus bulgaricus*. *L. bulgaricus* will ferment the remaining lactose in the milk. *Lactobacillus* is a more acid-tolerant bacterium and can withstand the already-present lactic acid. The second step continues until the temperature drops below the temperature range of *L. bulgaricus*, which halts the production of lactic acid and other minor fermentation products. The low pH will inhibit the growth of most other types of bacteria.

#### **MATERIALS NEEDED:** (per group of 4)

~700 ml of milk

Yogurt starter (container of plain Dannon yogurt with active bacteria)

Hot plate

Beaker in which to stir the milk

Glass stirring rod and/or magnetic stir bar

Thermometer

Plastic spoon (1 per student)

Styrofoam cup (1 per student)

Plastic wrap

Aluminum foil

#### **PROCEDURE:**

1. Use pH paper to measure the pH of the milk
2. Heat the milk in the beaker on medium-high to high heat, stirring constantly. Do not scorch! When the milk just begins to boil, turn off the heat. Cover with plastic wrap, allowing the plastic to touch the milk to prevent a film from developing on the milk.
3. Allow the milk to cool to 55°C. Note: An ice water bath may be used to cool the milk more rapidly.



4. Remove the plastic wrap and stir the milk. Pour the milk into 4 Styrofoam cups (one for each student in the group).
5. Add one teaspoon of the starter yogurt culture to each cup of milk. Stir carefully, but thoroughly, to distribute the starter throughout the milk.
6. Cover the cup with plastic wrap and cover the plastic wrap with the cup lid or aluminum foil. Incubate the yogurt cups at 45°C for at least 6 hours (overnight is fine).
7. Remove the cups, and check to see that the milk is solid. Refrigerate.
8. Use pH paper to measure the pH of the yogurt.

**RESULTS:**

Record your observations of the milk/yogurt before and after fermentation:

	pH	Texture	Appearance	Color
Milk				
Yogurt				

**STUDY QUESTIONS:**

1. Critical Thinking Question: Many people are unable to consume dairy products such as milk and cheese because they are lactose-intolerant. However, these same people often have no trouble eating yogurt. Based on your knowledge of the yogurt-making process, why do you think this is possible?

## EXERCISE #32: WATER MICROBIOLOGY

Contaminated water is a major route of transmission of infectious disease. When a body of water becomes contaminated with organic waste materials, it becomes a breeding ground for microorganisms, both infectious and non-infectious. Testing water for the presence of bacteria typically involves testing for the presence of fecal coliforms, which are considered **indicator organisms**. The presence of fecal coliforms **indicates** the water has been contaminated with human or animal feces. The presence of fecal coliforms correlates with presence of enteric bacterial pathogens such as *Vibrio cholerae*, *Escherichia coli*, and *Salmonella* and *Shigella* species; and enteric viral pathogens such as enterovirus, rotavirus, and Norwalk virus. Three tests are used to determine if a water sample is contaminated with coliform bacteria: a presumptive test, a confirmed test, and a completed test.

In the **presumptive test** a series of tubes of phenol red lactose broth are inoculated with measured amounts of water to see if the water contains any lactose-fermenting bacteria that produce gas. If, after incubation, fermentation plus gas is seen in the lactose broth, it is **presumed** that coliforms are present in the water sample. By counting the number of tubes positive for gas production at each dilution, the “most probable number” (MPN) of coliforms is statistically determined using a standardized chart. Based on MPN results, the EPA sets specific limits on the number of fecal coliforms for different types of water, such as drinking water and recreational water, to determine the threshold for contamination. Positive presumptive tests require follow-up with the confirmed and completed tests.

**Confirmed tests** involve plating the bacteria from the lactose broth tube onto differential media such as EMB or MacConkey to confirm the presence of fecal coliforms. **Completed tests** involve testing for various biochemical properties (MRVP, citrate, urease, indole, etc.) to determine the genus and species of the coliforms present. In today’s lab exercise, we will be performing a modified version of the **presumptive test** with samples from tap water, an aquarium, and sewage water.

### **MEDIA NEEDED** (*per group of 4*):

Single Strength Lactose broth with Durham tubes (6)  
Double Strength Lactose broth with Durham tubes (3)

### **CULTURES NEEDED:**

250 ml bottle Tap Water  
250 ml bottle Aquarium or Pond Water  
250 ml bottle Sewage Water

### **PROCEDURE:**

1. Each group will select one water sample to test for the presence of fecal coliforms. Coordinate with other groups so that all 3 water samples are tested.
2. Label each tube with the water sample source and the amount of sample to be added to the tube (i.e. 10 ml, 1 ml, or 0.1 ml).
3. Using a sterile pipette, add **10 ml** of the water sample to 3 **double-strength** Lactose broth tubes.
4. Using a sterile pipette, add **1 ml** of the water sample to 3 **single-strength** Lactose broth tubes.

5. Using a sterile pipette, add **0.1 ml** of the water sample to the other 3 **single-strength** Lactose broth tubes.
6. Make sure all caps are on *loosely*. (Hint: completely tighten, then un-screw half a turn.)
7. Incubate all tubes at 37 °C.

**RESULTS:**

1. Observe the tubes for the presence of gas in the Durham tube. Even a few tiny bubbles in the Durham tube is considered positive for gas.
2. Count the number of single-strength and double-strength lactose broth tubes which contain gas, and refer to the attached chart to determine the MPN.

3. Results:

Sample	# DS-lactose w/ gas (10 ml water)	# SS-lactose w/ gas (1 ml water)	# SS-lactose w/ gas (0.1 ml water)	MPN
Tap Water				
Aquarium Water or Pond Water				
Sewage Water				

MPN Determination from Multiple Tube Test: (EPA, 2003)

Number of Tubes Giving Positive Reactions Out Of			MPN Index per 100 ml	95% Confidence Limits	
3 of 10 ml each	3 of 1 ml each	3 of 0.1 ml each		Lower	Upper
0	0	0	0	0	0
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210

3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800
3	3	3	>1100	n/a	n/a

**STUDY QUESTIONS:**

1. Why are fecal coliforms considered indicator organisms?

### **EXERCISE #33: FOOD MICROBIOLOGY**

Contaminated food is another major route of transmission of infectious disease. Meat products such as beef, pork, and chicken often become contaminated during the slaughtering process, and individuals become infected when the meat is not properly cooked. Some of the primary bacterial pathogens associated with contaminated meat include *Escherichia coli* and other fecal coliforms, *Campylobacter*, *Salmonella* and *Shigella*. In today's lab, we will be testing chicken and ground meat from the supermarket for the presence of Gram-negative bacterial contaminants using *Salmonella-Shigella* agar plates.

*Salmonella-Shigella* agar plates contain lactose, bile salts, ferric citrate, and neutral red. The bile salts in agar make the media selective for Gram-negative enteric bacteria by inhibiting the growth of Gram-positive organisms. The lactose in the media is a carbohydrate substrate which allows the differentiation between lactose fermentors and non-lactose fermentors. If lactose is fermented, the acid end-products will react with the neutral red pH indicator, giving the bacterial growth a pinkish-red color. The ferric citrate in the media acts as an indicator of hydrogen sulfide production, producing a black precipitate when H<sub>2</sub>S is present.

**MEDIA NEEDED:** (per group of four)  
*Salmonella-Shigella* agar plates (SS plates)

**CULTURES NEEDED:**  
Chicken parts  
Sausage or ground beef

**PROCEDURE:**

1. With a permanent marker, divide the SS plate in half. On one half write "chicken" and on the other half write "sausage" or "ground beef". (Also label with your group's name/initials, lab period, and date.)
2. Swab over the chicken with a sterile cotton-tipped swab and rub over the "chicken" half of the SS plate.
3. Swab over the sausage with a sterile cotton-tipped swab and rub over the "sausage" half of the SS plate.
4. Incubate the plates at 37 °C.

**RESULTS:**

1. Observe SS plates for bacterial growth. Determine which bacteria are growing on the plate using the following criteria:

*Escherichia* and other fecal coliforms such as *K. pneumoniae*: ferment lactose and produce reddish pink colonies on the agar.

*Salmonella*: do not ferment lactose, but do produce hydrogen sulfide. Colonies will either be black, or clear with a black dot in the center.

*Shigella*: do not ferment lactose, and do not produce hydrogen sulfide. Colonies will be clear.

2. Record results in the table below.

Microorganism	Chicken	Sausage / Ground Beef
<i>Escherichia</i> / coliforms		
<i>Salmonella</i>		
<i>Shigella</i>		

**STUDY QUESTIONS:**

1. Critical Thinking Question: Will swabbing and streaking on SS plates identify **all** bacteria which could be chicken contaminants? Why or why not? Name at least one organism which commonly contaminates chicken which cannot be identified by this test.  
*Hint: Refer to lecture notes if you get stuck!*

# Appendix A: Unknowns

## UNKNOWN INSTRUCTIONS

### DAY 1:

Today you will be given a broth tube containing two unknown microorganisms: a Gram-positive coccus and a Gram-negative rod. Your ultimate task will be to separate the two organisms and determine the identity of each organism by performing various biochemical tests previously learned in this lab course. During the whole unknown project, you will be able to refer to your notes and consult your classmates for assistance. (However, remember that this is an **individual** project – a student **should not** rely completely on another student to tell them what to do!) You will also get to ask your instructor 3 “free” questions without any adverse effect on your grade.

For TODAY you will be doing 4 things:

1. Streaking out the broth culture onto a Nutrient Agar plate using a 4-way or T-streak isolation streak.
2. Streaking out the broth culture onto a MacConkey agar plate using a 4-way or T-streak isolation streak.
3. Streaking out the broth culture onto a C-CNA agar plate using a 4-way or T-streak isolation streak.
4. Performing a Gram stain on the culture to verify the presence of both a Gram-positive coccus and a Gram-negative rod.

\*\*Incubate all plates at 37°C. There will be Aneropak box on the front table if you wish to incubate one of your plates in an aerobic environment enriched with CO<sub>2</sub>.

### *Helpful hints:*

Think . . . why are we using 3 different kinds of plates to separate and isolate the two unknown organisms?

Label, label, label! Label the type of media, your unknown #, your name and class period, etc. Be sure you write down your unknown number!!

One of your Gram stains will be graded, so if you're happy with the results on this one, it's a good idea to get this hurdle out of the way!

Your isolation streaks will also be graded, so make sure your lab instructor can identify which plates belong to you!

### DAY 2:

Today you will be doing the following:

1. Examining your streak plates from the previous lab period for the presence of isolated colonies.
2. Using a sterile needle, pick **a small part** of one putative Gram-positive colony and perform a Gram stain to confirm its identity.
3. Once you've confirmed the organism is a Gram-positive coccus, transfer the remainder of the above colony to a nutrient agar slant. This is going to be your working stock culture from which to inoculate the rest of your Gram-positive tests. Make sure you label this slant with your unknown # AND with “Gram+” so you don't get it mixed up with your Gram-negative!
4. Using a sterile needle, pick **a small part** of one putative Gram-negative colony and perform a Gram stain to confirm its identity.
5. Once you've confirmed the organism is a Gram-negative rod, transfer the remainder of the above colony to a nutrient agar slant. This is going to be your working stock culture



from which to inoculate the rest of your Gram-negative tests. Make sure you label this slant with your unknown # AND with “Gram-” so you don’t get it mixed up with your Gram-positive!

6. Incubate all nutrient agar slants at 37°C.

*Helpful hints:*

Label, label, label!

If you didn’t have your Gram stain graded during the previous lab period, select one of your Gram stains from today to be graded.

DAY 3

Today you will be inoculating your isolated Gram-negative and Gram-positive unknowns onto the appropriate microbiological media in order to determine their identity.

1. Perform a Gram stain on bacterial growth from each nutrient agar slant to confirm that your cultures are pure.
2. Determine which genus your Gram-positive isolate belongs to: is it *Staphylococcus* or *Streptococcus/Enterococcus*?
3. Once you determine the genus, inoculate your Gram-positive isolate onto either the Staph ID media or the Strep ID media. DO NOT INOCULATE BOTH!!
4. Inoculate your Gram-negative isolate onto the 7 pieces of Gram-negative ID media.

*Helpful hints:*

Label, label, label!

Antibiotic disks (Novobiocin, SXT, Bacitracin) are available on the front cart.

An Anaeropak box is available at the front table if you wish to incubate anything aerobically in the presence of CO<sub>2</sub>.

DAY 4

Today you will be reading the results of the tests you inoculated during the previous lab period.

*Helpful hints:*

Don’t forget that some Gram-negative tests require you to add additional reagents – Read your notes!

Rulers are available.

Don’t discard anything until you are confident that you have interpreted all of your results correctly!

Name: \_\_\_\_\_

Unknown #: \_\_\_\_\_

Record the results of your unknown tests on this worksheet. Be sure to include this worksheet in your unknown report!

**Gram Stain of Mixed Culture: (Observations)**

**Isolation Streaks:**

Media used for Isolation	Observations	Gram Stain Results (if applicable)
Nutrient Agar		
MacConkey		
Columbia CNA Agar		

**Gram Positive Identification:**

Catalase Result: \_\_\_\_\_

	Result		Result
MSA fermentation	_____	Hemolysis	_____
DNase	_____	Bacitracin	_____
Novobiocin	_____	SXT	_____
		Bile esculin	_____
		MSA growth	_____

**Gram Negative Identification:**

MacConkey: Fermentation \_\_\_\_\_

TSI: Slant \_\_\_\_\_ Butt \_\_\_\_\_ Gas \_\_\_\_\_ H<sub>2</sub>S \_\_\_\_\_

Urease: \_\_\_\_\_

Citrate: \_\_\_\_\_

SIM: Indole \_\_\_\_\_ H<sub>2</sub>S \_\_\_\_\_

MR/VP: MR \_\_\_\_\_ VP \_\_\_\_\_

Nitrate: + or -

LIA: Deamination \_\_\_\_\_ Decarboxylation \_\_\_\_\_

Name \_\_\_\_\_ Unknown # \_\_\_\_\_

Unknown Deductions

You may be deducted points for the following:	Ded. Pts	Instr. Initial
1. Incorrect 4-way streak procedure (5 pts)	_____	_____
2. Gram stain (0-5 pts)	_____	_____
3. Microscopy (5 pts) – can't focus microscope	_____	_____
4. Using the wrong media _____ (10 pts)	_____	_____
5. Using extra media b/c of inoculation errors (8 pts)	_____	_____
6. Omitting or using the wrong disc on Staph or Strep ID (5 pts)	_____	_____
7. Not labeling tubes and plates correctly (1 pt per piece)	_____	_____
8. Incorrect ID of microbe (10 pts)	_____	_____
9. Each additional question after your 3 permitted (2 pts each)	_____	_____
10. Forgetting your Unknown Work sheet (5 pts)	_____	_____
11. Not bringing your instructions (5 pts)	_____	_____
12. Picking up extra media without instructor's permission (5 pts)	_____	_____
13. Viewing organism under wrong objective (5 pts)	_____	_____
14. Not cleaning oil off of microscope objectives (5 pts)	_____	_____
15. Not cleaning table at the end of the lab period (5 pts)	_____	_____
16. Failing to invert agar plates during incubation (5 pts)	_____	_____
17. Incorrectly interpreting results _____ (___ pts)	_____	_____
18. Incorrectly inoculating media _____ (3 pts each)	_____	_____
19.	_____	_____
20.	_____	_____

NOTES TO REMEMBER:

1. Each student is allowed to ask 3 questions throughout the Unknown Identification process, whether you ask a lab instructor or a lab assistant. After that, for every question asked, you are penalized 2 pts.
2. You must refer to your notes for directions and procedures. You may also ask your fellow students if you have questions. Be sure to bring your protocols to each class.
3. You are also required to bring this Unknown Identification Work Sheet each time you come to lab. Points will be deducted if you do not.
4. You must be able to independently find your microbes under the microscope.
5. Instructors will NOT interpret results for you or tell you the identity of your unknowns. It is your responsibility to correctly interpret the results of each test and to determine the identity of each unknown once you have obtained your test results.

NOTES:

# Appendix B: Unknown Reports

## **GENERAL:**

Unknown reports in microbiology are written in scientific format. Scientific writing is different from other types of writing in that the results of the exercise or experiment are being showcased, not the writing. The purpose of scientific writing is not to entertain, but to inform. The writing should be simple and easy to understand. There is a specific style that must be followed when writing scientific reports. Scientific writing is typically written in the passive voice. The pronouns "I", "We" and "They" are not used. For example, instead of writing "I used a TSA agar plate to isolate my unknown," it is customary to write, "A trypticase soy agar (TSA) plate was used to isolate the unknown." It is also customary to write in the past tense for most of the report. This includes the introduction, the summary, the description of the materials and methods, and the results. The present tense is reserved for the conclusions about the results.

**Microbial nomenclature:** The name of the bacterium should be written out and spelled correctly. The name should be italicized, e.g. *Staphylococcus aureus*. The genus is capitalized but the species is not. After the full genus name is given in the paper, it can be abbreviated as *S. aureus*, but it is still italicized. This is as long as there is no other genus in the paper that starts with the same letter. (For example, if you have a *Staphylococcus* species and a *Salmonella* species as your unknowns, you cannot abbreviate!)

**MECHANICS:** The lab report should be typed using a 12 point normal font such as Times New Roman or Arial, and should be single-spaced. The lab report should be presented **bound in a report folder**. Reports will be graded on content as well as spelling, grammar, punctuation, and organization.

## **SECTIONS OF THE UNKNOWN LAB REPORT:**

(Note: Other than the title page, the pages of the report must be numbered)

### **TITLE PAGE**

The title page should include the following information:

UNKNOWN NUMBER

YOUR NAME

DATE (the due date)

LAB INSTRUCTOR'S NAME

COURSE NAME / SECTION NUMBER

SEMESTER / YEAR

### **INTRODUCTION**

This section introduces the reader to the study and why the study was done. This should only be a few sentences long. Example: "There are many reasons for knowing the identity of microorganisms. The reasons range from . . . [explain 2-3 reasons why you may want to identify an unknown bacterium]. This study was done by applying all of the methods that have been learned in the microbiology laboratory class for the identification of an unknown bacterium."

### **MATERIALS & METHODS**

This is where the details of the study are listed. The materials and methods section should contain three paragraphs: (1) a description of how the two unknowns were separated and isolated (2) a description of the identification of the Gram-positive isolate and (3) a description of the identification of the Gram-negative isolate.

Be specific, but concise: do not rewrite the lab manual! The most common way is to mention the names of the materials used and **why** they were used, and reference the lab manual for the procedure or method. See example 1.

**Example 1:** "An unknown labeled number 37 was selected. The methods that have been learned in microbiology laboratory were applied to identify this unknown. Procedures were followed as stated in the course laboratory manual (Cain et al, 2015) unless otherwise noted. The first procedure that was performed was streaking the unknown out on a nutrient agar plate, using the T streak method described in the lab manual. This was done in order to . . . [explain why]. After the plates were incubated and grown, the morphology was observed and recorded and a Gram stain was performed. After determining the Gram reaction, specific biochemical tests were performed. The biochemical tests were chosen from the lab manual. Since unknown 37 was determined to be a Gram-positive rod, an endospore stain was also performed. The following types of media were inoculated with unknown 37: starch, lipid, and casein agars, mannitol salt agar, a gelatin deep, a bile esculin agar slant, and a nitrate broth. Table 1 lists the test, purpose, reagents and media components, and results."

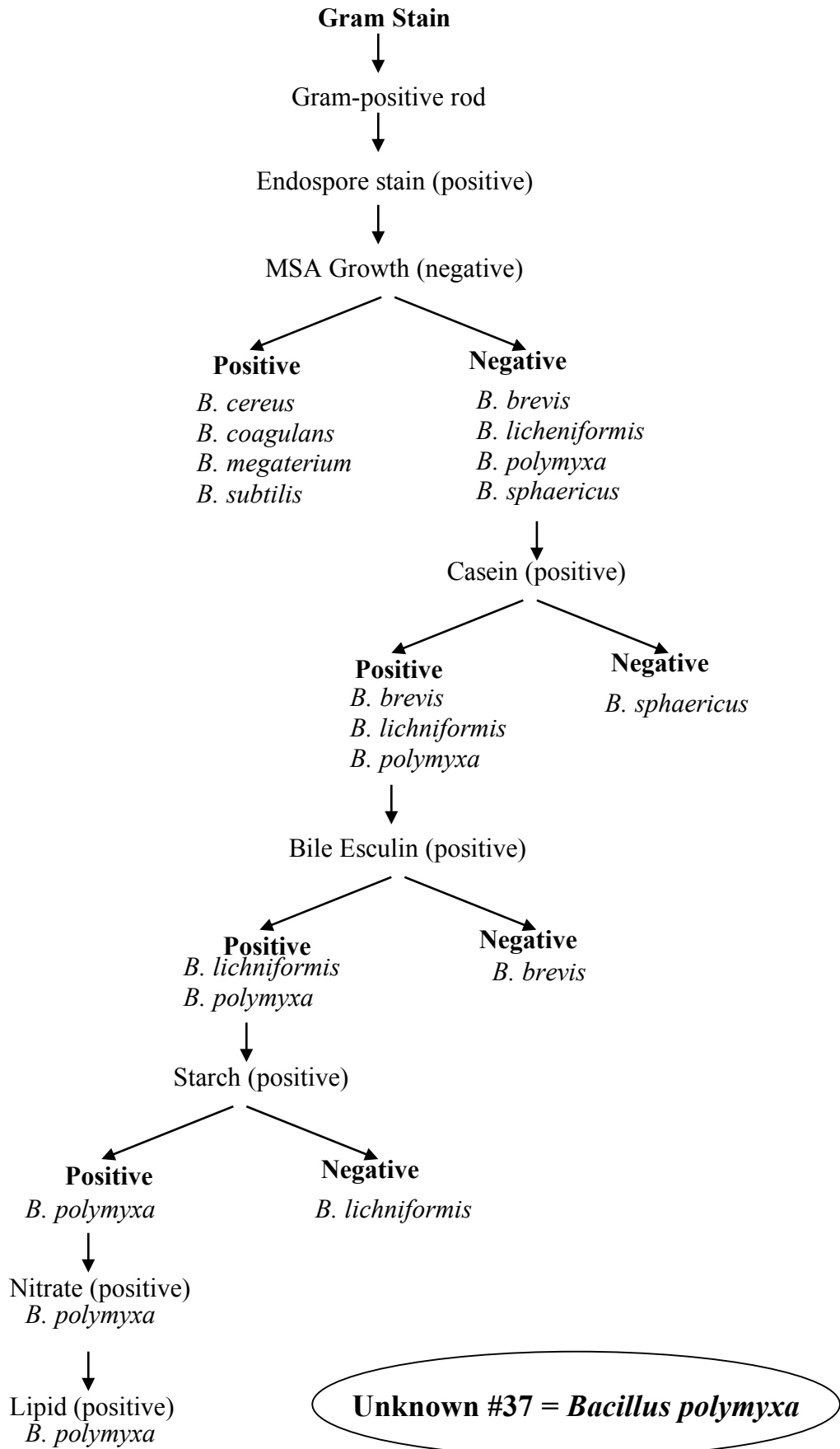
## **RESULTS**

This is where the results are summarized. The results should be presented in 2 tables, using a separate table for each unknown, i.e. one table for your Gram-positive, and another table for your Gram-negative (see example below). This is also where the flow charts showing how you arrived at your answers are presented. The flow charts included in the report should trace the route you followed on your flow chart to identify your unknown. A short paragraph explaining how the results are presented should also be included. Example: "Unknown 37 appeared as a large, mucoid, cream-colored colony on the nutrient agar plate. A Gram stain revealed that the organism was a Gram-positive rod, and an endospore stain demonstrated the presence of subterminal endospores. A stock of unknown 37 was grown on a nutrient agar slant and used to inoculate the tests listed in Table 1. The results of all tests are presented in Table 1 and shown in flow chart form."

**Table 1: Gram-positive Identification**

Test	Purpose	Media Components /Reagents	Observations	Results	Interpretations
Gram Stain	Determine cell wall properties	Crystal violet, iodine, alcohol, safranin	Purple rods	Gram-positive rods	Organism has thick peptidoglycan layer, no outer membrane
Endospore Stain	Determine if spore producer	Malachite green, safranin	Pink rods with small green endospores between the center and the end	Positive for spore formation	Organism produces endospores
Starch	Determine if organism can hydrolyze starch	Starch in plates Added iodine after incubation	Zone of clearing	Positive	Organism has enzymes maltase and amylase needed to hydrolyze starch
Lipid	Determine if organism can hydrolyze lipids	Tributylin in plates	No zone of clearing	Negative	Organism does not possess lipases and cannot hydrolyze lipids
Gelatin	Determine if organism can hydrolyze gelatin	Gelatin in tubes	Tube was still solid after incubating	Negative	Organism does not produce gelatinase

**FLOWCHART: UNKNOWN #37 (GRAM-POSITIVE)**





## DISCUSSION / CONCLUSION

This section interprets the meaning of the results. Again, you should have a separate paragraph for each identified isolate. The following questions should be answered here:

How did the test results lead to identification? What problems were encountered, if any? In this section, you should also include 1-2 paragraphs of background information on each bacterium you identified, including its characteristics and significance.

Example of a discussion: “After several differential tests, it was concluded that the Gram-positive organism in unknown 37 was *Bacillus polymyxa*. After performing the Gram stain, it was determined that the unknown was a Gram-positive rod. The results of the endospore stain showed that the organism produced subterminal endospores, indicating it belongs in the genus *Bacillus*. The organism was grown on a TSA slant for use in inoculating the rest of the biochemical tests. All of the biochemical tests worked well except for the test for gelatin hydrolysis. It gave a false negative result at first, which was inconsistent with the rest of the results. Using the *Bacillus* identification dichotomous key, it was concluded that the unknown was *Bacillus polymyxa*

*Bacillus polymyxa* is a Gram-positive endospore-forming bacillus (Bauman, 2006). It is a motile bacterium, and possesses peritrichous flagella (Todar, 2005). *B. polymyxa* can be found in a wide range of environmental habitats, including acidic environments. It is commonly found in soil, water, and decaying vegetables (Stedman, 2006). Because it can grow in both moderate and cold temperatures, *B. polymyxa* is classified as a psychrotroph. *B. polymyxa* produces a thick polysaccharide capsule, and is also a facultative anaerobe (Todar, 2005). Although *B. polymyxa* does not cause disease in animals or humans, it is of medical significance because it produces the antibiotics polymyxin and colistin (Madigan and Martinko, 2006).

## REFERENCES:

Note: the **minimum** number of references is three, which can include the lab manual and the textbook. You may **not** use wikis (such as Wikipedia or MicrobeWiki) as sources! References should be listed in **APA format**, and **parenthetical in-text citations** must be used where appropriate. Note: Any information presented that is not your original idea or your own data must be credited with an in-text citation!

Use this format to cite the lab manual on your reference page:

Cain, D., Hanks, H., Weis, M., Bottoms, C., and Lawson, J. (2017) *Microbiology Laboratory Manual B2420*. Collin County Community College District, McKinney, TX.

The corresponding in-text citation would be (Cain et al., 2017).

## UNKNOWN WORKSHEET

Attach your completed unknown worksheet.

Modified from: <http://www.sci.sdsu.edu/classes/biology/bio210/lab/demers/Howtowriteanunknownlabrepor.pdf>