# Table of Contents

**Getting Started** ................................................................................................................................................. 3  
  - Microbiology Laboratory Safety Rules ................................................................................................................ 3  
  - Needed for Each Lab .............................................................................................................................................. 3  
  - Things to Know About the Lab ............................................................................................................................. 3  
  - Biosafety Levels .................................................................................................................................................... 4  

**LAB #1: Ubiquity of Bacteria and Microscopy** ..................................................................................................... 5  
  - Lab Exercise 1-1 (Proper Hand-Washing Technique) ............................................................................................. 6  
  - Lab Exercise 1-2 (Bacteria are Ubiquitous) ............................................................................................................ 7  
  - Lab Exercise 1-3 (Introduction to the Light Microscope) ...................................................................................... 8  

**LAB #2: Survey of Microorganisms and Culturing Bacteria** .............................................................................. 14  
  - Lab Exercise 2-1 (Survey of Microorganisms) .................................................................................................... 15  
  - Lab Exercise 2-2 (Aseptic Technique and Culturing of Microorganisms) ............................................................... 19  
  - Lab Exercise 2-3 (Quadrant Streak Plate Method for Colony Isolation) ............................................................... 22  

**LAB #3: Staining I-Positive and Negative Staining of Bacteria** ........................................................................ 25  

**LAB #4: Staining II-Differential Staining** .......................................................................................................... 31  
  - Preparation for the next lab (Lab 5: Selective/Differential Media) .................................................................. 35  

**LAB #5: Selective/Differential Media** ................................................................................................................ 38  
  - Lab Exercise 5-1 (Phenylethyl Alcohol Agar-PEA) ............................................................................................. 40  
  - Lab Exercise 5-2 (Columbia CNA with 5% Sheep Blood Agar-Columbia CNA) .................................................... 40  
  - Lab Exercise 5-3 (Mannitol Salt Agar-MSA) .......................................................................................................... 42  
  - Lab Exercise 5-4 (MacConkey Agar-MAC) ............................................................................................................. 42  
  - Lab Exercise 5-5 (Eosin Methylene Blue Agar-EMB) ............................................................................................ 43  
  - Lab Exercise 5-6 (Hektoen Enteric Agar-HEA) ....................................................................................................... 44  
  - Preparation for the next lab (Lab 6: Physical Growth Factors for Bacterial Growth) .......................................... 47  

**LAB #6: Physical Growth Factors for Bacterial Growth** ...................................................................................... 51  
  - Lab Exercise 6-1 (Fluid Thioglycollate Media) .................................................................................................... 52  
  - Lab Exercise 6-2 (Effect of Temperature on Bacterial Pigments) ........................................................................ 54  
  - Lab Exercise 6-3 (Effect of Temperature on Bacterial Growth) ........................................................................... 54  
  - Lab Exercises 6-4 to 6-8 (Hydrolysis Tests) .......................................................................................................... 55  
    - Lab Exercise 6-4 (Starch Hydrolysis Test) ........................................................................................................ 56  
    - Lab Exercise 6-5 (DNA Hydrolysis Test) ......................................................................................................... 56  
    - Lab Exercise 6-6 (Tributyrin Hydrolysis Test) .................................................................................................. 56  
    - Lab Exercise 6-7 (Casein Hydrolysis Test) ....................................................................................................... 56  
    - Lab Exercise 6-8 (Esculin Hydrolysis Test) ...................................................................................................... 57
Preparation for the next lab (Lab 7: Introduction to Biochemical Test Media).................................57

LAB #7: Introduction to Biochemical Test Media...........................................................................60
Lab Exercise 7-1 (Catalase Test).................................................................................................61
Lab Exercise 7-2 (Oxidase Test)................................................................................................62
Lab Exercise 7-3 (Nitrate Reduction Test) ..................................................................................62
Lab Exercise 7-4 (Phenylalanine Deaminase Test)......................................................................64
Lab Exercise 7-5 (The Kligler Iron Agar Test) .........................................................................65
Lab Exercise 7-6 (Coagulase Test)...........................................................................................68

Preparation for the next lab (Lab 8: Culture and Sensitivity-Identification of an Unknown Enterobacteriaceae and Antibiotic Susceptibility Test).............................................69

LAB #8: Analysis of Culture and Sensitivity (Identification of the Unknown Bacteria) and Analysis of Antibiotic Susceptibility ......................................................................................72
Lab Exercise 8-1 (Simmons Citrate Test)....................................................................................73
Lab Exercise 8-2 (Phenol Red Test)............................................................................................74
Lab Exercise 8-3 (Decarboxylase Test)......................................................................................75
Lab Exercise 8-4 (Urea Hydrolysis Test)....................................................................................76
Lab Exercise 8-5 (Gelatin Test) ................................................................................................76
Lab Exercise 8-6 (SIM Test)........................................................................................................77
Lab Exercise 8-7 (MRVP Test)...................................................................................................78
Lab Exercise 8-8 (Antibiotic Susceptibility Test-Kirby-Bauer Method)....................................79

LAB #9: DNA: Transformation and DNA Damage by Ultraviolet Light .....................................81
Lab Exercise 9-1 (The Lethal Effect of Ultraviolet Light on Microbial Growth)..........................82
Lab Exercise 9-2 (Bacterial Transformation-The pGLO System) ..............................................83

LAB #10: Immunology: ELISA Test ...........................................................................................87
Lab Exercise 10-1 (ELISA)........................................................................................................88
Lab Exercise 10-2 (Epidemiology Simulation) ..........................................................................90

LAB #11: Differential Blood Cell Count.....................................................................................93

Appendix A: Unknown Identification Guide (Guides obtained from Microbiology: Laboratory Theory & Application by Leboffe and Pierce).................................................................97

Appendix B: Zone of Inhibition Interpretation Table (Guide obtained from Microbiology: Laboratory Theory & Application by Leboffe and Pierce)..............................................................100

Appendix C: pH Indicator Guide .............................................................................................100
Appendix D: Example Lab Exam #1 .......................................................................................101
Appendix E: Example Lab Exam #2 .......................................................................................106
References..............................................................................................................................112
Getting Started

Microbiology Laboratory Safety Rules

All students must abide by the following rules to keep the lab experience safe for everyone.

1. No eating, drinking, application of makeup, chewing gum/tobacco in the lab.
2. All students will be wearing lab coats (buttoned) at all times while in the lab, even at times of examination.
3. Students cannot come to lab with open-toed shoes or sandals or flip-flops.
4. While working with bacteria, students must wear their lab coats, goggles, and gloves (sometimes masks).
5. Students must disinfect their workbench at the beginning of lab and at the end of lab.
6. Students must handle all glass tubes by holding the glass tube, not the cap.
7. Students with long hair must have their hair tied.
8. Students must handle the gas burner with extreme care.
9. No mouth pipetting.
10. All students must wash their hands with soap and water before leaving the lab.
11. Restroom use: Students must remove gloves/lab coat before leaving the room to use the restroom.
12. Report any injury to your instructor.

Needed for Each Lab

To participate in the lab, you must have the following items:

1. Your lab coat (will be provided to you by Valencia)-must be kept in the lab.
2. Black Sharpie-you need to purchase one. The Sharpie has to remain in the lab with your lab coat.
4. Closed toe shoes.
5. Pen/Pencil and Notebook.
6. Goggles, masks, and gloves are provided to you by Valencia College

Things to Know About the Lab

Pay close attention to your instructor introducing each of the following items:

- Security telephone (dial 1000 on west campus for security)
- Disinfection solution for lab benches
- Antiseptic had washing solution
- Fire extinguisher
- Emergency Shower an Eyewash station, Fire Blanket
- MSDS folder
- Biohazardous waste containers
- Biohazardous sharps containers
- Broken glassware container
- “Kill Cart”
- Incubators
- Refrigerated cold room
- Biological Safety Cabinet
- Vortex mixers
- Microscopes
- First Aid Kit; Chemical Spill Kit
- Inoculating loops, flint strikers, plastic pipettes, microscope lens cleaner and lens paper, ethanol, distilled water, sterile swabs, test tube racks
- Autoclave (In the Preparation Room)
- Fire Alarm
- Escape routes
- Gas shut off

Biosafety Levels

Biosafety levels (BSL) are precautionary criteria used to contain microorganisms and to prevent harm to personnel. There are 4 BSL levels and they are as follows:

**BSL-1** - Criteria used when handling microorganisms that pose minimal harm, normally do not cause disease. No special equipment needed. Work on open benches approved. Wearing gloves, mask, goggles, lab coat required. No eating or drinking. Standard procedure used to dispose any contaminated material. Disinfection of countertops.

**BSL-2** - Criteria used when handling microorganisms that pose moderate harm, may cause disease. All the standard procedures used in BSL-1 applies in addition to other precautions. These other precautions include special training, limited access, care with sharp items, and the use of biological safety cabinets.

**BSL-3** - Criteria used when handling microorganisms that pose serious harm, cause disease and are potentially lethal. All the standard procedures used in BSL-1 and BSL-2 applies in addition to other precautions. The additional precautions include medically testing personnel as well specific criteria that the facility must fulfill.

**BSL-4** - Criteria used when handling microorganisms that we do not have vaccination or a means to treat. They cause severe disease and death. All the standard procedures used in BSL-1, BSL-2, and BSL-3 applies in addition to other precautions. The additional precautions include the use special protective suits, a decontamination protocol upon personnel exit from the facility, extreme access restriction, among others.

**BSL-1 Bacteria Used in the Lab:** Aquaspirillum itersonii; Aspergillus niger; Bacillus cereus; Bacillus stearothermophilus; Citrobacter freundii; Clostridium sporogenes; Escherichia coli; Enterobacter aerogenes; Micrococcus luteus; Pseudomonas fluorescens; Salmonella typhimurium; Serratia marcescens; Staphylococcus epidermidis

**BSL-2 Bacteria Used in the Lab:** Enterococcus faecalis; Morganella morganii; Proteus mirabilis; Pseudomonas aeruginosa; Shigella flexneri; Staphylococcus aureus; Streptococcus mitis

*No BSL-3 or BSL-4 agents used in this lab.*

Special Note: All images used in this lab manual that are marked with 📷 are images produced by Valencia College students for Valencia College students. Students registered in Dr. Saad’s Microbiology (MCB2010C) classes from summer 2018 to summer 2019 helped participate in producing these images.
LAB #1: Ubiquity of Bacteria and Microscopy

**Lab Objectives:**

1. To learn a proper hand washing technique
2. To show evidence that bacteria are ubiquitous.
3. To become familiar with all the parts of a microscope and their function, as well as the proper use of the microscope.

**Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions):**

1. What is the meaning of the word ubiquitous?

2. What is the meaning of a general-purpose media?

3. Which two parts of the microscope allows for focusing?

4. What is the relationship between magnification and field of view?

5. What is the relationship between magnification and brightness?

6. What is the formula used to calculate total magnification?

7. What is resolution?

8. What is contrast?

9. What is the formula used to calculate resolving power?

10. What are the names of the lenses used to magnify the specimen?
Lab Exercise 1-1 (Proper Hand-Washing Technique)

**Background Information**—Proper hand washing is essential as a preventive measure to contracting disease causing pathogens and illness. We all have to be aware of our surroundings and what we are handling with our hands. Working in the medical field, you will have to become even more aware of your surroundings. You will be expected to wash your hands on a regular basis for example before/after patient contact, before/after procedures, and after coming into contact with body fluids. In the microbiology laboratory, you will also need to take the same precautions, since you will be directly working with culture tubes and plates. In this exercise, you will learn a proper hand-washing technique that you will be expected to follow throughout the semester as you clean up prior to you departing from lab.

**Proper Hand-Washing Technique:**

1. Turn on faucet with disposable paper towel.
2. Wet hands under running water.
3. Apply soap to the palm of your hands and rub them together for at least 20-30 seconds making sure you get every part of your hands, fingers, and nails.
4. Rinse your hands thoroughly under running water.
5. Dry your hands with a disposable paper towel.
6. Use that towel to close the faucet.

**Lab Exercise:**

1. Students will work in pairs.
2. Student #1 will place two drops of the GLO-Solution into the palm of his/her hands (The solution WILL NOT harm you, but do not get on your clothing).
3. Student #2 will handle the UV light source to show student #1 where the GLO-Solution is on his/her hands. The glow observed from the GLO-Solution under UV will be used to mimic a bacterial contamination on your hands.
4. Use the above technique to wash off the GLO-Solution from your hands.
5. Student #2 will use the UV light to show you if you were successful in removing the GLO-Solution.
6. Repeat the hand washing procedure as needed.
7. Record how many times you had to wash your hands to remove GLO-Solution.
8. You and your lab partner will now switch roles.

<table>
<thead>
<tr>
<th># of Hand Washes</th>
<th>Student #1</th>
<th>Student #2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Lab Exercise 1-2 (Bacteria are Ubiquitous)

**Background Information:** The ubiquity (abundance) of bacteria (Figure 1-1) may not be an obvious concept to you because of course you cannot see them all around you due to their microscopic size. In fact, the number of bacteria that live on you and in you outnumber the number of cells that make you who you are. Bacteria are all around us. They are on our skin and in our gut. They are on your desk, bench, doorknobs, clothes, backpack, in the air, in the soil, etc. The goal of this exercise is to do a quick experiment that shows us that bacteria are ubiquitous.

**Lab Exercise:** You will be working with your lab partners to inoculate Trypticase Soy Agar (TSA) plates using samples from the air, the desk, your fingertip, your scalp, and from your throat through coughing. TSA is a general-purpose media used to support the growth of a wide variety of bacteria. The use of general-purpose media is highly recommended when the microbiologist does not know which bacteria he/she is culturing. We also use TSA for general culturing, culture storage, isolation of pure cultures, among other use. You and your lab partners will work together to inoculate the plates today, but analyze them next week. When you examine your plates next week you will see the bacteria growing as colonies on the plate. A colony is a mound of bacteria that are clones of each other. A progenitor bacterium initiated the process by undergoing binary fission to replicate. The resulting clones of the original cell will also undergo binary fission. Binary fission repeats multiple times during the incubation step producing the mound of bacteria. Different species of bacteria can produce different types of colonies that have different size, shape, color, edges, height, appearance, and texture.

**Materials:** 8 sterilized TSA (Recipe: 1.5% Pancreatic digest of Casein, 0.5% Panaic digest of Soybean, 0.5% Sodium Chloride, and 1.5% Agar - 1L Final Volume) plates and two sterile cotton swabs.

**Methods:** Label each plate with a number 1 through 8 using your Sharpie. Always label plates on the bottom half, NOT THE LID. You will also label each plate with your group number, ex. G2 (for group 2). You will always put your group number on everything you setup.

<table>
<thead>
<tr>
<th>SETUP</th>
<th>GROWTH (Yes or No) [Analyze Next Week]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate #1-30 minutes air exposure then incubate at 25°C.</td>
<td>__________________</td>
</tr>
<tr>
<td>Plate #2- Desk sample then incubate at 25°C.</td>
<td>__________________</td>
</tr>
<tr>
<td>Plate #3- Desk sample then incubate at 37°C.</td>
<td>__________________</td>
</tr>
<tr>
<td>Plate #4- Coughing sample then incubate at 37°C.</td>
<td>__________________</td>
</tr>
<tr>
<td>Plate #5- Fingertip sample then incubate at 37°C.</td>
<td>__________________</td>
</tr>
<tr>
<td>Plate #6- Head sample then incubate at 37°C.</td>
<td>__________________</td>
</tr>
<tr>
<td>Plate #7- Keep plate closed then incubate at 37°C.</td>
<td>__________________</td>
</tr>
<tr>
<td>Plate #8- Keep plate closed then incubate at 25°C.</td>
<td>__________________</td>
</tr>
</tbody>
</table>
**Incubation Instructions:**

**Students:** Tape plates #1, #2, and #8 together and place in the 25°C bin for your instructor to incubate. Tape plates #3, #4, #5, #6, and #7 together and place in the 37°C bin for your instructor to incubate. Always place your plate LID DOWN for incubation.

**Instructor:** Distribute plates based on their temperature requirements. A 48-hour incubation is required. Subsequently, place in the cold room until class time.

**Cleanup Instructions:** Dispose the cotton swabs in the sharps container. **NEXT WEEK**, you will be discarding the plates in the orange biohazard bag AFTER you and your group analyzes them.

**Questions**—After you have had a chance to analyze your plates, make a conclusion statement based on your results.

**Statement:** ________________________________________________.

**Lab Exercise 1-3 (Introduction to the Light Microscope)**

**Background Information**—In a microbiology lab, students will use the microscope to view microscopic organisms. The microscope provides magnification, contrast, and resolution.

**Magnification**—The ocular and objective lenses work together to help enlarge the specimen. The ocular lens for the microscopes that we use in the lab has a magnification power of 10X. For the objective lens, you will be able to choose from one of four lenses. The scanning objective has a magnification power of 4X. The low-power objective has a magnification of 10X. The high-power (Dry) has a magnification power of 40X and the oil-immersion lens has a magnification power of 100X. You will have to use an oil with a high refractive index when using the oil immersion objective lens otherwise you will not be able to get the proper resolution to produce a clear image. To calculate total magnification you will have to multiply the magnification of the ocular lens with the magnification of the selected objective lens. Another important feature of microscopy that you will become familiar with as you use the microscope is the relationship between magnification and the size of the diameter of the field of view as well as the level of brightness. As you magnify the image, you will notice that the image is enlarging at the same time you see less of the specimen because the size of the field of view will be getting smaller. The image you will be viewing will also become dimmer as you magnify due to less light reaching the specimen.

**Contrast**—The relative darkness between the specimen and the background surrounding the specimen on the slide is the definition of contrast. To be able to view and distinguish between different aspects of the specimen, the microscope has to have contrast. To achieve contrast using our microscopes, you can do one or a combination of the following:

a. Decrease the brightness using the iris diaphragm lever and/or the rheostat.

b. Stain the specimen.

c. Manipulate the height of the condenser.
Resolution: The microscope will produce a clear image only if it has resolving power, seeing two close objects as two separate objects. Without resolving power, the microscope will produce a blurred image. To calculate the resolving power, use the following formula (NA = Numerical Aperture):

\[
\text{Resolving Power} = \frac{\text{Wavelength of the Light Used (nm)}}{\left[ \text{NA \ objective} + \text{NA \ Condenser} \right]}
\]

The Compound Light Microscope: This type of microscope uses multiple lenses to magnify the specimen (an ocular lens and an objective lens). The microscope that you will be using uses parfocal lenses, which allows for minor focus adjustments after the initial focusing is done, using the scanning objective. The microscope will also allow you to adjust the eyepieces for your specific inter-pupillary distance. The eyepiece will also have a focusing knob that helps correct for a difference in focusing power that you might have between your right and left eye. To use the...
**oil immersion lens** you will have to add an oil with a **high-refractive index** onto the slide prevent the refraction of light that would otherwise result in the loss of light and the lack of visibility. The microscope image that you see in Figure 1-2 will introduce you to all the parts of the microscope and their function.

**Experiment**—Each student in your group will use his/her own microscope to become familiar with all the parts of the microscope and their functions.

**Materials**—One compound light microscope and a prepared letter “e” slide.

**Methods**—

1. Make sure you go over all the different parts of the microscope and their functions.

**Lab Exercise**—With the microscope in front of you, fulfill the following written exercises (NA=Numerical Aperture).

<table>
<thead>
<tr>
<th>Lens Name</th>
<th>Ocular Lens Magnification</th>
<th>Objective Lens Magnification</th>
<th>Total Magnification</th>
<th>Condenser (NA)</th>
<th>Objective (NA)</th>
<th>Resolving Power (nm) @ 500nm Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scanning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-Power</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-Power (Dry)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oil immersion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Use the microscope to focus the letter “e” slide using the scanning objective and the low power, and high-power (dry) objectives. **MAKE SURE YOU PLACE THE LETTER “e” SLIDE RIGHT SIDE UP.**

**Procedure to focus the microscope:**

a. Plug in your microscope.
b. Turned it on.
c. Open the iris diaphragm to allow the light to get to the condenser.
d. Make sure you position the scanning objective in place.
e. Drop the stage all the way down using the course-focusing knob.
f. Place the slide flat onto the stage and make sure that the stage clip is holding the slide in place. This will allow you to use the stage manipulator knobs to position your slide over the condenser.
g. Once you have the specimen positioned over the condenser, slowly move the stage upwards using the course-focusing knob until you see the image through the ocular lens.
h. Use the fine focusing knob to do any final focusing adjustments.
i. When you increase the magnification, DO NOT touch the course focus knob, use only the fine focus knob when you are using the 10X, 40X, and 100X objectives.
Lab Exercise - Draw the images that you see using the letter “e” slide using the scanning, low-power, and high-power objectives.

Questions:

1. Compare the letter “e” image that you are viewing compared to the right side up letter “e” on the slide?

2. What happened to the letter “e” image as you moved from the scanning to the low-power and from the low-power to the high-power (Dry)?

3. What is happening that explain why you see only part of the letter “e” under the High-Power objective versus the scanning objective?

Procedure to preparing the microscope for storage:

a. Turn off the microscope.
b. Remove your slide and put the scanning objective in place.
c. Bring the stage up to give you room to wrap the cord in front of the illuminator snugly.
d. Position the plug under the wire to hold it in place and then drop the stage all the way down and push the stage all the way back.
e. Dial down the rheostat all the way.
f. Clean all the lenses with lens cleaning solution and lens cleaning paper. MAKE SURE YOU TAKE YOUR TIME CLEANING, ESPECIALLY WITH THE OBJECTIVE LENSES THAT HAVE OIL ON THEM.
g. Store the microscope in the cabinet with the stage facing in and the microscope’s arm facing out.
h. If you used prepared slides, clean them and place them back in their designated cassette (holder).
i. If you prepared slides, clean or discard them following your instructor’s directions.
Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):

Letter Choice-
A. Arm
B. Base
C. Coarse Adjustment
D. Diaphragm
E. Diaphragm Lever
F. Fine Adjustment
G. High Power Objective
H. Illuminator or Lamp
I. Inversion
J. Low Power Objective
K. Nosepiece
L. Ocular Lens
M. Scanning Objective
N. Stage
O. Stage Clips
P. Condenser

Numbered Options-

1. Contains a magnifying lens which you look into.
2. Holds the slide in place.
3. Holds the objectives and can be rotated to change the magnification.
4. Moves the body tube slightly to sharpen the image.
5. Moves the body tube to focus the image.
6. Occurs when the image is reversed in the field of view.
7. Open and closes the diaphragm.
8. Produces light or reflects light up towards the eyepiece.
9. Provides a magnification of 10X.
10. Provides a magnification of 40X.
11. Provides a magnification of 4X.
12. Regulates the amount of light passing up toward the eyepiece.
13. Supports the body tube.
14. Supports the microscope.
15. Supports the slide being-observed.
16. Focuses the light onto the slide.

17. A microscope with an ocular lens (15X) was used to magnify a specimen on a slide at each objective lens magnification (4x, 10x, 20x, 40x, 60x, 100x). What is the total magnification achieved at each setting:

4x lens: __________________________ 10x: __________________________
20x lens: __________________________ 40x: __________________________
60x lens: __________________________ 100x: __________________________
18. Using a wavelength of light (520nm), which of the four objective lenses would provide you with good resolving power to produce a clear image of specimens that are 285nm apart? Give all that apply and show proof (Give Values).

4x objective: ________________________________
10x objective: ________________________________
40x objective: ________________________________
100x objective: ________________________________

19. Convert 320 nm (nanometer) into μm (micrometer) ___________________.
   Convert 3 mm (millimeter) into nm (nanometer) ___________________.
   Convert 20 μm (micrometer) into mm (millimeter) ___________________.
   Convert 4000 μm (micrometer) into nm (nanometer) ___________________.
   Convert 4 mm (millimeter) into μm (micrometer) ___________________.
   Convert 1 cm (centimeter) into μm (micrometer) ___________________.

Conversion Factors: 1 cm = 10 mm; 1 mm = 1000 μm; 1 μm = 1000 nm

20. What is the maximum magnification achieved using the microscope that you are using in the lab?

21. What is the maximum resolving power achieved using the microscope that you are using in the lab (assume a wavelength of 500nm)?

22. In lab exercise 1-2-Bacteria are Ubiquitous, we used undefined media to grow bacteria. We also call this type of media complex media or a general-purpose media, because we do not know the exact composition of the media. What benefit did that type of media provide you with respect to growing bacteria?
LAB #2: Survey of Microorganisms and Culturing Bacteria

Lab Objectives:
1. To survey a variety of different types of microorganisms.
2. To learn the aseptic technique and the culturing of bacteria.
3. To learn how to do a quadrant streak plate to isolate bacteria from a mixed culture into pure colonies.

Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions):
1. What is the difference between nutrient broth and nutrient agar?

2. What is the difference between defined and undefined media?

3. What does aseptically mean?

4. What will you be doing to the inoculating loop for you to sterilize it?

5. What is a culture?

6. Describe the difference between a pure culture and a mixed culture.

7. Explain why we are going to do the quadrant streak plate.

8. What is a colony?

9. What physical state must your media be in for a colony to form?

10. What are the different ways of categorizing media?
**Reminder:** Before you start lab #2, please work with your lab partners to analyze the plates that you inoculated for lab exercise 1-2 from last week.

**Lab Exercise 2-1 (Survey of Microorganisms)**

**Background Information**—We can categorize a cell as either **prokaryotic** or **eukaryotic**. The main difference is that prokaryotic cells do not have membrane bound organelles, whereas eukaryotic cells have membrane bound organelles. That is why prokaryotic cells do not have a true-nucleus, whereas eukaryotic cells do. A few prokaryotic cells have cellular structures with membranes surrounding the cellular structures making them exceptions to the rule. Eukaryotic cells also have other organelles like the mitochondria, chloroplast, lysosome, Golgi apparatus, endoplasmic reticulum, among others. The only prokaryotes are bacteria and archaea, both of which are microscopic and unicellular organisms. Among the eukaryotes, there are unicellular as well as multicellular representatives that are both microscopic and non-microscopic. The different types of eukaryotic microorganisms include fungi, protozoa, algae, and helminths (worms). The last type of microorganisms are viruses, which are neither prokaryotic nor eukaryotic.

**Prokaryotes:**

**Bacteria (Figure 2-1):** All bacteria are unicellular and microscopic. Bacteria are so diverse that they make up one of the three domains of life. Most bacteria have a cell wall made up of peptidoglycan. In some bacteria, the cell wall is thick, whereas in others the cell wall is thin. The cell wall provides protection for the bacteria. The bacterial cell with a thin cell wall will also have an outer membrane that works to help further protect it. The smallest known bacteria, mycoplasma, does not have a cell wall but its cell membrane is tough enough to protect the cell. Most bacteria do not have intracellular membranes. There are a few examples of bacterial species that do form intracellular membranes. Some of the intracellular membranes are involved in photosynthesis (in photosynthetic bacteria), while others wrap around storage units. Some of the intracellular membranes are composed of the standard phospholipid, whereas others are composed of proteins forming bacterial micro-compartments. Most bacteria have at least one or two circular chromosomes and some also have an extrachromosomal (not part of the main chromosome) circular DNA called a plasmid. Some bacteria are able to transfer a copy of their plasmid into a recipient bacterium through the formation of a pilus by a process called conjugation. The ribosomes used for protein synthesis in bacteria are 70S in size. Some bacteria have flagella for motility. Some bacteria have a capsule for protection. Some bacteria have the ability to form endospores under harsh environmental conditions. Fimbriae are hair-like structures used for attachment by the bacteria that possess them. Of course, some bacteria are pathogenic, whereas others are non-pathogenic and actually serve very important functions that are beneficial to us.

**Archaea:** Like bacteria, archaea are also highly diverse that they form another domain of life. Some archaea live in normal environments and other live in and prefer harsh environments (extremophiles). Some of these extreme environments include high salt (halophiles), low pH
(acidophiles), high pressure (barophiles), extreme hot temperatures (hyperthermophiles), and low temperatures (psychrophiles). They share some features with bacteria, but they also share some features with eukaryotic organisms. Their cell wall is composed of a protein layer called an S-layer others have what appears to be a structure like a peptidoglycan but it is not a true peptidoglycan (pseudo-peptidoglycan). There are some photosynthetic archaea. Similar to bacteria, archaea do not have membrane bound organelles. To date, archaea has not been associated with any human disease.

**Eukaryotes:**

**Fungi (Figure 2-2):** There are fungi that are microscopic (yeast and mold) and other fungi are macroscopic (mushrooms). These eukaryotic organisms play a very important role in recycling by helping decompose dead matter alongside with bacteria. They do contain a cell wall, but its chemical composition is different from that of the peptidoglycan of bacteria and the cellulose of plant cells. Yeast divides by budding to form attached cells called pseudo-hyphae. Other fungi form a network of branches (hyphae) that collectively form a structure referred to as a mycelium. These fungi can replicate both sexually and asexually. Some types of fungi do cause disease in humans like ringworm and athlete’s foot. Others can infect plants, while other fungi live with plants in a symbiotic relationship. We benefit greatly from fungi. We use fungi to make bread, wine, beer, food flavoring, and antibiotic production.

**Protozoa (Figure 2-3):** Protozoans are unicellular organisms that live in fresh water habitats. Most protozoans require a close association with water, while others can form cysts and become dormant in dry environments. They do not have a cell wall, but instead have contractile vacuoles that store the water rushing into the cells and then squeezes it back out by contracting its vacuoles. These contractile vacuoles protects these organisms from lysing. With respect to their motility, protozoans use cilia, flagella, or pseudopodia. Other protozoans are non-motile. A number of protozoans are harmless, whereas others cause disease in humans like malaria, sleeping sickness, and toxoplasmosis.

**Algae (Figure 2-4):** Algae are photosynthetic organisms that have a cell wall. The main type of photosynthetic pigment is chlorophyll (green pigment), but some algae have different types of pigments (red, brown, and yellow). The different types of pigments will produce algae that exhibit different colors. Algae come in different forms that range from unicellular to multicellular. Other forms include colonies, while others form filaments. Algae play a major role in the aquatic food web. Algae...
also play a major role in contributing to the earth’s oxygen alongside with the other photosynthetic microorganisms (photosynthetic bacteria).

**Helminths (Figure 2-5):** Helminths are parasitic worms. A number of worms infect the intestines. Some helminths infect blood vessels, the eye, and deep layers of the skin. Some example diseases include pinworm and river blindness.

**Neither Prokaryotic nor Eukaryotic-Acellular:**

**Viruses:** Viruses are made up of a protein container (capsid) filled with either DNA or RNA. Outside of the host organism, viruses are inactive. For viruses to become active, they need to infect a host cell. For this reason, viruses are obligate intracellular parasites. A virus will show host specificity as well as cell specificity. Once the host cell is infected, the host cell will become a manufacturing site for new viral copies. During this process, the host organism will become diseased. Viruses play a major role in human disease as in AIDS, influenza, genital herpes, and chickenpox. They can also infect bacteria, plants, and other organisms.

**Experiment:** Survey a variety of microorganisms using the microscope.

**Materials:** Microscope and prepared slides.

**Methods:**

1. Obtain different slides representing different types of microorganisms (Bacteria, Fungi, Protozoa, Algae, and Helminths).

2. Place each slide on the stage and initially focus using the 4X objective.

3. Magnify using either the 10X or 40X (your choice).

4. Do your best to draw what you see.

5. After you are done, place the slides back in their respective cassettes and prepare the microscope for storage.

**Lab Exercise:** Drawings of images of your chosen slides. Give the name of the microorganism and the total magnification used and the resolving power (**assume a 500nm wavelength of light**).
<table>
<thead>
<tr>
<th>Name of Microorganism:</th>
<th>______________________</th>
<th>______________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Magnification:</td>
<td>______________________</td>
<td>______________________</td>
</tr>
<tr>
<td>Resolving Power:</td>
<td>______________________</td>
<td>______________________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Microorganism:</th>
<th>______________________</th>
<th>______________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Magnification:</td>
<td>______________________</td>
<td>______________________</td>
</tr>
<tr>
<td>Resolving Power:</td>
<td>______________________</td>
<td>______________________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Microorganism:</th>
<th>______________________</th>
<th>______________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Magnification:</td>
<td>______________________</td>
<td>______________________</td>
</tr>
<tr>
<td>Resolving Power:</td>
<td>______________________</td>
<td>______________________</td>
</tr>
</tbody>
</table>
Lab Exercise 2-2 (Aseptic Technique and Culturing of Microorganisms)

**Background Information:** Culturing bacteria requires the use of media as a source of nutrients to support their growth. Media will provide bacteria adequate amounts of amino acids, sugar, salt, pH, among other important nutrients. The microbiologist uses water to dissolve media then distributes the media into tubes/flasks prior to autoclaving it to achieve sterilization. Liquid media, broth, is one physical state used to grow bacteria. The microbiologist may choose to use media in a solidified state or even in a semi-solid state. The microbiologist achieves this by adding agar into the media prior to autoclaving. The amount of agar added is the determining factor between the solid to semi-solid state. The microbiologist pours
media containing agar into tubes placed vertically until they cool and solidify. This provides the microbiologist tubes used for stabbing with the **inoculating needle**. The microbiologist places the agar media to cool at a 45° angle to get a **slant tube** or pours agar media into sterile culture plates to get **agar plates**.

Transferring an inoculum (sample) from a tube containing a culture into a sterile tube/flask or plate containing media requires the use of the **aseptic technique** (Figure 2-7). The aseptic technique helps prevent **contamination**. To use the aseptic technique, all students will follow the procedure given below:

Through the observation of the **bacterial growth in broth** (Figure 2-8), the microbiologist will describe the growth as pellicle, sediment, turbid, or flocculent. A **pellicle** growth pattern is a bacterial growth in broth media that is located only on the top of the broth. Bacteria that produce pellicle growth grow on top of the water-based media because they produce a hydrophobic substance in their cell wall. Due to the hydrophobicity of this substance, these bacterial species can only grow on top of the broth similar to how oil stays on top when added to water. A **sediment** growth pattern is a bacterial growth in broth that is strictly on the bottom of the broth. These bacterial species have no means of locomotion so they settle to the bottom as long as the tube is left without shaking during incubation. A **turbid** (cloudy) growth is a bacterial growth in broth media that is all throughout the entire tube. Bacterial species containing flagella will produce turbid growth due to their movement throughout the broth media. A **flocculent** growth pattern of bacteria appears as clumps of bacteria sticking to each other.

We characterize bacterial growth in slant tubes using a number of different properties. The texture property has two options, which are moist versus dry (**friable**). Another property used to describe the growth is based on the growth’s optical properties, which are either opaque versus translucent. Margins (Figure 2-9) is another property used in describing the growth on top of a slant. Margins include smooth-even (**filiform**), spreading (**diffuse**), beaded, root-like (**rhizoid**), spiny-pointed (**echinulate**), and branched (**arborescent**). Finally, color or pigmentation is another property that used to characterize the bacterial growth since some bacteria are able to produce...
pigments and others are non-pigmented. The pigments produced by different bacteria can differ in color.

**Experiment**-Using the aseptic technique, you will be practicing the transfer of an inoculum from a culture tube (broth/slant) or agar plate into a sterile broth/slant tube. You will also obtain a sample from your surroundings (1 sample/group) to inoculate an agar plate.

**Materials**-4 nutrient broth tubes (NBT-Recipe: 0.5% Peptone, 0.1% Meat Extract, 0.2% Yeast Extract, 1.5% Agar, 0.5% Sodium Chloride-Final Volume 1L); 4 nutrient agar slants (NAS-Recipe: 0.5% Peptone, 0.3% Beef Extract/Yeast Extract, 1.5% Agar, 0.5% Sodium Chloride-Final Volume 1 L); 1 TSA plate. Cultures: *Micrococcus luteus* in broth and on a slant and on an agar plate. *Escherichia coli* on a slant. 1 tube with sterile water. 1 sterile cotton swab. Inoculating loop and Bunsen burner.

**Methods**-This procedure is designed for a group of 4 students. If you have less than 4 students, you will just have to work together to get all of them done. Each student should do one combination as follows:

**Student #1**: Inoculates one NBT and one NAS with the *Micrococcus luteus* in broth. Label your NBT tube NBT #1 and your NAS tube NAS #1.

**Student #2**: Inoculates one NBT and one NAS with the *Micrococcus luteus* in slant. Label your NBT tube NBT #2 and your NAS tube NAS #2.

**Student #3**: Inoculates one NBT and one NAS with the *Escherichia coli* in slant. Label your NBT tube NBT #3 and your NAS tube NAS #3.

**Student #4**: Inoculates one NBT and one NAS with the *Micrococcus luteus* on agar plate. Label your NBT tube NBT #4 and your NAS tube NAS #4.

-Pick one location from your surroundings to sample with a wet cotton swab and inoculate one agar plate. This is your environmental sample. Label your plate with which sample your group chose to do.

**Incubation Instructions**: All tubes/plate incubated at 37°C by your instructor. You will analyze them the following week. A 48-hour incubation is required. Subsequently, place in the cold room until class time.

**Lab Exercise**: Examine your tubes and fulfill the following exercise (During the next lab):

<table>
<thead>
<tr>
<th></th>
<th>Does Your Tube Show Growth?</th>
<th>Characterize the Growth?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT #1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBT #2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBT #3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBT #4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAS #1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAS #2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAS #3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAS #4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Cleanup Instructions:** Remove all labels using a paper towel moistened with ethanol. Place your cultures on the cart near the door. Separate the liquid tube media from the slant tubes.

**Lab Exercise 2-3 (Quadrant Streak Plate Method for Colony Isolation)**

**Background Information** - The 5 I's of microbiology include inoculation, incubation, isolation, inspection, and identification. You already did three of them inoculation, incubation, and inspection. Isolation of bacteria is very important in producing a pure culture, also referred to as an axenic culture. There are different laboratory techniques used to produce a pure culture, which include the quadrant-streak-plate method, the loop-dilution method, and the spread technique. In this lab, we will use the more convenient quadrant-streak-plate method (Figure 2-10). This technique requires you to follow the steps precisely to achieve good results, but it also requires some practice.

**Experiment** - Each student will produce a quadrant streak plate using a mixed culture containing two species of bacteria.

**Materials** - 1 agar plate per student; 1 empty sterile tube; 2 sterile plastic pipettes; 1 broth culture tube of *Staphylococcus epidermidis*; 1 broth culture tube of *Serratia marcescens*. Inoculating loop and Bunsen burner.

**Methods** -

1. One lab partner will intentionally mix 1 ml of each culture, *Staphylococcus epidermidis* and *Serratia marcescens*, into the empty sterile tube using the sterile plastic pipettes.

2. All your lab partners will use the same mixed tube to produce their own quadrant streak plate.

3. Use the following quadrant streak plate method (Figure 2-11) to produce your plate:

4. **Incubation Instructions:** Incubate your plate at 37°C. A 48-hour incubation is required. Subsequently, place in the cold room until the next lab.
Lab Exercise - Sketch a representation of your quadrant streak plate (Next Week).

Did you get isolation? ________________

In which quadrant did you get your best isolation? ________________

Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):

1. What is the main difference between prokaryotic cells and eukaryotic cells?

2. Complete the following table:

<table>
<thead>
<tr>
<th>Domain</th>
<th>Bacteria</th>
<th>Archaea</th>
<th>Eukarya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Cell? (prokaryote or eukaryote)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus Present? (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria Present? (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptidoglycan in Cell Wall Present? (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroplast Present? (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscopic Only? (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unicellular Only? (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extremophile? (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Viruses are NOT prokaryotic nor are they eukaryotic, Why?
4. Give an example of a photosynthetic eukaryotic cell.

5. Which of the microorganisms have a cell wall?

6. Why is the aseptic technique important to do when you are inoculating tubes?

7. What is the purpose of the quadrant-streak-plate method?

8. What are the 5 I’s of microbiology?

9. What do you have to add to broth media to solidify it?

10. What do we do to sterilize media?

11. List and describe all the different growth patterns observed on slants and in broth media.

12. Which technique would you use if your goal were to isolate single colonies of different types of bacteria from an overnight culture with mixed bacteria?

13. Why do some cultures grown in broth appear to be turbid while others appear to produce a sediment at the bottom of the broth tube?
LAB #3: Staining I-Positive and Negative Staining of Bacteria

Lab Objectives:
1. To learn and practice how to focus using the oil immersion lens.
2. To learn and practice how to produce a fixed bacterial smear slide to use for staining.
3. To learn and practice how to do positive staining of bacteria.
4. To learn and practice how to do negative staining of bacteria.
5. To distinguish between the most common shapes and arrangements of bacteria.

Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions):
1. List and describe all the different bacterial shapes.

2. List and describe all the different bacterial arrangements.

3. Write the steps involved in making a bacterial smear.

4. Describe, how a basic stain works?

5. Describe, how a negative stain works?

6. Give 2 examples of basic stains.

7. Give 1 example of an acidic stain.

8. You will need to use the 100X objective lens today to get the highest magnification possible. What do you have to use along with this objective to be able to get a focused image.

9. Give two means by which you can manipulate the microscope or specimen to increase contrast.
Reminder: Before you start lab #3, please work with your lab partners to analyze the plates that you inoculated for lab exercise 2-2 and 2-3 from last week.

Background Information-Bacteria come in three main shapes along with a number of different arrangements (Figure 3-1). The main bacterial shapes are coccus (spherical/round), bacillus (rod-shaped), spirillum (rigid-spirals), and spirochetes (flexible-spirals). Bacterial arrangements result when bacterial cells stay attached to each other at the end of binary fission. Coccus shaped bacteria can form an arrangement of two-diplo, packets of four-tetrad, a chain-strepto, irregular cluster-staphylo, and cubical packets-sarcinae. Bacillus shaped bacteria can form an arrangement of two, as well as chains like coccus bacteria. Bacillus shaped bacteria can also form a picket-fence appearance-palisades.

To observe these bacterial shapes and arrangements, the microbiologist will need to use the objective with the best magnification and resolving power to produce clear images of these microscopic organisms. The use of the oil-immersion lens will be required when viewing bacteria due to their small size. When using this objective lens, the microbiologist is required to add oil with a high-refractive index directly onto the slide. The microbiologist focuses the image as he/she progresses through all the other objectives from the lowest to the highest magnification. The microbiologist will then rotate the nosepiece and positions it in between the highest magnification lens and the oil-immersion lens. This gives the opportunity to add a few drops of oil onto the slide. The microbiologist positions the oil-immersion lens in place and fine-focuses the image for viewing.

Another critical feature of microscopy is contrast. To view specimens using the microscope, the microbiologist has to adjust contrast, which is the darkness of the background in relationship to the specimen. Achieving contrast is through the manipulation of the lighting using the intensity knob, the iris diaphragm, and/or the height of the condenser. Another means of achieving contrast is through staining of the specimen itself. A staining procedure called positive (simple) staining (Figure 3-2) uses a cationic (positively charged) stain to increase sample contrast. There are a number of different kinds of cationic stains, but the ones that you will be using are Methylene Blue, Crystal Violet, and Safranin. Following a standard procedure (described later), the microbiologist adds the stain to cells fixed onto the slide. The positively charged stain will interact electrostatically with the overall negatively charged environment of the cell achieved. The negatively charged environment is the result of a predominant number of molecules that are negatively charged located within the cell’s interior (DNA and certain proteins). The microbiologist removes excess stain from the background through a wash step. To wash the slide without losing the specimen, the specimen must be heat-fixed onto the slide. A step that dehydrates the cells and helps them attach to the glass slide.
There is yet another staining procedure used called **negative staining** (Figure 3-3). In this procedure, the microbiologist uses anionic (negatively charged) stain **Nigrosin** that is repelled by the negatively charged cell while sticking to the glass slide (the background). In this procedure, there is no heat-fixing step needed, because there is no need to do a wash step. The background will stain, but not the cells. Negative staining is ideal at times when the microbiologist needs to measure cell size accurately. This is because there will be no dehydration of the cell that would otherwise occur due to fixing, hence, no alteration in cell size.

**Experiment**-Each student will produce two stained bacterial slides, one positive stain and one negative stain. At least one member of your group will produce a stained slide using human cheek cells.

**Materials**-Each lab member will have three glass slides. A choice of positive stain (Methylene Blue, Crystal Violet, or Safranin) and culture tube (**Micrococcus luteus**, **Bacillus cereus**, **Aquaspirillum itersonii**). The negative stain Nigrosin. Inoculating loop and Bunsen burner. Water bottle dropper. Paper towel. Staining tray and slide rack. Clothes pin. Bibulous paper. Microscope, lens cleaning paper, and lens cleaning solution. High-refractive index oil. One toothpick.

**Methods**-

**Student 1**: Use one of the glass slides to make a positive stain of **Micrococcus luteus** with ONE of the stains (Methylene Blue or Crystal Violet or Safranin). The same student will use the other two glass slides to make a negative stain slide of **Micrococcus luteus** with Nigrosin. After the staining procedure, place slide on the microscope and view under oil-immersion and share with your lab partners. Each student should view the coccus shaped bacteria using your prepared slides under positive and negative staining.

**Student 2**: Use one of the glass slides to make a positive stain of **Bacillus cereus** with ONE of the stains (Methylene Blue or Crystal Violet or Safranin). The same student will use the other two glass slides to make a negative stain slide of **Bacillus cereus** with Nigrosin. After the staining procedure, place slide on the microscope and view under oil-immersion and share with your lab partners. Each student should view the bacillus shaped bacteria using your prepared slides under positive and negative staining.

**Student 3**: Use one of the glass slides to make a positive stain of **Aquaspirillum itersonii** with ONE of the stains (Methylene Blue or Crystal Violet or Safranin). The same student will use the other two glass slides to make a negative stain slide of **Aquaspirillum itersonii** with Nigrosin. After the staining procedure, place slide on the microscope and view under oil-immersion and share with your lab partners. Each student should view the spiral shaped bacteria using your prepared slides under positive and negative staining.

**Student 4**: Use one of the glass slides to make a positive stain your choice of **Micrococcus luteus**, **Bacillus cereus**, or **Aquaspirillum itersonii** with ONE of the stains (Methylene Blue or Crystal Violet or Safranin). The same student will use the other two glass slides to make a negative stain slide of your choice of **Micrococcus luteus**, **Bacillus cereus**, or **Aquaspirillum itersonii**.
itersonii with Nigrosin. After the staining procedure, place slide on the microscope and view under oil-immersion and share with your lab partners.

One member of the group-Scrape your gum line to obtain a sample of cheek cells and oral bacteria using a clean toothpick. Place on your sample on the slide and stain with Carbolfuchsin.

Use the following images as a guide on how to produce the positive and negative stained slides.

**Procedure for positive staining (Figure 3-4):**

1. Using your water bottle, place a single drop on your inoculating loop and transfer the drop of water into the center of the slide.

2. Use the inoculating loop to transfer a sample amount of bacteria onto the slide into the drop of water and swirl the bacteria around to get a large smear.

3. Spread the smear wide enough to get the cells to separate and the sample to air dry easy.

4. Heat fix the slide by quickly passing the slide through the flame 2 or three times. Use clothes pin to hold the slide while heat fixing the slide.

5. Place the slide on top of the slide rack inside the staining tray. Add your stain of choice onto the sample for 1 minute.

6. Hold slide at 45° angle and use the water bottle to wash slide.

7. Blot dry the slide using bibulous paper.

8. View using the 100X objective with oil.

9. **Slide clean up instructions:** Scrub slide with a damp paper towel containing detergent, rinse once, place slide in the acid dish.

**Procedure for negative staining (Figure 3-5):**

1. Using one slide, place a single drop of Nigrosin towards the short side of the slide.

2. Using your inoculating loop, transfer a small amount of bacteria directly in the stain.

3. Using your other glass slide at a 45° angle, touch the sample using the small edge of the slide and drag-smear the sample all the way down the long-length of the slide.

4. Leave slide to air dry; view slide using the 100X objective with oil.

5. **Slide clean up instructions:** Discard both slides in the sharps container.
**Other Clean-Up Instructions:** It is very important to clean each objective lens of your microscope thoroughly using lens cleaning paper and lens cleaning solution. Get your microscope ready for check out by your instructor.

❖ **Lab Exercise**- Draw a sketch of each of the three shapes using **positive staining (LEFT PANEL)** and **negative staining (RIGHT PANEL)**. Give total magnification and resolving Power (assume a 500nm wavelength of light).

<table>
<thead>
<tr>
<th>Name of Microorganism</th>
<th>Total Magnification</th>
<th>Resolving Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micrococcus luteus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aquaspirillum itersonii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aquaspirillum itersonii</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):

1. Which staining procedure would you use if you wanted to measure the size of the cell accurately? Why?

2. What is the difference between the strepto- and staphylo- arrangements?

3. What is a sarcinae arrangement?

4. How does a microbiologist achieve contrast, when using a microscope?

5. What is a palisade arrangement?

Sample: Human Cheek Cells
Total Magnification: ____________________
Resolving Power: ____________________
LAB #4: Staining II-Differential Staining

Lab Objectives:
1. To learn about different types of differential staining procedures.
2. To practice the Gram staining procedure.
3. To produce a Gram slide that is free of clumping and that clearly shows both Gram-positive and Gram-negative cells under oil immersion.
4. View acid-fast and endospore prepared slides.
5. To setup the plates for Lab #5 (Selective/Differential Media).

Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions):
1. What type of staining is Gram staining?
2. What is the main difference between a Gram\(^+\) and Gram\(^-\) bacterium?
3. What type of staining is Acid-Fast staining?
4. What is the main difference between an Acid-Fast and non-Acid-Fast bacterium?
5. What type of staining is endospore staining?
6. When do endospore-forming bacteria produce endospires?
7. List all the stains and/or reagents used in the Gram staining procedure. List them in order of use.
8. Make a systematic brief protocol that you will be using to do the Gram staining.
Background Information-Differential staining procedures help distinguish between different bacterial species. The three different types of differential staining procedure that we will focus on are **Gram staining**, **acid-fast staining**, and **endospore staining**. Both Gram staining and acid-fast staining are based on a difference that if found in the cell envelope of different bacterial species. Endospore staining looks for the presence or absence of an endospore.

**Gram Staining**—The cell envelope of a number of bacterial species is made up of three layers: the cell membrane (bilayer of phospholipids), the cell wall (made up of peptidoglycan), and an outer membrane (bilayer of phospholipids and lipopolysaccharides in the outer leaflet). In other bacterial species, only the cell membrane and the peptidoglycan cell wall make up the cell envelope. One interesting difference between these two different types of bacteria is the thickness of the cell wall. Bacterial species that have a three-layered cell envelope have a thin cell wall, whereas the bacterial species with a two-layered cell envelope have a thick cell wall. The bacteriologist **Hans Christian Gram** took advantage of the difference in the cell wall’s thickness and developed a staining technique. The Gram staining technique helps distinguish between the two different types of bacterial species based on the thickness of the cell wall. The **Gram-positive** bacterial species are those with a thick cell wall, whereas **Gram-negative** bacterial species are those with a thin cell wall. The staining procedure starts with the staining of both the Gram-positive and Gram-negative bacteria with **crystal violet**. They will both stain purple. The second step involves adding **Gram’s iodine**. This step helps trap the crystal violet in the Gram-positive cell wall. There will be less trapping of the crystal violet in the Gram-negative bacteria because its cell wall is much thinner than that of the Gram-positive bacterial species. The third step involved the use of the **decolorizer (ethanol)**. In this step, the stain of the Gram-negative cells is lost much easier than that of the Gram-positive cells. Furthermore, the ethanol destroys the outer membrane of Gram-negative cells. The effects of the ethanol on the Gram-negative cells causes them to lose their purple color and become colorless once again, while the Gram-positive cells retain the purple color. The final step is adding the **counterstain (safranin)**, which now stains the Gram-negative cells pink/red (Figure 4-4). Upon viewing a bacterial smear with both Gram-positive and Gram-negative cells, the Gram-positive cells will be purple in color and the Gram-negative will be pink/red (Figure 4-1).

**Acid-Fast Staining**—Some bacterial species cannot be Gram stained due to the presence of a
hydrophobic molecule called **mycolic acid**. For these bacterial species, the **Ziehl-Neelsen method** is used instead. The Ziehl-Neelsen method is a special procedure that differentiates between bacterial species that either have or not have the mycolic acid. Acid-fast cells are those bacterial species that have the mycolic acid, whereas non-acid fast cells are those bacterial species that do not have mycolic acid. The acid-fast cells will be red in color due to the stain **carbol fuchsin**. The non-acid fast cells will be blue in color due to the counterstain **methylene blue**. Just like the Gram staining procedure, the acid-fast staining method also differentiates based on a difference in the cell wall. In the Gram staining procedure, the difference is the thickness of the cell wall. In the acid-fast staining procedure, the difference is the presence or absence of the mycolic acid, which is a component of the cell wall in certain bacterial species.

**Endospore Staining**—Some bacterial species form a resistant endospore at times when the environment becomes too harsh for the vegetative cell (the metabolically active bacterial cell). These endospores remain behind long after the vegetative cell has died. Once the nutrients return, the endospore germinates forming a new vegetative cell becoming metabolically active once again. The endospore stains a green color due to the stain **malachite green**, while the vegetative cell stains red due to **safranin**.

**Experiment**—Each student will produce his/her own Gram stain slide and view the slide using the microscope (oil immersion). You will also need to view an acid-fast prepared slide and an endospore prepared slide.

**Materials**—1 prepared acid-fast slide; 1 prepared endospore slide; 1 blank slide; *Micrococcus luteus* and *Escherichia coli* slant culture tubes; Bunsen burner; Crystal violet; Gram’s Iodine; Decolorizer; Safranin; Staining rack; water bottle; clothes pin; inoculating loop; Bibulous paper; microscope; high-refractive index oil; lens cleaner; lens cleaner paper.

**Methods**—Use the following diagram as a guide for setting up your Gram stain slide.

1. Use your blank slide to prepare a bacterial smear (similar to what you did during the previous lab), but this time you will use two different bacterial species. You will intentionally mix *Micrococcus luteus* and *Escherichia coli* onto the same slide.
2. Dry and heat fix the slide (similar to what you did during the previous lab).

3. The staining procedure used here is different from the positive stain that you did during the previous lab. **You will need to perform the following staining steps carefully** (Figure 4-4):

   a. Place the fixed slide onto the rack inside the staining tray.
   
   b. Flood the fixed slide with crystal violet (cover the entire smear).
   
   c. Let stand for exactly 1 minute.
   
   d. Hold the slide at a slant and use the wash bottle to wash the stain off the slide.
   
   e. Flood the fixed slide with Gram’s iodine (cover the entire smear).
   
   f. Repeat steps c and d, then go to step g.
   
   g. In this step, you will use the decolorizer. **Be careful**; if you over use the decolorizer the stains that you added will wash off and your Gram stain will not work. Hold the slide at a slant and use the decolorizer as a wash for 5 to 10 seconds (**no longer**). If the run off becomes colorless prior to the 10 seconds, stop the decolorizing earlier and go to step h.
   
   h. **IMMEDIATELY**, rinse the slide using water. Hold the slide at a slant and wash off the decolorizer.
   
   i. Flood the fixed slide with safranin (cover the entire smear).
   
   j. Repeat steps c and d, then go to step k.
   
   k. Place the stained slide on bibulous paper. Fold the bibulous paper over the slide to blot off the excess fluids.
   
   l. View the slide using your microscope and focus at 4X, 10X, 40X, and then 100X (use oil).

4. Using your microscope, view the acid-fast prepared slide and the endospore prepared slide.

   ✤ **Lab Exercise**-Draw a sketch of the image you viewed under oil immersion for each slide that you viewed.

   ![Sketch of microorganisms](image)

   **Name of Microorganisms:** *Micrococcus luteus and Escherichia coli*

   **Total Magnification:** 100X

   **Staining Procedure:** Gram Staining
Answer the following questions:

1. What is the shape and color of the Gram-positive bacteria?

2. Give the name of the bacteria that you think is Gram-positive.

3. Give the name of the bacteria that you think is Gram-negative.

4. What is the shape and color of the Gram-negative bacteria?

5. What is the main difference between these two different bacterial species that we take advantage of when we do the Gram staining procedure?

<table>
<thead>
<tr>
<th>Name of Microorganism:</th>
<th>Mycobacterium smegmatis</th>
<th>Clostridium botulinum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Magnification:</td>
<td>100X</td>
<td>100X</td>
</tr>
<tr>
<td>Staining Procedure:</td>
<td>Ziehl-Neelsen acid-fast staining</td>
<td>Endospore Staining</td>
</tr>
</tbody>
</table>

Cleanup Instructions: Cleanup the prepared slides from any oil using the lens cleaner and lens cleaning paper. Place them back in their appropriate holders. For the Gram staining slide, scrub the slide with a damp paper towel containing detergent, rinse once, place slide in the acid dish.

Preparation for the next lab (Lab 5: Selective/Differential Media)

Instructions to Students-Inoculate your plates using the aseptic technique. Use the following schematic (Figure 4-5) to prepare the plates for analysis during the next lab. Inoculate your
plates using a small “Z” like motion using your inoculating loop. Place the inoculum in the center of the designated area closer towards the edge of the plate than center of the designated area.

**Materials:** Six TSA control plates. 1 Phenylethyl Alcohol Agar Plate (PEA). 1 Columbia CNA with 5% Sheep Blood Plate (CNA). 1 Mannitol Salt Agar Plate (MSA). 1 MacConkey Agar Plate (MAC). 1 Eosin-Methylene Blue Agar Plate (EMB). 1 Hektoen Enteric Agar Plate (HEA).

**Methods:** Use the following diagram as a guide for your inoculations.

---

**Incubation Instructions:**

**Students:** Tape all your TSA plates together. Separate your Columbia CNA plate from the other experimental plates. Tape all the other experimental plates (5-plates) together.

**Instructor:** All plates incubated at 37°C. Incubate the Columbia CNA inside an anaerobic jar. A 48-hour incubation is required. Subsequently, store in cold room until class time.
Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):

1. In Diagram form, show the similarities and differences between a cell-envelope found in a Gram-positive bacterium versus the cell envelope found in a Gram-negative bacterium.

2. What is the purpose of the decolorizer in the Gram staining procedure?

3. What would be the color of the cells of a Gram stain if you forget to use the ethanol?

4. Acid-fast cells do not stain well using the Gram staining method, why?

5. Under what conditions does bacteria produce an endospore?

6. Are endospores hard to kill? If yes, what do we do to destroy them?
LAB #5: Selective/Differential Media

Lab Objectives:
1. To learn the use, composition, and purpose of example selective media.
2. To learn the use, composition, and purpose of example differential media.
3. To evaluate and record data from six different types of selective/differential media.
4. To setup the plates/tubes for Lab #6 (Bacterial Growth Factors and Hydrolysis Tests).

Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions):
1. Use the following table to fill in the essential information that you will need to analyze the selective/differential plates that your group setup during the last lab.

SELECTIVE COMPONENT

<table>
<thead>
<tr>
<th>Test</th>
<th>Selective (Yes or No)</th>
<th>Selective Agent</th>
<th>Inhibits Growth (Yes or No)</th>
<th>Promotes Growth (Yes or No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex. 5-1: PhenylEthyl Alcohol Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-2: Columbia CNA w/ 5% Sheep Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-3: Mannitol Salt Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-4: MacConkey Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-5: Eosin Methylene Blue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-6: Hektoen Enteric Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Differential Component

<table>
<thead>
<tr>
<th>Test</th>
<th>Differential (Yes or No)</th>
<th>Differential Agent</th>
<th>Basis of Differentiation</th>
<th>pH Indicator Name (ranges and colors)-Appendix C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex. 5-1: PhenylEthyl Alcohol Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-2: Columbia CNA w/ 5% Sheep Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-3: Mannitol Salt Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-4: MacConkey Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-5: Eosin Methylene Blue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex. 5-6: Hektoen Enteric Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Background Information** - The addition of various components to media allows the microbiologist to either select between two different bacterial species or differentiate between them or both. The microbiologist makes the media **selective** by adding a selective agent like a dye, sugar, antibiotic, or salt. How the selective agent works to inhibit growth is dependent on the agent of choice. Among some of the ways these selective agents work is by interfering with the...
organism’s metabolism or enzymatic activity. Certain selective agents inhibit the growth of Gram-negative bacteria, but not the Gram-positive bacteria. Other selective agents inhibit Gram-positive, but not Gram-negative. Another selective agent, high-salt concentration, inhibits the growth of most bacteria with the exception of Staphylococcus species.

The microbiologist adds a differential agent to media to make differential media. The use of various sugars are common examples of differential agents.

**Lab Exercise 5-1 (Phenylethyl Alcohol Agar-PEA)**

**Recipe:** 1.5% Pancreatic Digest of Casein, 0.50% Papaic Digest of Soybean Meal, 0.50% Sodium Chloride, 0.25% β-Phenylethyl Alcohol (PEA), pH 7.1-7.5 at 25°C (1L Final Volume).

**Selective Media:** The phenylethyl alcohol agar plate (Figure 5-1) is a selective media only. The selective agent is the phenylethyl alcohol itself. The selective agent kills or at least significantly decreases the growth of many Gram-negative bacteria, but has no effect on most, but not all, Gram-positive bacteria. To isolate Staphylococci, Streptococci, Enterococci, and Lactococci bacterial species.

**Mode of Action of the Selective Agent:** The alcohol dissolves the outer membrane of Gram-negative bacteria. The thin cell wall of Gram-negative bacteria will allow the alcohol to gain entry into the cell, denature proteins, and inhibit DNA synthesis killing the Gram-negative bacteria. The thick cell wall of most, but not all, Gram-positive bacteria protects the cell from the effect of the alcohol.

**Lab Exercise 5-2 (Columbia CNA with 5% Sheep Blood Agar-Columbia CNA)**

**Recipe:** 1.2% Pancreatic Digest of Casein, 0.5% Peptic Digest of Animal Tissue, 0.3% Yeast Extract, 0.3% Beef Extract, 0.1% Corn Starch, 0.5% Sodium Chloride, 1.35% Agar, 5% Sheep Blood, 0.001% Colistin, 0.001% Nalidixic Acid, pH 7.1-7.5 at 25°C (1L Final Volume).

**Selective Media:** The Columbia CNA media (Figure 5-2) has two separate selective agents, colistin and nalidixic acid. These selective agents are antibiotics that are highly effective against a number of Gram-negative bacteria (Enterobacteriaceae and Pseudomonas), but...
much less effective against Gram-positive bacteria (Streptococci, Staphylococci, and Pneumococci). The selective agents are not effective against all Gram-negative bacteria.

The combination of different factors play a role in achieving selection. These factors include the presence of both antibiotics in the media at the same time, the concentration of the antibiotics in the media, mode of action of both antibiotics, and the differences between Gram-negative and Gram-positive bacteria.

**Mode of Action of the Selective Agents:** The antibiotic colistin binds to the Gram-negative outer membrane (lipopolysaccharide) disrupting the membrane. The disrupted outer membrane will allow the colistin to damage the cell membrane. The weakened cell membrane will now allow the nalidixic to enter the cell and interfere with DNA replication causing the cell to die. The thick cell wall of the Gram-positive bacterium provides it protection from the actions of both of these antibiotics.

**Differential Media (Basis of Differentiation):** The Columbia CNA media containing 5% sheep blood serves the function of distinguishing between those bacterial species that do survive the selective agents. The degree at which bacterial colonies are capable of lysing red blood cells (hemolysis) is the basis of differentiation used for this type of media. The following describes each type:

*Alpha-hemolysis:* $\alpha$-hemolytics comprise the type of bacteria that result in *incomplete or partial lysis* of red-blood cell. These bacteria release *hydrogen peroxide*, which oxidizes the hemoglobin found within the red blood cell turning its color to *green*. The presence of this green color and minimal lysis on the Columbia CNA plate containing sheep blood clearly identifies that bacterial species as $\alpha$-hemolytic.

*Beta-hemolysis:* $\beta$-hemolytics comprise the type of bacteria that result in *complete lysis* of red blood cells. These bacteria release *hemolysins* made up of lipids or proteins. The hemolysins may weaken the membrane by altering the phospholipids causing the cells to lyse, or they may form pores through which the cell contents leak out. Some of these hemolysins are oxygen-sensitive (not stable in the presence of oxygen); therefore, not effective in the presence of oxygen. Other hemolysins are oxygen-stable. The type of hemolysin produced by bacteria is species dependent. $\beta$-hemolysics growing on the Columbia CNA plate containing sheep blood will produce a clear area called, the *zone of transparency*. The zone of transparency looks like a “halo” all around the bacterial colonies in the region where all the red blood cells have lysed. The extent of hemolysin diffusion correlates with the width of the “halo” resulting from the loss of red blood cells.

*Gamma-hemolysis:* $\gamma$-hemolytics comprise the type of bacteria that do not produce hemolysin. These bacteria will grow normally on the Columbia CNA plate, but will have no effect on the red blood cells around them. All the red blood cells will remain intact.
Lab Exercise 5-3 (Mannitol Salt Agar-MSA)

**Recipe:** 0.1% Beef Extract, 1.0% Peptone, 7.5% Sodium Chloride, 1.0% D-Mannitol, 0.0025% Phenol Red, and 1.5% Agar, pH 7.1-7.5 at 25°C (1L Final Volume).

**Selective Media:** The selective agent in the Mannitol Salt Agar (Figure 5-3) is the high concentration of salt. The high salt concentration is inhibitory for most bacteria (Gram-negative and Gram-positive) except for the Gram-positive *Staphylococcus* and *Micrococcaceae*.

**Mode of Action of the Selective Agent:** The high salt concentration draws out water from most bacteria causing them to dehydrate and die. Halophilic (salt-loving) bacteria like *Staphylococcus* regulates its intracellular ionic concentration allowing it to survive in a hypertonic environment.

**Differential Media (Basis of Differentiation):** The Mannitol Salt Agar is also differential. The differential agent is the sugar alcohol mannitol. Some species of *Staphylococcus* are able to ferment mannitol and produce an acid as a byproduct. The acidic byproduct causes a decrease in the media’s pH, which changes the media color to yellow. The pH indicator in the media changes from its normal color (red) to yellow under acidic pH. Other species of *Staphylococcus* do not ferment the mannitol. The phenol red (media) retains its normal red color.

Lab Exercise 5-4 (MacConkey Agar-MAC)

**Recipe:** 1.7% Pancreatic Digest of Gelatin, 0.15% Pancreatic Digest of Casein, 0.15% Peptic Digest of Animal Tissue, 1.0% Lactose, 0.15% Bile Salts, 0.5% Sodium Chloride, 0.003% Neutral Red, 0.0001% Crystal Violet, and 1.35% Agar, pH 7.1-7.5 at 25°C (1L Final Volume).

**Selective Media:** The bile salts and crystal violet added to the MacConkey agar (Figure 5-4) are the selective agents. Gram-positive bacteria are susceptible to the effects of the bile salts and crystal violet. Gram-negative bacteria are resistant to the effects of the bile salts and crystal violet.
**Mode of Action of the Selective Agent:** The bile salts act upon the lipoteichoic acid that helps link the cell wall to the cell membrane. The bile salts; therefore, acts as a detergent weakening the cell wall and cell membrane of the Gram-positive bacteria. The crystal violet binds directly to the peptidoglycan and interferes with its maintenance further weakening the cell wall. The combined effects of the bile salts and crystal violet kills Gram-positive bacteria. The outer membrane of Gram-negative bacteria protects them from the effects of the bile salts and crystal violet.

**Differential Media (Basis of Differentiation):** The differential agent added to the MacConkey agar is the lactose. The coliform bacterial species that ferment the lactose will produce an acid as a byproduct. The lowering of pH will cause the pH indicator, neutral red, to produce a pink color. Therefore, fermentation of lactose is the basis of differentiation in the MacConkey agar plate. The non-coliform bacterial species that do not ferment the lactose will not be red or pink.

**Lab Exercise 5-5 (Eosin Methylene Blue Agar-EMB)**

**Recipe:** 1.0% Pancreatic Digest of Gealtin, 0.2% Dipotassium Phosphate, 1.0% Lactose, 0.04% Eosin-Y, 0.0065% Methylene Blue, 1.5% Agar, pH 6.9-7.3 at 25°C (Some formulations have 0.5% Lactose and 0.5% Sucrose, instead of 1.0% Lactose)-1L Final Volume.

**Selective Media:** The eosin-Y and methylene blue are dyes that serve as selective agents in the eosin-methylene blue agar (Figure 5-5). Together they inhibit the growth of most Gram-positive bacteria (except for some *Enterococcus* and *Staphylococcus* species), but have no effect on the growth of most Gram-negative bacteria. These two dyes will also serve as pH indicators in this media.

**Mode of Action of the Selective Agent:** Eosin-Y produces toxins (reactive oxygen species) that oxidize proteins, nucleic acids, and lipids. Methylene blue breaks down disulfide bridges found in the cell walls of most Gram-positive bacteria. The outer membrane of most Gram-negative bacteria prevents the dyes from accessing the cell and therefore providing them protection from the effects of eosin and methylene blue.

**Differential Media (Basis of Differentiation):** The sugar serves as a source of fermentable carbohydrate in the eosin-methylene blue agar test. In this test, the degree of fermentation can also be determined based on the coloration. The eosin-Y and methylene blue pH indicators will react with each other in the presence of the acid to produce a purple/black color and at times a green sheen color. This color identifies an aggressive fermenter. On the
other hand, a moderate to weak fermenter will produce colonies with pink coloration. Colonies that are not fermenting will retain their normal coloration (colorless).

Lab Exercise 5-6 (Hektoen Enteric Agar-HEA)

**Recipe:** 1.2% Peptic Digest of Animal Tissue, 0.3% Yeast Extract, 0.9% Bile Salts, 1.2% Lactose, 1.2% Sucrose, 0.2% Salicin, 0.5% Sodium Chloride, 0.5% Sodium Thiosulfate, 0.15% Ferric Ammonium Citrate, 0.0064% Bromothymol Blue, 0.01% Acid Fuchsin, 1.35% Agar, pH 7.4-7.8 at 25°C (1L Final Volume).

**Selective Media:** The bile salts found in the hektoen enteric agar (Figure 5-6) inhibits the growth of most Gram-positive bacteria and do not affect the Gram-negative bacteria. The hektoen enteric agar helps to isolate *Salmonella* and *Shigella* species from other enteric species (fermenting coliform from non-fermenting coliform, as well as sulfur reducers).

**Mode of Action of the Selective Agent:** Bile salts kill most Gram-positive bacteria through its detergent actions, disrupting membranes. These salts also result in protein coagulation, altered RNA structure, and DNA damage. Gram-negative bacteria are more inherently resistant to the effects of bile salts through their adaption to the environment of the gastrointestinal tract.

**Differential Media (Basis of Differentiation):** Fermentation of lactose, sucrose, or salicin is the basis of differentiation in the hektoen enteric agar. Fermentation of these sugars will produce a yellow to salmon colored colonies, whereas non-fermenters will have a blue-green coloration. Another basis of differentiation used in this agar is sulfur reduction to form hydrogen sulfide. The sodium thiosulfate provides the sulfur and produces hydrogen sulfide gas when it reacts with the ferric ammonium citrate. The iron of ferric ammonium citrate will form a black precipitate upon the formation of hydrogen sulfide.

❖ **Lab Exercise:**

All members of the group need to work together to analyze the plates that were streaked during the last lab. Make sure everyone in the group is involved in the analysis. Each person needs to understand how to read these results and what these results mean.

**Phenotypes Information:** Use the limited phenotype information given below alongside your plates to complete the data-gathering table for this experiment.

- *Escherichia coli:* Gram Negative and Fermenter
- *Staphylococcus epidermidis:* Gram Positive and Non-Fermenter
**Streptococcus mitis:** Gram Positive and α-Hemolytic  
**Enterococcus faecalis:** Gram Positive and γ-Hemolytic  
**Staphylococcus aureus:** Gram Positive and Fermenter and β-Hemolytic  
**Salmonella typhimurium:** Gram Negative and Non-Fermenter. Sulfur Reducer (H$_2$S producer)  
**Enterobacter aerogenes:** Gram Negative and Low to Moderate Fermenter  
**Shigella flexneri:** Gram Negative and Non-Fermenter. Non-Sulfur Reducer (H$_2$S producer)

**Selective/Differential Student Data Table:**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Control Plate Growth (Yes or No)</th>
<th>Experimental Media</th>
<th>Experimental Plate Growth (Yes or No)</th>
<th>Change in color of media compared to control plate? (Yes or No); If YES, give color.</th>
<th>Change in color of bacteria compared to control plate? (Yes or No); If YES, give color.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli (1)</td>
<td></td>
<td>PEA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus (3)</td>
<td></td>
<td>PEA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis (4)</td>
<td></td>
<td>PEA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli (1)</td>
<td></td>
<td>Columbia CNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus (3)</td>
<td></td>
<td>Columbia CNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis (4)</td>
<td></td>
<td>Columbia CNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. mitis (5)</td>
<td></td>
<td>Columbia CNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli (1)</td>
<td></td>
<td>MSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. epidermidis (2)</td>
<td></td>
<td>MSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus (3)</td>
<td></td>
<td>MSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli (1)</td>
<td></td>
<td>MAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis (4)</td>
<td></td>
<td>MAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. typhimurium (6)</td>
<td></td>
<td>MAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>Control Plate Growth (Yes or No)</td>
<td>Experimental Media</td>
<td>Experimental Plate Growth (Yes or No)</td>
<td>Change in color of media compared to control plate? (Yes or No); If YES, give color.</td>
<td>Change in color of bacteria compared to control plate? (Yes or No); If YES, give color.</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------------------</td>
<td>--------------------</td>
<td>--------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>E. coli</em> (1)</td>
<td></td>
<td>EMB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> (4)</td>
<td></td>
<td>EMB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurium</em> (6)</td>
<td></td>
<td>EMB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. aerogenes</em> (7)</td>
<td></td>
<td>EMB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> (1)</td>
<td></td>
<td>HEA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> (4)</td>
<td></td>
<td>HEA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurium</em> (6)</td>
<td></td>
<td>HEA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. flexneri</em> (8)</td>
<td></td>
<td>HEA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Describe what is happening in the PEA plate.

2. Describe what is happening in the Columbia CNA plate.

3. Describe what is happening in the MSA plate.
4. Describe what is happening in the MAC plate.

5. Describe what is happening in the EMB plate.

6. Describe what is happening in the HEA plate.

**Cleanup Instructions:** Simply discard all of your group’s plate in the biohazard container.

**Preparation for the next lab (Lab 6: Physical Growth Factors for Bacterial Growth)**

**Special Note to Instructor**-Instructor will place the FTM into the 100°C water bath for 20 minutes and then transfer them into the 50°C water bath prior to giving the tube to your group. When ready, get the fluid thioglycollate media, a black cap tube, from the hot water bath. The heat helps remove the oxygen from the media prior to inoculating the tube.

**Instructions to Students**-Inoculate your plates/tubes using the aseptic technique. Use the following schematic (Figure 5-7) to prepare the plates for analysis during the next lab. Inoculate your hydrolysis plates using a small “Z” like motion using your inoculating loop. Place the inoculum in the center of the designated area closer towards the edge of the plate than center of the designated area. For the TSA plates inoculated with *Serratia marcescens* (Ex. 6-2), you can be creative with your inoculation and draw or write with your inoculating loop. The tubes for exercise 6-1 will be located in the hot water bath prior to you using them. For exercise 6-3, your instructor will assign your group with ONE bacterial species to use for your group’s inoculation. Only use the assigned bacteria to inoculate all the broth tubes supplied to you for this exercise.

**Materials**-Four FTM tubes. 2 TSA plates. 4 Broth tubes. 1 Starch Agar Plate. 1 DNA Hydrolysis Plate. 1 Tributyrin Hydrolysis Plate. 1 Milk Agar Plate. 1 Esclalin Hydrolysis Plate. Cultures of *Bacillus cereus*, *Escherichia coli*, *Serratia marcescens*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*. 
**Methods**—Use the following diagram (Figure 5-7) as a guide for your inoculations.

**Incubation Instructions:**

**Students:** Tape all your hydrolysis plates together along with the 37°C Serratia plate from exercise 6-2. Separate the other Serratia plate to place it at 25°C. Keep your FTM together and place them on a rack for incubation by your instructor. Distribute your different temperature tube by placing them in a rack labeled with the appropriate temperature.

**Instructor:** Place all plates and tubes in the cold room. Only place the Esculin plates in the incubator just **one day prior** to class. 48-hour incubation for all other plates and tubes. All plates at 37°C, except for the 25°C Serratia marcescens, which goes in the 25°C incubator. Except for the *Pseudomonas* tube, all the other FTM tubes go at 37°C. Distribute the temperature growth tubes into their corresponding incubators.

**Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):**

1. In the selective/differential agar exercises, we used a TSA plate with each experimental plate. What was the purpose of the TSA plate?
2. Using the Columbia CNA Test as an example, give a purpose or benefit achieved by setting up this type of selective/differential plate? In other words, what does this test help you accomplish?

3. You streak the same bacteria on a TSA plate and an experimental plate. What would you conclude about the type of agar (in general) that you are using for the experimental plate if the bacteria grew on the TSA plate, but not the experimental plate?

4. What would you conclude about the type of agar (in general) being used for an experimental plate if upon observing the various streaks on the plate you notice that one streak is red in color and the other is yellow in color?

5. Determine what would be the expected result for the following experiment (Which ones would grow and which ones would not grow and WHY?):

- Phenylethyl Agar plate streaked with *S. mitis, E. aerogenes, S. flexneri,* and *S. epidermidis.*

6. Using the information that was supplied to you, determine what would be the expected result for the following experiment (Which ones would grow and which ones would not grow and WHY?) (What color will the bacteria exhibit and WHY?):

- MacConkey Agar plate streaked with *S. epidermidis, E. coli,* and *S. typhimurium.*
7. Describe the difference between selective media and differential media.

8. In the mannitol salt agar, a yellow color indicates fermentation. In the MacConkey Agar, a red or pink color indicates fermentation. Explain why there is a difference in color for these two types of media.
LAB #6: Physical Growth Factors for Bacterial Growth

Lab Objectives:
1. To learn the use, composition, and purpose of the thioglycollate media (aerotolerance).
2. To assess the effects of temperature on bacterial pigment production.
3. To assess the effects of temperature on bacterial growth.
4. To understand the various categories of bacteria based on their temperature requirements.
5. To learn the use, composition, and purpose of example hydrolysis media namely starch, DNA, tributyrin, casein, and esculin.
6. To learn the use and purpose of the catalase test.
7. To learn the use and purpose of the oxidase test.
8. To setup the tubes for Lab #7 (Introduction to Biochemical Test Media).

Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions):
1. Complete the following table BEFORE you come to lab to analyze your plates. This table will then serve as your predictions that you will then compare your actual results against. You will have to do some research (internet) to find out how each of the bacterial species inoculated should behave in the media/test examined.

Ex. 6-1: Fluid Thioglycollate

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Location of Growth</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium sporogenes</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ex. 6-2: Effect of Temperature on Pigment Production

Prediction: *S. marcescens* at 25°C produced more or less red pigment? ___________________
Ex. 6-3: The Effect of Temperature on Bacterial Growth

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Escherichia coli</th>
<th>Serratia marcescens</th>
<th>Bacillus stearothermophilus</th>
<th>Pseudomonas fluorescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>5°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conclusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Hydrolysis Tests:**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Ex. 6-4</th>
<th>Ex. 65</th>
<th>Ex. 6-6</th>
<th>Ex. 6-7</th>
<th>Ex. 6-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (1)</td>
<td>Starch Agar</td>
<td>DNA Hydrolysis</td>
<td>Tributyrin Agar Plate</td>
<td>Casein Hydrolysis</td>
<td>Bile Esculin Test</td>
</tr>
<tr>
<td>Bacillus cereus (2)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Escherichia coli (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serratia marcescens (4)</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Eterococcus faecalis (5)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (6)</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Enzyme Released by Bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

X means we did not inoculate the bacterial species for that particular test.

**Background Information** - This lab examines the growth requirements (environmental and nutritional) that different bacteria demand in order to thrive. When culturing bacteria, a microbiologist must provide the proper environmental and nutritional needs required by the bacterial culture. The utilization of oxygen and the effect of temperature are two environmental factors that greatly influence the degree of bacterial growth. Utilization of starch, DNA, tributyrin, casein, and esculin are among the various nutritional factors that bacteria utilize to obtain key nutrients to support their growth. The tests performed in this lab will examine all of those factors, environmental and nutritional, by examining a variety of different bacterial species.

**Lab Exercise 6-1 (Fluid Thioglycollate Media)**

**Recipe**: 0.5% Yeast Extract, 1.5% Pancreatic Digest of Casein, 0.55% Dextrose, 0.25% Sodium Chloride, 0.05% Sodium Thioglycollate, 0.05% L-cystine, 0.075% Agar, 0.0001% Resazurin (1L Final Volume).
Oxygen Requirements: Oxygen can influence bacterial growth in a variety of ways depending on the species of bacteria. For obligate aerobes, the presence of oxygen in mandatory. For obligate anaerobes, even low-levels of oxygen is fatal; therefore, the absence of oxygen is mandatory. Other species of bacteria are called facultative anaerobes, which survive in the presence or absence of oxygen (they prefer oxygen). Aerotolerant anaerobe species of bacteria can tolerate the presence of oxygen, but do not use the oxygen. Microaerophiles species of bacteria can only live in low-levels of oxygen, some of which also prefer carbon dioxide (capnophiles).

Concept of Test: To help remove the diffused oxygen we place the tube in a hot water bath just prior to inoculating it. We add agar into the media at a low percentage to help slow the diffusion of oxygen back into the media, as the tube is inoculated/incubated. The added sodium thioglycollate and L-cystine help get rid of the oxygen by converting it into water. The resazurin will turn pink in the presence of oxygen and behaves as an oxygen indicator in this media. The fluid thioglycollate medium tube will have a gradation of oxygen levels as you go from the top (high concentration of oxygen-aerobic) to the bottom (no oxygen-anaerobic). As seen in figure 6-1, aerobic bacteria will grow near the top of the media, whereas anaerobic species will grow in the anaerobic region of the tube (bottom). Facultative anaerobes will grow all the way through, but better near the top. Microaerophiles (not pictured) will grow in the region where oxygen is low, but before the oxygen concentration drops to zero. Aerotolerant (not pictured) bacteria will grow all the way through the tube equally.

Lab Exercise: Analyze all four tubes and determine if what you predicted is correct for each of the bacterial species with respect to their oxygen requirements.

Answer the Following Questions:

Were your predictions the same as your actual results? ________________.

The control tube has a light red color on top, why?

With respect to its oxygen requirements, what is Pseudomonas fluorescens? ______________

With respect to its oxygen requirements, what is Clostridium sporogenes? ______________

With respect to its oxygen requirements, what is Escherichia coli? _____________________
Lab Exercise 6-2 (Effect of Temperature on Bacterial Pigmets)

Chromobacteria are bacteria that produce pigments. The synthesis of these pigments is highly dependent on a number of environmental factors like pH, temperature, and light. The pigment color produced by bacteria is species dependent, but collectively chromobacteria can produce a whole array of colors. Each pigment will have its own function. Some of the functions associated with the pigments include photosynthesis, as well as cell protection from ultraviolet light damage or reactive oxygen species damage. Other pigments behave as antibiotics. The red pigment prodigiosin, produced by Serratia marcescens, has antibiotic properties. Interestingly, its synthesis is sensitive to fluctuations in temperature (Figure 6-2). Temperatures within the range of 24°C to 28°C are optimal for prodigiosin synthesis, whereas a significant decrease in prodigiosin production is observed at temperatures lower than 16°C and higher than 30°C.

Lab Exercise: Compare the growth of Serratia marcescens at 25°C to the growth of Serratia marcescens at 37°C. Write a quick observation statement describing what the main difference between these plates.

Student Statement:
__________________________________________________________________________________________

Lab Exercise 6-3 (Effect of Temperature on Bacterial Growth)

The temperatures at which a given bacterium can thrive at can fall within a range of temperatures. However, the bacterium will grow optimally at a given temperature. Bacteria known as psychrophiles grow best (optimally) below 20°C. Psychrotrophs are bacteria that are able to thrive in habitats that have temperature fluctuations between 0°C to 30°C. Mesophiles thrive between the temperatures of 15°C to 45°C. Thermophiles are bacteria that live about 40°C. Obligate thermophiles cannot live at temperatures below 40°C, whereas facultative thermophiles are able to live below 40°C and above. Finally, extreme thermophiles live above 80°C.

Lab Exercise: Analyze all four tubes and determine if what you predicted is correct for each of the bacterial species with respect to the effect of temperature on bacterial growth. Since your instructor only assigned one bacterial species for your group to do, you and your group members will have to visit the other groups to gather data about the other assigned bacterial species.

Answer the Following Questions:

Were your predictions the same as your actual results? ________________.

With respect to its temperature requirements, what is Escherichia coli?

With respect to its temperature requirements, what is Serratia marcescens?
With respect to its temperature requirements, what is *Bacillus stearothermophilus*?

With respect to its temperature requirements, what is *Pseudomonas fluorescens*?

**Lab Exercises 6-4 to 6-8 (Hydrolysis Tests)**

**Bacterial Acquisition of Nutrients by Hydrolysis of Biomolecules:** To survive, bacteria have to be able to transport molecules, found within its immediate environment, into the cell. These molecules, once inside the cell, are nutrients used by the cell to support bacterial cell growth. The concern arises when the biomolecule found in the environment is a macromolecule or a molecule that cannot easily enter the cell. Bacteria will then release exoenzymes, enzymes released by a living bacterium to hydrolyze biomolecules found in its environment, to digest these macromolecules (Figure 6-3). After digestion of the macromolecules, their monomeric forms can now easily enter the cell. Some bacteria are able to produce and release amylase to digest exogenous starch into its monomers, glucose (Exercise 6-4). Other bacteria release DNase to digest DNA into its monomers, nucleotides (Exercise 6-5). Some bacteria release lipase to digest lipids (Exercise 6-6). Other bacteria are capable of releasing the proteolytic enzyme, casease, to digest the milk protein casein to obtain its amino acid monomers (Exercise 6-7). Some bacteria release esculinase to breakdown the glycoside esculin to obtain the glucose (Exercise 6-8).

![Figure 6-3: Example hydrolysis test plates. The “halo” around the culture in the “+” quadrant for the starch, DNA, tributyrin, and casein tests indicates that the bacterial culture in its vicinity is releasing the corresponding exoenzyme for each of the hydrolysis test. For the bile esculin test the “+” culture is the one that produces the black coloration.](image)

**Recipes:**

**Starch Hydrolysis Media:** Recipe: 0.3% Beef Extract, 1.0% Soluble Starch, 1.2% Agar, pH 7.3-7.7 at 25°C (1L final volume). **Reagents Needed for Testing:** Gram Iodine from the Gram Staining Kit.

**DNA Hydrolysis Media:** Recipe: 2.0% Tryptone, 0.2% DNA, 0.5% Sodium Chloride, 1.5% Agar, 0.005% Methyl Green, pH 7.1-7.5 at 25°C (1L final volume).
**Tributyrin Hydrolysis Media:** Recipe: 0.3% Beef Extract, 0.5% Peptone, 1.5% Agar, 10ml of Tributyrin Oil, pH 5.8-6.2 at 25°C (1L final volume).

**Casein Hydrolysis Media:** Recipe: 0.45% Pancreatic Digest of Casein, 0.23% Yeast Extract, 9.09% Powdered Nonfat Milk, 0.091% Glucose, 1.364% Agar, pH 6.9-7.1 at 25°C (1.1L Final Volume).

**Bile Esculin Hydrolysis Media:** Recipe: 0.5% Pancreatic Digest of Gelatin, 0.3% Beef Extract, 2.0% Oxball (ox bile), 0.05% Ferric Citrate, 0.1% Esculin, 1.4% Aga, pH 6.6-7.0 at 25°C (1L Final Volume).

**Lab Exercises-Hydrolysis Tests**

Lab Exercise 6-4 (Starch Hydrolysis Test)-Open your starch hydrolysis plate and add 2ml of iodine directly on top of the agar. Swirl the plate around to cover your entire plate. When iodine interacts with starch, it will turn the media into a brownish to blueish/purplish color. The presence of that color around the culture on the plate indicates that the bacterial culture is not releasing amylase and therefore not digest the starch. The absence of that color around the culture indicates that the bacterial culture is releasing amylase and is therefore digesting the starch to obtain the glucose.

Which bacterial species was positive for amylase? ________________________.

Were your predictions the same as your actual results? ____________________.

Lab Exercise 6-5 (DNA Hydrolysis Test)-In the DNA hydrolysis test, the methyl green dye is used as an indicator of DNase activity. Conjugation (attaching) the methyl green dye to the DNA in the media gives the blue-green color that is typical for the DNA hydrolysis plate. The methyl green dye does not conjugate to the monomers of DNA, the nucleotides. Therefore, the loss of the blue-green indicates that the DNA in the media lost its conjugation to the methyl green due to its hydrolysis by the enzyme. To analyze your plate, identify the bacterial culture that loses the blue-green color in its vicinity. That bacterial culture released DNase, hydrolyzed the DNA away from the methyl green, and the media as a consequence loses its blue-green color around the bacterial culture. The DNase negative bacterial culture will still have the blue-green color surrounding it.

Which bacterial species was positive for DNase? ________________________.

Were your predictions the same as your actual results? ____________________.

Lab Exercise 6-6 (Tributyrin Hydrolysis Test)-The tributyrin hydrolysis test is used to identify lipolytic bacterial species. Lipolytic bacteria release lipase to digest lipids in the immediate environment. To analyze your plate, identify the bacterial culture that produces a clear zone around it. The clear zone results from the release of lipase and digestion of the lipids in the media. The bacterial culture that is not releasing lipase will not have the clear zone around it.

Which bacterial species was positive for lipase? ________________________.

Were your predictions the same as your actual results? ____________________.

Lab Exercise 6-7 (Casein Hydrolysis Test)-Casein is the most abundant protein found in milk and it gives milk its distinctive white color. Casein will lose its white color and becomes clear when
hydrolyzed. Some bacteria will release the enzyme *casease* to digest the protein to obtain the amino acid monomers. To analyze your plate, identify the bacterial culture that produces a clear zone around it. The *clear zone* results from the release of casease and digestion of the casein in the media. The bacterial culture that is not releasing casease will not have the clear zone around it.

Which bacterial species was positive for casease? ________________________.
Were your predictions the same as your actual results? ________________.

**Lab Exercise 6-8 (Esculin Hydrolysis Test)**-**Esculin** is a glycoside composed of glucose bonded to esculetin through a glycosidic bond. A number of bacterial species release *esculinase* to break the glycosidic bond to release and attain the glucose. Only a subset of bacteria, *Enterococci* and Group D *Streptococci*, are able to hydrolyze the esculin in the presence of bile salt in the media. Therefore, the bile esculin test helps in the identification of those bacterial species from other bacterial species. Bacteria that release esculinase to digest the glycoside esculin will cause the release of *esculetin* into the media as it attains the glucose. The released esculetin will react with the ferric citrate producing a brown/black precipitate or coloration in the media. To analyze your plate, identify the bacterial culture that produces the *brown/black coloration* around the bacterial culture. The presence of this color around the bacterial culture is indicative of the bacterial culture being capable of hydrolyzing the esculin in the presence of the bile salts. The lack of the brown/black coloration is indicative that this bacterial culture is not able to digest esculin in the presence of the bile salts.

Which bacterial species was positive for esculinase? ________________________.
Were your predictions the same as your actual results? ________________.

**EXAM NOTE:** Your lab exam #1 will cover all the content from the start of lab #1 up until this point, the esculin hydrolysis test.

**Preparation for the next lab (Lab 7: Introduction to Biochemical Test Media)**

**Note to Student:**
Work with your group to setup and finish the catalase test and the oxidase test today. Record your results. These two tests, catalase and oxidase, are discussed in more detail in the lab 7 background information section.
**Instructions to Students:** Work with your group to inoculate your tubes for analysis during the next lab. All tubes inoculated using the aseptic technique. Inoculate the broth tubes and slant tubes as we have done before. For the KIA tubes, streak the slant using the inoculating loop and stab the butt region of the tube using the inoculating needle. Make sure you only add one bacterial species per tube. Do not cross contaminate.

**Materials:** Glass slide. Hydrogen Peroxide. 2 cotton swabs (sterile). Oxidase reagent. 4 nitrate reduction broth media tubes with Durham tubes. 2 phenylalanine Deaminase slant agar tubes. 4 Kligler Iron Agar slant tubes. 2 rabbit plasma tubes. Inoculating loop and needle. Bunsen burner.

**Methods:** Use the following diagrams (Figures 6-4 and 6-5) as guides for your inoculations.

**Answer Questions:**

1. Which bacteria produced bubbles in the catalase test (Ex. 7-1)?

2. Which bacteria produced a purple color in the oxidase test (Ex. 7-2)?

**Note to students:** Remember these answers for the next lab. When you read the background information for lab # 7, you will understand the meaning of these two results (catalase test and oxidase test).

**Incubation Instructions:**

**Students:** Prepare your tube for incubation by placing all of them on a rack.

**Instructor:** Incubate all tubes in the 37°C incubator for 2 days.
Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):

1. What does hydrolysis mean?

2. An ______________ is released by bacteria in order to hydrolyze a macromolecule that is located in the media.

3. Give the enzyme responsible for hydrolyzing the following molecules or macromolecules (Use your lab book):
   
   Starch: ___________  DNA: ___________  Lipid: ___________
   
   Casein: ___________  Esculin: ___________

4. _______________ is added to the starch hydrolysis plate prior to its evaluation.

5. What is the main purpose behind using the fluid thioglycollate media?

6. How is oxygen removed from the fluid thioglycollate media (Give two ways)?

7. What is added to the fluid thioglycollate media to indicate the presence of oxygen?

8. List all the categories that bacteria are placed in based on their temperature requirements?

9. Take in consideration a human’s body temperature. Based on their optimal temperature requirements, what is the category of our gut bacteria?
LAB #7: Introduction to Biochemical Test Media

**Lab Objectives:**
1. To learn the use and purpose of the catalase test used in microbiology.
2. To learn the use and purpose of the oxidase test used in microbiology.
3. To learn the use, composition, and purpose of the nitrate reduction test used in microbiology.
4. To learn the use, composition, and purpose of the phenylalanine deaminase test used in microbiology.
5. To learn the use, composition, and purpose of the Kligler iron agar test used in microbiology.
6. To learn the use, composition, and purpose of the coagulase test used in microbiology.
7. To setup the tubes/plates for Lab #8 (Culture and Sensitivity-Identification of an Unknown Enterobacteriaceae and Antibiotic Susceptibility Test).

**Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions):**
1. What is the substrate that catalase acts upon?

2. What are the two by-products of the reaction between catalase and the substrate that you gave in question #1?

3. What is the main purpose of the electron transport chain?

4. What is the final by-product at the end of denitrification?

5. What is the name of the tube that we will place up-side-down into the main tube that helps trap gas produced by the bacteria (Nitrate Reduction Test)?

6. Give the name of the enzyme that removes the amine functional group from amino acids?

7. In the KIA test, if the inoculated bacteria metabolize proteins only, what color will the media be?
Background Information and Lab Questions/Exercises - 

Lab Exercise 7-1 (Catalase Test) The electron transport chain, used during oxidative phosphorylation in aerobes and in some facultative anaerobes, transfers the electrons to oxygen (the final electron acceptor). The transfer of these electrons to oxygen and oxygen reacting with hydrogen results in the reduction of oxygen into water. Most of the time the electrons go through a series of complexes before they finally reach the oxygen. However, the transfer of the electrons sometimes derails and goes directly to oxygen forming a **superoxide ion**. Superoxide ions are highly destructive and toxic to the cell. For that reason, organisms that consume oxygen will express an enzyme (**superoxide dismutase**) that converts the superoxide ion to a slightly lesser toxin, **hydrogen peroxide**. Oxygen consuming organisms will also express another enzyme (**catalase**) that will breakdown the hydrogen peroxide into water and oxygen gas. Addition of the substrate (hydrogen peroxide) to bacterial smears on a slide helps identify those species that produce catalase. The addition of the hydrogen peroxide substrate (3%) directly onto a bacterial smear expressing catalase will result in the formation of the **oxygen gas bubbles** immediately. Obligate aerobes and some facultative anaerobes will produce a positive test result, whereas obligate anaerobes and some facultative anaerobes will produce a negative result. The test is useful to identify catalase-positive *Staphylococci* from catalase-negative *Streptococci*.

- Answer the following questions based on the observations during the last lab when you and your lab partners actually performed this experiment:
  1. What did you have to add to the bacteria in the catalase test (name the substrate)?
  2. Why did one of them bubble? Write the reaction.
  3. Which enzyme found in the cell makes the substrate that you gave in #1?
  4. The enzyme you gave in #3, converts superoxide ion into the substrate that you added in #1. Describe how cells form superoxide ion.
  5. Why does the cell have to get rid of the superoxide ion?
Lab Exercise 7-2 (Oxidase Test) In a number of aerobically respiring organisms, the fourth complex of the electron transport chain, cytochrome c oxidase, helps transfer the electrons by oxidizing cytochrome c and reducing oxygen. Cytochrome c is the molecule that proceeds cytochrome c oxidase, whereas oxygen (the final electron acceptor) comes after cytochrome c oxidase in the chain of events. Some bacterial species do not have cytochrome c oxidase at all since they do not utilize oxygen, while other bacterial species use a different type of cytochrome to transfer the electrons to oxygen. Most enteric bacteria are oxidase negative. To determine if a bacterial species is oxidase positive or negative, we directly add an oxidase reagent to a sample of the culture. The oxidase reagent mimics the function of cytochrome c. In the presence of cytochrome c oxidase, the oxidase reagent will lose electrons to cytochrome c oxidase. The reagent oxidizes as it reduces cytochrome c oxidase. The oxidized reagent forms a purple color indicating the presence of cytochrome c oxidase. If cytochrome c oxidase is not present, the reagent will retain its reduced form. The reduced form of the reagent is colorless. Therefore, lack of a color change to purple will indicate that cytochrome c oxidase is not present.

Answer the following questions based on the observations during the last lab when you and your lab partners actually performed this experiment:

1. What did you have to add to the bacteria in the oxidase test?

2. What does the purple color indicate? How about the lack of purple color?

Lab Exercise 7-3 (Nitrate Reduction Test)

Recipe: 0.3% Beef Extract, 0.5% Peptone, 0.1% Potassium Nitrate, pH 6.8-7.2 at 25°C.

Reagents Needed for Testing: Nitrate Reduction Test Reagent A (0.8% sulfanilic acid in 5N acetic acid), Nitrate Reduction Test Reagent B (0.6% N,N-Dimethyl-α-naphthylamine in 5N acetic acid), Zinc Powder.

Concept of Test: In aerobic cellular respiration, oxygen behaves as the final electron acceptor receiving electrons from complex IV of the electron transport chain. Reduction of oxygen leads to the production of water. In anaerobic cellular respiration, nitrate, in some cases, will substitute for oxygen. Reduction of nitrate may result in the production of different nitrogen containing products depending on the bacterial species in question. Some bacteria reduce the nitrate to produce nitrite. Other bacteria, denitrifiers, are able to reduce the nitrate to nitrite and then further reduce the nitrite to produce nitrogen gas through a multi-step process known as denitrification. Some bacteria initiate the denitrification process, but stop short of producing the nitrogen gas. Furthermore, other bacteria are able to reduce the nitrate to nitrite and reduce the
nitrite to make ammonia to use for amino acid synthesis. The nitrate reduction test determines how a particular bacterial species utilizes the nitrate.

Possible Outcomes:

1. Nitrate to nitrite then to nitrogen gas (Figure 7-3)- We use the Durham tube to trap the nitrogen produced by the denitrifiers. Look for the trapped gas bubble inside the Durham tube. **The presence of the gas indicates that the bacterial species is a denitrifier.**

2. Nitrate to nitrite (Figure 7-4)- The nitrate reduction reagent A and reagent B are used to identify those bacterial species that reduce the nitrate to nitrite. **A red color formed after the addition of reagents A and B indicates that the bacterial species is positive for nitrate reduction to nitrite.**

3. Nitrate not used at all (Figure 7-5)- We add a small amount of zinc powder to the bacterial species that lack a red color formation after the addition of reagents A and B. If the nitrate is still present, the **zinc powder will convert the nitrate to nitrite.** The nitrite produced by the zinc powder addition will react with reagents A and B and produce a red color. **Therefore, the red coloration after the zinc powder addition indicates that the bacterial species does not use nitrate.**

4. Nitrate to Nitrite then to either ammonia or another nitrogen compound (Figure 7-6)- **Another possible outcome is that the media does not produce any color change after the addition of reagents A and B and zinc powder.** One possible explanation to this outcome is that the bacterial species is utilizing the nitrate to produce ammonia to use to make amino acids. One other possibility is the bacterial species is reducing the nitrate to nitrite and is then attempting to denitrify, but is stopping short of producing the nitrogen gas.

Directions to Students-

1. Record the presence or absence of gas in each tube.

2. To the tubes that do not have gas bubbles, add 8 drops of the nitrate-reduction-test reagent A and 8 drops of the nitrate-reduction-test reagent B.

3. Look for a red color after reagent A and B addition and record (yes or no).

4. To the tubes that do not turn red after the addition of reagent A and B, add a very small amount of zinc powder.

5. Look for a red color after adding the zinc powder and record (yes or no).

6. Write your conclusions for each type of bacteria.
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Gas (Yes or No)</th>
<th>Red Color After Reagents A and B Addition</th>
<th>Color After the Addition of Zinc</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lab Exercise 7-4 (Phenylalanine Deaminase Test)

**Recipe:** 0.2% DL-Phenylalanine, 0.3% Yeast Extract, 0.5% Sodium Chloride, 0.1% Sodium Phosphate, 1.2% Agar, pH 7.1-7.5 at 25°C.

**Reagents Needed for Testing:** *Phenylalanine Deaminase test Reagent* (1.2% Ferric Chloride, 2.5% Concentrated Hydrochloric Acid, Final volume 100ml with Water).

**Concept of Test:** Catabolism of amino acids by bacteria requires the synthesis of amino acid specific enzymes that carry out specialized reactions. *Phenylalanine deaminase* is one example. This enzyme has specificity for the amino acid *phenylalanine*. The specialized reaction that it carries out is a *deamination reaction*, removal of the amine group from, in this case, phenylalanine. Removal of the amine group results in the formation of *ammonia*. It also results in the formation of *phenylpyruvic acid*. The presence of the phenylpyruvic acid is a clear indication that the bacterial species in question is producing phenylalanine deaminase and is catabolizing phenylalanine. Phenylpyruvic acid is colorless. The addition of *ferric chloride* to the phenylpyruvic acid produced by deaminating the phenylalanine will produce a green color (Figure 7-7). *Therefore, the formation of the green color is indicative that the bacterial species is phenylalanine deaminase positive.* Members of the genera *Proteus*,

![Figure 7-7: Example phenylalanine deaminase test.](image-url)
Morganella, and Providencia are phenylalanine deaminase positive, whereas other Enterobacteriaceae are negative.

**Directions to Students**—add about 5 drops of ferric chloride (FeCl₃) directly to the slant region of each culture and look for a color change (green color).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Result (Color)</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Morganella morganii</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Make a Statement to Explain Your Results:

Lab Exercise 7-5 (The Kligler Iron Agar Test)

**Recipe:** 1.0% Pancreatic Digest of Casein, 1.0% Peptic Digest of Animal Tissue, 0.1% Dextrose (glucose), 1.0% Lactose, 0.05% Ferrous Ammonium Citrate, 0.5% Sodium Chloride, 0.05% Sodium Thiosulfate, 1.5% Agar, 0.0025% Phenol Red, pH 7.2-7.6 at 25°C.

**Concept of Test:** The Kligler iron agar helps distinguish between bacterial species that ferment sugar and/or reduce sulfur. The media provides two different types of sugars (glucose and lactose) because some bacteria can ferment only one of these sugars, while others can ferment both of these sugars. The media also provides an oxidized source of sulfur to identify bacterial species that reduce sulfur. The Kligler iron agar media also has a source of protein for those bacterial species that cannot reduce sulfur or ferment any of the sugars added in the media. Therefore, the Kligler iron agar helps distinguish between the various Enterobacteriaceae based on sulfur reduction, sugar fermentation, and protein metabolism.

**Possible Outcomes:**

Some bacterial species ferment glucose and produce acid byproducts causing a lowering of the media’s pH. Confirmation of fermentation is through the production of the yellow color produced by the phenol red pH indicator. The low supply of glucose in the media forces the bacterial culture to utilize other nutrients to survive upon the consumption of the glucose. Some bacterial species will start consuming the other sugar provided (lactose). Some bacterial species will not consume the sugars, but instead metabolize, aerobically and/or
anaerobically, the protein in the media. Other bacteria will anaerobically respire and reduce sulfur in the process. Interestingly, some bacterial species will be able utilize one type of nutrient in the slant region of the tube, while it utilizes a different nutrient in the butt region of the tube. The following describes all the possible outcomes:

1. **Fermentation of Glucose and Lactose (Slant/Acid; Butt/Acid)** - Bacteria that ferment the glucose initially and then switch to fermenting the lactose will produce a Kligler iron agar tube that is yellow in color all the way through, in the slant and butt regions. Again, this is because of the drop in pH caused by the acid byproduct of fermentation and the phenol red pH indicator. Even though the fermentation occurs in the butt region (anaerobic environment), the acid production is so high that it diffuses all the way through the tube (butt and slant regions) changing the media color to yellow all the way through. You may also notice fissures (cracks) in the media indicating the production of gas as a byproduct of fermentation besides the production of the acid.

2. **Glucose Fermentation Only/Protein Metabolism (Slant/Alkaline; Butt/Acid)** - Bacteria that only ferment the glucose sugar, but not lactose, will switch to protein metabolism upon the consumption of the glucose. Restricting the amount of glucose in the media will ensure that the switch from glucose fermentation to protein metabolism takes place. The bacterial species will ferment the glucose in the butt region (anaerobic) releasing the acid into the butt region changing its color to yellow. Upon the consumption of the glucose, the bacterial species will switch to protein metabolism. Utilization of protein will take place primarily in the slant region due to the presence of oxygen near the slant region. Protein metabolism will result in the release of ammonia (basic), which will increase the pH causing a change in the media color to red.

3. **Glucose Fermentation Only/Protein Metabolism/Sulfur Reducer (Slant/Alkaline; Butt/Acid and Black Precipitate in Butt)** - Some bacterial species will behave the same as the bacterial species described in #2, but they are also capable of sulfur reduction within the butt region (anaerobically). The glucose fermentation in the butt region will produce a yellow color, while the protein metabolism will produce a red color in the slant region (aerobically). Additionally, these bacteria will also begin to reduce the sodium thiosulfate in the acidic environment of the butt region (anaerobically). The hydrogen sulfide (H\(_2\)S) produced by sulfur reduction will react with the ferrous ammonium citrate to produce a black color in the butt region. The black color produced in the butt region will eventually mask the yellow color produced by glucose fermentation. We do not have to see the yellow color to confirm that the bacterial species fermented the glucose, since the acid produced by fermentation has to be present for sulfur reduction to begin.

4. **Glucose and Lactose Fermentation/Sulfur Reducer (Slant/Acid; Butt/Sulfur Precipitate)** - Some bacterial species will behave the same as the bacterial species described in #1, but they are also capable of sulfur reduction within the butt region (anaerobically). The glucose and lactose fermentation in the butt region will produce a yellow color that will also overwhelm the slant region and turns the slant region yellow as well. Additionally, these bacteria will also begin to reduce the sodium thiosulfate in the acidic environment of the butt region (anaerobically). The hydrogen sulfide (H\(_2\)S) produced, as a reduction of the sulfur reduction, will react with the ferrous ammonium citrate to produce a black color in the butt region. The black color produced in the butt region will eventually mask the yellow color produced by glucose/lactose fermentation. We do not have to see the
yellow color to confirm that the bacterial species fermented the glucose and lactose, since the acid produced by fermentation has to be present for sulfur reduction to begin. The black precipitate will not form in the slant region, since sulfur reduction requires an anaerobic environment. The slant region will remain yellow in color.

5. **Protein Metabolism Only (Slant/Alkaline; Butt/No Color Change or Slant/Alkaline; Butt/Alkaline)**-Bacteria that do not consume the glucose or lactose provided to them in the Kligler iron agar media are able to survive only if they are able to metabolize protein. These bacteria may metabolize the protein in the slant region (aerobically) and/or in the butt region (anaerobically). If the bacterial species only aerobically metabolizes the protein then only the slant region will be red in color. If the bacterial species aerobically and anaerobically metabolizes the protein then both the slant and the butt region will be red in color. Either situation would confirm that the bacterial species is not an *Enterobacteriaceae*.

*No color change in both the slant and butt regions indicates that the bacterial species is growing very slowly or not at all, which is also indicative that it is not *Enterobacteriaceae*.

**Directions to Students**-

1. Record the color found within the slant region of the tube.
2. Record the color found within the butt region of the tube.
3. Record the presence of gas in the Durham tube (yes or no).
4. Record the presence of sulfur, black color (yes or no).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Slant Region Color</th>
<th>Butt Region Color</th>
<th>Gas (Yes or No)</th>
<th>H₂S Production</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Morganella morganii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Questions:

1. Which bacteria was a dentrifier? ________________________________________________

2. Which bacteria reduced the nitrate to nitrite and did not reduce the nitrite?
   ______________________________________________________

3. Which bacteria did not reduce the nitrite? ____________________________

Lab Exercise 7-6 (Coagulase Test)

Recipe: Tubes of rabbit plasma in EDTA.

Concept of Test: Some bacterial species (example, Staphylococcus aureus) are capable of releasing factors that help them resist the immune response as well as the effects of antibiotics. One of these factors is the enzyme coagulase. There are two forms of this enzyme, a bound form (attached on the cell wall) and a free form (released by the cell into the nearby environment, exoenzyme). Bacterial species that produce coagulase are able to evade the host’s immune response and the effects of antibiotics by forming a protective barrier around a single bacterial cell or around a group of bacterial cells. These bacteria produce the protective barrier through the actions of the coagulase they produce and the proteins found in blood plasma. The coagulase causes the coagulation of these plasma proteins near the bacterial species producing the enzyme. The reaction can result in the formation of a barrier around single bacterial cells or a group of cells. The bound form causes clumping of the bacterial cells. We use the tube-rabbit-plasma coagulase test to determine if the bacterial species is producing coagulase or not, but it will not determine if it is the bound or free form. Coagulation of the rabbit plasma proteins in the tube after inoculation is indicative of the presence of coagulase, whereas non-coagulated plasma proteins after inoculation indicates the lack of coagulase (Figure 7-9).

Directions to Students-Slightly shake each tube and record in the table which one coagulated and which one is still liquid.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Result (Coagulation)</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Make a Statement to Explain Your Results:
Preparation for the next lab (Lab 8: Culture and Sensitivity-Identification of an Unknown Enterobacteriaceae and Antibiotic Susceptibility Test)

**Instructions to Students**-Your instructor will give your group a plate labeled with a single letter, A thru E, which will become your assigned unknown *Enterobacteriaceae*. Your objective is to identify the bacterial species assigned to your group. You will do this by inoculating different types of media to figure out a list of characteristics about your assigned unknown. You will then use a chart of phenotypic characteristics to identify your unknown. The other experiment that you will setup has to do with antibiotic resistance (nothing to do with the unknown identification).


**Methods**-Use the following diagrams (Figures 7-10 and 7-11) as guides for your inoculations.

Which unknown did the instructor assign to your group? Letter on Plate: ____________.

Use the aseptic technique to inoculate your assigned unknown bacteria into each of test given below. **Use the following inoculating directions:**

- **Ex. 8-1, Simmons Citrate:** Streak the slant region with inoculating NEEDLE.
- **Ex. 8-2, Phenol Red Tubes:** Inoculate the broth with inoculating loop.
- **Ex. 8-3, Decarboxylase Test:** Inoculate the broth with inoculating loop and then layer with 1 to 2 ml using oil and sterile pipette.
- **Ex. 8-4, Urea Hydrolysis Test:** Inoculate the broth with inoculating loop.
- **Ex. 8-5, SIM Test:** Use the inoculating needle to stab the media.
- **Ex. 8-6, Gelatin Test:** Use the inoculating needle to stab the media.
- **Ex. 8-7, MRVP Test:** Inoculate the broth with inoculating loop.
Procedure for Inoculating the TSA Plates for the Antibiotic Susceptibility Test (Ex. 8-8):

Ex. 8-8 Antimicrobial Susceptibility Test (Kirby Bauer Method)

*Use a cotton swab and the bacterial culture to cover the whole plate. You have to produce what is called a bacterial lawn before you place the antimicrobial discs on top of it.

*Place the antimicrobial discs for Penicillin, Chloramphenicol, Trimethoprim, and Ciprofloxacin

Penicillin (Cell wall), Chloramphenicol (Protein synthesis), Trimethoprim (synthesis of AGCT and U), Ciprofloxacin (DNA replication)

Figure 7-11
**Incubation Instructions:**

**Students**—Place all your unknown identification tests together on one rack. Tape the antibiotic susceptibility test plates together.

**Instructor**—Incubate all plates and tubes at 37°C for 48 hours, except for the gelatin hydrolysis test (incubate at 25°C). Incubate the antibiotic susceptibility plates immediately.

**Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):**

1. Cytochrome C Oxidase is a component of ________________ and is involved in the production of the important energy molecule called ________________.

2. Explain the negative consequence of oxygen utilization by aerobic bacteria that is overcome by the bacteria producing catalase.

3. Explain how this is possible: Bacteria growing in a Nitrate Reduction Test tube does not produce nitrogen gas, does not turn red after the addition of the reagents, and does not turn red after the addition of zinc.

4. Explain what bacteria are utilizing for energy when you observe a Kligler Iron Agar tube that has a red-slant and a yellow-butt.

5. How does the coagulase released by *S. aureus* benefit the bacterium?
LAB #8: Analysis of Culture and Sensitivity (Identification of the Unknown Bacteria) and Analysis of Antibiotic Susceptibility

**Lab Objectives:**

1. To learn the use and purpose of the Simmons Citrate test to gather data to use in the identification of an unknown bacteria.
2. To learn the use and purpose of various phenol red fermentation tests to gather data to use in the identification of an unknown bacteria.
3. To learn the use and purpose of the lysine and ornithine decarboxylase tests to gather data to use in the identification of an unknown bacteria.
4. To learn the use and purpose of the urea hydrolysis test to gather data to use in the identification of an unknown bacteria.
5. To learn the use and purpose of the gelatin hydrolysis test to gather data to use in the identification of an unknown bacteria.
6. To learn the use and purpose of the SIM test to gather data to use in the identification of an unknown bacteria.
7. To learn the use and purpose of the MRVP tests to gather data to use in the identification of an unknown bacteria.
8. To use the supplied guide in the identification of the unknown bacteria.
9. To learn the use and purpose of the Kirby-Bauer method in the analysis of antibiotic susceptibility.

**Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions):**

1. What does "ZONE OF INHIBITION" mean?

2. What is the SIM test used for?

3. What is the Simmons Citrate Agar testing for?

4. Explain why culturing of a urease positive bacteria in the urea hydrolysis media turns the media pink.
5. Why do you incubate the gelatin hydrolysis tube at 25°C, but not at 37°C, after you have inoculated it with your bacteria?

**Background Information:** Identification of an unknown bacterium is one of the 5 I’s of microbiology. The microbiologist has to establish a set of characteristics about the unknown bacterium through experimentations. Visual and microscopic inspection helps to some extent, but are not enough to narrow down the identification to a single bacterial species. A collection of biochemical testing of the unknown bacterial species are performed help in the identification process. The set of experiments that we will be carrying out will help us identify an unknown bacterial species from among the members of the *Enterobacteriaceae*.

**Lab Exercise 8-1 (Simmons Citrate Test)**

**Recipe:** 0.1% Ammonium Dihydrogen Phosphate, 0.1% Dipotassium Phosphate, 0.5% Sodium Chloride, 0.2% Sodium Citrate, 0.02% Magnesium Sulfate, 1.5% Agar, 0.008% Bromothymol Blue, pH 6.7-7.1 at 25°C.

**Concept of Test:** In the Simmons citrate agar, the sole carbon source added is *citrate*. Bacterial species that cannot transport that sole carbon resource into the cell will die on the Simmons citrate media. Bacterial species that do survive on the Simmons citrate media survive because they are able to bring in that valuable carbon resource. Interestingly, the cell does not actually transport the citrate. *Pyruvate* is what actually provides the cell the carbon source. The actions of two enzymes, *citrate lyase* and *citrate permease*, are involved in this process. Citrate lyase helps in the breakdown of citrate into *oxaloacetate* (occurs outside the cell). Citrate permease, which is a *membrane protein (enzyme)*, helps transport the oxaloacetate into the cell. The same enzyme, citrate permease, also *decarboxylates* the oxaloacetate into pyruvate during the transport. Therefore, pyruvate is what is actually entering the cell and providing the cell the needed carbon. Using pyruvate, the bacterial cell can form a variety of products to support its nutritional needs. To assay for the presence of citrate permease, which is what this test is testing for, we look for byproducts of metabolism produced by those surviving bacterial species. One of these byproducts will include ammonia produced by the utilization of the ammonium dihydrogen phosphate found in the media. The alkalinity of ammonia will cause the pH to increase. The basic pH conditions will change the color of the media from its original color (green) to blue, due to the presence of the pH indicator bromothymol blue. Therefore, a blue coloration in the tube is indicative of the presence of citrate permease (Figure 8-1). A green tube with no obvious bacterial growth would indicate that the bacterial species died due to the lack of carbon, because it is citrate permease negative. However, a green tube with obvious bacterial growth indicates that the bacterial species is positive for citrate permease. This is why we use the inoculating needle when we are preparing these tubes so that we do not confuse growth with a heavy inoculum.

Figure 8-1: Example Simmons citrate test results.
**Exercise**-Observe your Simmons Citrate tube and record your unknown’s Simmons Citrate Test Result:

- Color: ____________________  Conclusion: ____________________

**Lab Exercise 8-2 (Phenol Red Test)**

**Recipe:** 1.0% Pancreatic Digest of Casein, 0.5% Sodium Chloride, 0.5% Carbohydrate (Choice of ONE in each tube: Lactose, Glucose, Xylose, Arabinose, or Mannitol), 0.0018% Phenol Red, pH 7.1-7.5 at 25°C.

**Concept of Test:** The phenol red media (Figure 8-2) helps us examine if a particular bacterial species is able to ferment certain sugars. We add a single sugar in the media to determine if the bacterial species is capable of fermenting it. A byproduct of fermentation, in many cases, is the production of an acid. The acid will lower the pH and change the media color to yellow because of phenol red, the pH indicator, turns yellow in an acidic environment. **The yellow color clearly indicated that the bacterial species ferments the sugar added in the tube.** Another potential byproduct of fermentation is the gas carbon dioxide (among other gases). Inside the phenol red tube, we add a Durham tube to help trap any gas produced by fermentation. **The presence of a gas bubble inside the Durham tube helps in the confirmation of fermentation.** A phenol red tube that is red in color and without any gas in the Durham tube indicates that the bacterial species is not fermenting the sugar added in that particular tube.

**Exercise**-Record your unknown’s Phenol Red Test Results:

- **Arabinose:**
  - Color: ________
  - Gas: ______
  - Conclusion: ____________________

- **Glucose:**
  - Color: ________
  - Gas: ______
  - Conclusion: ____________________

- **Lactose:**
  - Color: ________
  - Gas: ______
  - Conclusion: ____________________

- **Mannitol:**
  - Color: ________
  - Gas: ______
  - Conclusion: ____________________

- **Xylose:**
  - Color: ________
  - Gas: ______
  - Conclusion: ____________________
Lab Exercise 8-3 (Decarboxylase Test)

**Recipe:** 0.5% Peptone, 0.5% Beef Extract, 0.05% Glucose (Dextrose), 0.001% Bromocresol Purple, 0.0005% Cresol Red, 0.0005% Pyridoxal, 1.0% Amino Acid (Choice of ONE per tube: L-Lysine or L-Ornithine), pH 5.8-6.2 at 25°C.

**Concept of Test:** Some members of the *Enterobacteriaceae* are able to metabolize amino acids by removing the amino acid’s carboxyl group, utilizing specialized enzymes called decarboxylases. These decarboxylases are substrate specific so that the lysine decarboxylase and the ornithine decarboxylase only decarboxylate lysine and ornithine, respectively. Interestingly, activation of the decarboxylase gene expression requires a decrease in pH (acidic environment). Furthermore, not all members of the *Enterobacteriaceae* express all the different types of decarboxylase genes.

To differentiate between the members of the *Enterobacteriaceae*, the decarboxylase media will always contain glucose along with one type of amino acid, in this case either lysine or ornithine. The sterile oil added to the inoculated media blocks the availability of oxygen to the media. This anaerobic environment helps induce the fermentation of the glucose by the bacterial species. This step is critical to lower the media’s pH to activate the decarboxylase gene for the particular amino acid added in the media (if the bacterial species has that particular gene). The pH indicator found in the media, bromocresol, produces a yellow coloration in the presence of acid (Figure 8-3). **Yellow coloration** in the media after incubation indicates that the bacterial species is only able to ferment glucose and does not have the particular decarboxylase gene for the amino acid added in the media. If the bacterial species did have the decarboxylase gene, the acidic pH will activate its expression to produce the amino acid specific decarboxylase. The enzyme will then remove the carboxyl group and leave behind the amine group attached to the rest of the amino acid. The amine group is basic, which will cause an increase in the media’s pH. Bromocresol will produce a **purple coloration** under basic pH. Therefore, a purple color after incubation indicates that the bacterial species produces the amino acid specific decarboxylase to metabolize the particular amino acid in the media.

**Exercise** Record your unknown’s Decarboxylase Test Results:

Lysine:
Color: __________ Conclusion: ____________________________

Ornithine:
Color: __________ Conclusion: ____________________________
Lab Exercise 8-4 (Urea Hydrolysis Test)

**Recipe:** 0.01% Yeast Extract, 0.91% Potassium Phosphate (Monobasic), 0.095% Potassium Phosphate (Dibasic), 2.0% Urea, 0.001% Phenol Red, pH 6.6-7.0 at 25°C.

**Concept of Test:** Some members of the *Enterobacteriaceae* express the enzyme **urease** to breakdown **urea** into **ammonia** and carbon dioxide. The bacterial species will utilize the ammonia produced by this reaction as a source of nitrogen. The formulation of the media (Figure 8-4) used in this test helps identify only those bacterial species that produce urease and hydrolyze the urea **rapidly** from those bacterial species that do not produce urease and therefore do not hydrolyze urea. Those bacterial species that produce urease will breakdown the urea to release ammonia, which will increase the media’s pH because ammonia is basic. The pH indicator, phenol red, produces a **pink coloration** in a basic environment. Therefore, a pink color indicates that the bacterial species produces urease. Bacterial species that do not produce urease will not cause a change in the media’s pH and the media will retain its original color.

**Exercise** - Record your unknown’s Urea Hydrolysis Test Result:

- Color: ________________
- Conclusion: ____________________

Lab Exercise 8-5 (Gelatin Test)

**Recipe:** 0.03% Beef Extract, 0.5% Peptone, 12% Gelatin, pH 6.6-7.0 at 25°C.

**Concept of Test:** Some members of the *Enterobacteriaceae* express an enzyme called **gelatinase** that metabolizes the protein **gelatin**. The bacterial species uses the amino acids derived from the breakdown of the gelatin as a source of nutrition. In this test, we use gelatin as the solidifying agent. That is why this media is a semisolid. The use of gelatin as the solidifying agent is critical to be able to differentiate between those members that produce gelatinase and those that do not. Those bacterial species that do produce gelatinase will breakdown the gelatin (solidifying agent) **liquefying the media** and those bacterial species that do not produce gelatinase will not liquefy the media. One important concern to remember is that above temperatures of 28°C gelatin loses its ability to solidify and the media will liquefy due the higher temperature. For that reason, we incubate the gelatin test media at 25°C to ensure that the liquefying of the media is because of the breakdown of gelatin by gelatinase and not simply because of an incubation temperature that is higher than 28°C.

**Exercise** - Record your unknown’s Gelatin Test Result:

- Semi-Solid or Liquid: ________________
- Conclusion: ____________________
Lab Exercise 8-6 (SIM Test)

**Recipe:** 2.0% Pancreatic Digest of Casein, 0.61% Peptic Digest of Animal Tissue, 0.02% Ferrous Ammonium Sulfate, 0.02% Sodium Thiosulfate, 0.35% Agar, pH 7.1-7.5 at 25°C.

**Reagents Needed for Testing:** **Kovac’s Reagent** (5% *p*-dimethylaminobenzaldehyde in a 3 to 1 ratio of Amyl Alcohol : Concentrated Hydrochloric Acid, respectively).

**Concept of Test:** The SIM test test actually three different tests setup all together in one tube (Figure 8-5). The tests examines if the bacterial species reduces sulfur (S), metabolizes tryptophan (I), and motility (M).

**Sulfur Reduction:** The conversion of cysteine to pyruvate releases the reduced form of sulfur, H₂S. Another way to produce H₂S is through anaerobic respiration. Those bacterial species that carry out either one of these reactions or both of them will produce a black precipitate in the SIM media tube. The black precipitate produced in media results from the H₂S interacting with the ferrous ammonium sulfate found in the media. **Therefore, a black coloration in the media indicates that the bacterial species is a sulfur reducer.**

**Tryptophan Metabolism:** Some bacterial species are capable of producing an enzyme called *tryptophanase* to metabolize the amino acid tryptophan into pyruvate, indole, and ammonia. To confirm if the inoculated bacterial species is capable of producing the enzyme tryptophanase, we look for the presence of the indole byproduct. The Kovac’s reagent reacts with indole, if present, and produces a red coloration. **Therefore, a red color after the addition of Kovac’s to the SIM test tube indicates that the bacterial species expresses tryptophanase.** The absence of a red color indicates that the bacterial species is negative for tryptophanase.

**Motility:** Inoculation of the semisolid SIM tube requires the use of the stab technique to help assess for motility after incubation. Bacterial species that are not motile will only grow within the stab region, whereas bacterial species that are motile will grow within the stab region and throughout the media. **Motile bacteria will produce agar that appears hazy after incubation.**

**Exercise**—Record your unknown’s SIM Test Result:

No addition of reagents required to examine for sulfur reduction (Visual Observation Only)-

“S”-Sulfur (Yes or No): ____________ Conclusion: _______________________

Kovac’s reagent (3-5 drops) added to the top of the semisolid media followed by visual observation-

“I”-Indole (Yes or No): ____________ Conclusion: _______________________

No addition of reagents required to examine for motility (Visual Observation Only)-

“M”-Motility (Yes or No): ____________ Conclusion: _______________________
Lab Exercise 8-7 (MRVP Test)

**Recipe:** 0.7% Buffered Peptone, 0.5% Dipotassium Phosphate, 0.5% Dextrose (Glucose), pH 6.7-7.1 at 25°C.

**Reagents Needed for Testing:**
- **Methyl Red Reagent** (0.02% Methyl Red Dye in 60% Ethanol in Water),
- **VP Reagent A** (0.5% α-naphthol in Absolute Alcohol),
- **VP Reagent B** (40% Potassium Hydroxide in Water).

**Concept of Test:** In an oxygen-deprived environment, a number of bacterial species produce a limited number of ATPs through glycolysis followed by fermentation of the pyruvate produced by glycolysis. Interestingly, the products of fermentation varies depending on the bacterial species. **Alcoholic fermenters** are bacterial species that ferment the pyruvate to produce ethanol and carbon dioxide, like yeast. **Homolactic fermenters** are bacteria that ferment the pyruvate to produce lactic acid only, similar to animal cells. **Heterolactic fermenters** are bacteria that ferment to produce lactic acid, ethanol, acetate, and carbon dioxide. Other bacteria, referred to as **2,3-butanediol fermenters**, ferment to produce ethanol, lactic acid, formic acid, carbon dioxide, hydrogen gas, and 2,3-butanediol (these bacterial species are identified by the Voges-Proskauer Test). A red coloration after the addition of the two VP reagents directly into the culture indicates the presence of 2,3-butanediol (Figure 8-6). Finally, **mixed acid fermenters** ferment to produce ethanol, gas (carbon dioxide and hydrogen), along with a mixture of acids to include acetic, lactic acid, succinic, and formic. The methyl red test identifies those mixed acid fermenters. A red coloration after the addition of methyl red directly into the culture indicates that the bacterial species is a mixed acid fermenter (Figure 8-6).

**Exercise**—Record your unknown’s MRVP Test Result:

- Place 2 ml of your culture into an empty sterile tube. We will call this tube, tube #1.

- Place another 2 ml of your culture into a second empty sterile tube. We will call this tube, tube #2.

- To tube #1, add 5 drops of methyl red and quickly mix. A red color is positive for mixed acid fermentation.

- To tube #2, add 15 drops of VP reagent A then mix. Then add 5 drops of VP reagent B then mix. Read within 10 to 60 minutes. A red color indicates that 2,3-butanediol is present.

“MR”-Methyl Red-Color: ___________ Conclusion: _______________________

“VP”-Voges-Proskauer-Color: ___________ Conclusion: _______________________
IDENTIFICATION OF THE UNKNOWN-

1. Collect your results from the “Indole”, “Methyl Red”, “Voges-Proskauer”, and “Citrate” tests to produce an IMVC code.

   Indole (Red) = “+”; Indole (Yellow) = “-”
   Methyl Red (Red) = “+”; Methyl Red (Yellow) = “-”
   Voges-Proskauer (Red) “+”; Voges-Proskauer (Yellow) = “-”
   Citrate (Blue) = “+”; Citrate (Green) “-”

   What is your unknown’s IMVC code?  ____  ____  ____  ____

2. Use the identification guide given in Appendix A to identify your unknown.

   What is the name of your Unknown? ____________________________

Lab Exercise 8-8 (Antibiotic Susceptibility Test-Kirby-Bauer Method)

Recipe: (Mueller-Hinton II Agar-4mm Depth) 0.2% Beef Extract, 1.75% Acid Hydrolysate of Casein, 0.15% Starch, 1.7% Agar, pH 7.2-7.4 at 25°C.

Background Information-Microorganisms produce agents (antibiotics) that either inhibit the growth or kill bacteria. Other antimicrobial agents are chemically modified antibiotics, while others are synthetic. The Mueller-Hinton II agar is a standardized media used to perform the Kirby-Bauer method (Figure 8-7). The bacterial species tested is inoculated onto the agar plate to produce a bacterial lawn after incubation. After inoculation, but prior to incubation, paper disk containing a specific antimicrobial agent at a specific concentration is placed directly onto the surface of the agar. During incubation of the plate, the antimicrobial will begin to diffuse throughout the media. The concentration of the antimicrobial will decrease the farther away it is from the disk. If the antimicrobial is bacteriostatic, it will inhibit the bacterial growth. If the antimicrobial is bactericidal, it will kill the bacteria. Regardless, a region of no growth or killing of the bacteria will form around the disk. This clear zone or region is called the zone of inhibition. At the outer edge of the zone of inhibition, the concentration of the antimicrobial is low enough as to not hinder bacterial growth. This antibiotic concentration is called the minimum inhibitory concentration. The minimum inhibitory concentration is not determined using the Kirby-Bauer method. The size of the zone of inhibition is important in determining the effectiveness of the antimicrobial agent against the bacterial species inoculated on the plate. Depending on the size of the zone of inhibition, the interpretations may range from the bacterial species being resistant to the antimicrobial agent to it being susceptible to the antimicrobial agent.
**Exercise**

Record the diameter of the zone of inhibitions in millimeters and use appendix B to help you interpret the results (R=Resistant; I=Intermediate; S=Susceptible):

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>E. coli</th>
<th>R, I, or S</th>
<th>S. aureus</th>
<th>R, I, or S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethoprim</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):**

1. A bacterial species that is positive for indole indicates that it is producing ____________________.

2. A positive methyl red test indicates that the bacterial species is able to produce ______________ by fermentation.

3. Sulfur reduction produces a _____________ coloration in the SIM test.

4. A bacterial species with a 15mm zone of inhibition for penicillin is considered to be ___________ to penicillin, whereas the same bacterial species with a 22mm zone of inhibition for chloramphenicol is considered to be ______________ to chloramphenicol.

5. You obtain a culture from two patients and do several tests to be able to identify the bacteria. Using your lab manual identify the following bacteria:

   A). Citrate (-); Xylose Fermentation (-); Indole (+); Ornithine Decarboxylase (-); Methyl Red (+); Vogas-Proskauer (-)

   B). Citrate (+); Ornithine Decarboxylase (+); Vogas-Proskauer (-); H₂S Production (+); Methyl Red (+); Lysine Decarboxylase (+); Indole (-); Glucose Fermentation (+)
LAB #9: DNA: Transformation and DNA Damage by Ultraviolet Light

Lab Objectives:
1. To learn the consequence of ultraviolet light on DNA and how some bacteria are able to fix the damaged DNA and survive.
2. To learn how DNA transformation benefits bacteria.
3. To learn how DNA transformation into bacteria benefits humans.
4. To learn how to perform DNA transformation procedure in a laboratory setting.

Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions)
1. How are we going to genetically mutate bacteria in this lab?
2. What happens to thymidines when they are exposed to the agent that you gave in question #1?
3. Which enzyme is used in photoreactivation?
4. Which macromolecule is being fixed by the enzyme that you gave in question #3?
5. List all the enzyme that are involved in excision repair. Describe what each enzyme does in the process in order of their appearance.
6. Which type of macromolecule are we transforming into the bacteria?
7. After transformation of the plasmid, addition of arabinose will induce the expression of ____________ protein.
8. What does competent bacterial cells mean?
9. How are we going to make the bacteria competent?
10. How will arabinose initiate gene expression in this experiment?
Lab Exercise 9-1 (The Lethal Effect of Ultraviolet Light on Microbial Growth)

**Background**—Ultraviolet light is a type of non-ionizing radiation. Upon exposure to UV, the pyrimidine bases found in a DNA molecule will start producing dimers, which is abnormal. Longer exposure results in more dimer formation. One type of dimer that can form is between a cytosine and another cytosine, another type of dimer is between a thymidine and a cytosine, but the most common type is between a thymidine and another thymidine. Unresolved **pyrimidine dimers** in bacteria results in inhibition of DNA replication, transcription, and cell death. Bacteria has repair mechanisms that help correct this type of DNA damage. The two repair mechanisms include **light repair (photoreactivation)** and **dark repair (excision repair)**. Some bacterial species are able to fix DNA damaged by UV using one or both of these DNA repair mechanisms. Bacterial species that do not adequately fix the damaged DNA will end up dying due to the damaged DNA, whereas bacterial species that employ a more aggressive approach towards fixing the damaged DNA will survive the UV exposure.

Some bacterial species use the enzyme **photolyase** to resolve the pyrimidine dimers that result from UV exposure. Visible light is required to activate the enzyme photolyase. This mechanism of DNA repair is called light repair or photoreactivation. The other mechanism of DNA repair is called dark repair or excision repair (visible light is not required). However, this type of repair mechanism is more involved. It does not simply resolve the dimer like in photoreactivation. Dark repair involves the use of four different enzymes because the repair mechanism will not only remove the mutation, but it will also remove the DNA segment around the mutation and then repair it. The first enzyme is an **endonuclease**, which cleaves the phosphodiester bonds upstream and downstream of the mutation site. The second enzyme is **helicase** to break down the hydrogen bonds that are holding the defective segment of DNA to the complementary strand. The combined actions of both the endonuclease and helicase will remove the defective segment. The third enzyme is **DNA polymerase I**, which will use the other strand as a template to build the new and correct sequence in the 5’ to 3’ direction. The fourth and last enzyme is **DNA ligase**, which will seal the final gap between the last nucleotide of the newly synthesized DNA segment and the neighboring nucleotide by forming a phosphodiester bond.

**Lab Procedure**

**Instructions to Students**—Inoculate your plates using the aseptic technique. Use the following schematic (Figure 9-1) to prepare the plates for analysis during the next lab. Inoculate your plates using a small “Z” like motion using your inoculating loop. Place the inoculum in the center of the designated area closer towards the edge of the plate than center of the designated area.

**Materials**—Five TSA plates. Four culture tubes (*Serratia marcescens; Escherichia coli; Aspergillus niger; Bacillus cereus*). Inoculating loop and Bunsen burner. UV light box.
**Methods** Use the following diagram (Figure 9-1) as a guide to inoculate the plates for next week’s evaluation. After inoculating your plates and exposing your plates to the UV light, group your plates together for incubation at 37°C.

![Setup diagram for the effects of ultraviolet light on bacterial growth.](image)

**Exercise (Next Week)** Record the level of growth for each bacterial species and each plate using the table below. Use your “control, no UV plate’ as your reference point. Use five “+++++” signs to indicate the level of growth on the control plate for each bacterial species. Compare the level of growth on all the subsequent plates to indicate the level of growth on those plates using “+++++”, to indicate the same level of growth as observed in the control plate. “++++”, “+++”, “++”, or “+”, to indicate a reduced level of growth compared to the control plate. “-“, to indicate no growth.

<table>
<thead>
<tr>
<th>Plate</th>
<th><em>Serratia marcescens</em></th>
<th><em>Escherichia coli</em></th>
<th><em>Aspergillus niger</em></th>
<th><em>Bacillus cereus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>No UV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min UV, Lid Off</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 min UV, Lid Off</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min UV, Lid Off</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min UV, Lid On</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Student Conclusion Statement:**

Lab Exercise 9-2 (Bacterial Transformation-The pGLO System)

**Background** Bacterial transformation is the acquisition of exogenous DNA by a competent bacterium. Competent bacteria are bacteria that are capable of taking in DNA from the environment. Transformation results in the production of a new recombinant, an organism that
expresses genes carried on DNA that was originally part of another organism’s genome. In the lab, we can make bacteria become competent through the combined effects of a chemical treatment followed by temperature shock. Treating bacterial cells with the transformation reagent, calcium chloride (CaCl₂), partially disrupts the cell membrane to allow the exogenous DNA to enter the cell during the heat shock step. We commonly use plasmid DNA as the exogenous DNA. The plasmid (in this lab-pGLO) will have the gene of choice (in this lab-the green fluorescent gene-GFP) under the control of a promoter (in this lab-the pBAD promoter). The plasmid will also have an antibiotic resistance gene (in this lab-ampicillin resistance) used to select for the bacterial cells that were transformed from those that were not transformed. The transformed bacteria will grow on an ampicillin selection plate, whereas the non-transformed bacteria will die on the selection plate.

The pBAD promoter is the promoter used by the arabinose inducible operon. The pBAD promoter will be in the “OFF” position when the protein called araC is bound to the promoter. The binding of araC to the promoter is not sufficient for RNA polymerase to bind to the promoter to initiate transcription. To initiate transcription, araC has to undergo a conformational change without it losing its binding to the promoter. Binding of the inducer, arabinose, to araC will cause the conformational change of araC, which will subsequently invite the RNA polymerase to bind to the promoter and initiate transcription. Normally, the RNA polymerase binding to the pBAD promoter, which is part of the arabinose operon, will initiate the transcription of genes that are involved in the metabolism of arabinose. However, the pGLO plasmid supplier engineered the plasmid to have a pBAD promoter upstream of the GFP gene. In this case, binding of the RNA polymerase to the plasmid’s pBAD promoter will initiate the transcription of the green fluorescent protein, but this will only happen when the inducer, arabinose, is added in the media. Bacterial colonies expressing the green fluorescent protein will fluoresce a green color upon exposure to a UV source (Figure 9-2).

Lab Exercise

Instructions to Students-Students will use the pGLO system to transform competent bacteria with plasmid DNA containing coding sequence for the GFP protein as well as an ampicillin resistant gene. Follow the stepwise procedure given to you under “methods” to produce your new recombinant strain.

**Methods**

1. Label one of the empty microfuge tubes with a “+ DNA” and the other tube with a “- DNA.” Label both tubes with your group number.

2. Place both tubes in the foam holder.

3. Using the micropipette and micropipette tips, transfer 250 \( \mu l \) of transformation reagent in both the “+” and “-“ DNA tubes.

4. Place both tubes back in the foam holder and place into the ice bath.

5. Transfer 3-5 *E. coli* colonies into each tube using a sterile plastic loop.

6. Mix the colonies thoroughly to disperse the colony throughout the transformation reagent.

7. Add 10 \( \mu l \) of pGLO DNA suspended in transformation reagent (Ask your instructor) to the “+” DNA tube, only.

8. Keep on ice for 10 minutes.

9. Quickly transfer the foam rack from the ice bath directly into the 42°C water bath for exactly 50 seconds (Heat Shock).

10. After the heat shock step is complete, place the foam holder directly back onto the ice bath for two minutes.

11. Remove the microfuge tubes from the ice bath and place them on the table at room temperature.

12. Add 250 \( \mu l \) of LB broth into each microfuge tube.

13. Leave tubes at room temperature for 10 minutes.

14. Aliquot the samples as follows using the micropipette and a different micropipette tip for each sample sample/plate:

   a. 100 ul of “-“ DNA onto the LB only plate.

   b. 100 ul of “-“ DNA onto the LB + Ampicillin plate.

   c. 100 ul of “+“ DNA onto the LB + Ampicillin plate.

   d. 100 ul of “+“ DNA onto the LB + Ampicillin + Arabinose plate.
15. Using a different plastic sterile loop for each sample/plate, spread the aliquot to cover the entire plate.

16. Gather your plates and give them to your instructor for incubation at 37°C.

**Exercise (Next Week)** - Fill out the following table. You will have to use a hand held UV pen to confirm expression of the green fluorescent protein.

<table>
<thead>
<tr>
<th></th>
<th>LB Only Plate</th>
<th>LB + Ampicillin</th>
<th>LB + Ampicillin + Arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (+ OR -)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial Growth (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP Expression (Yes or No)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):**

1. Which macromolecule does ultraviolet radiation damage?

2. What is a pyrimidine dimer?

3. What is the difference between the two types of repair light versus dark?

4. If the bacterial species does not repair the damage caused by UV radiation, what might be the consequence to that bacteria?

5. What is bacterial transformation?

6. What are competent bacterial cells?

7. How do we make the cells competent in a laboratory setting?
LAB #10: Immunology: ELISA Test

**Lab Objectives:**

1. To learn the usefulness and theory of the ELISA test.
2. To learn how to perform and interpret an ELISA test.
3. To simulate an epidemic.

**Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions)**

1. What is an antigen?
2. What does ELISA stand for?
3. When would you perform an ELISA?
4. What is the difference between the direct and indirect ELISA?
5. Define epidemiology.
Lab Exercise 10-1 (ELISA)

**Background**—For this lab, you will learn how to perform a very important test called enzyme linked immunosorbent assay (ELISA). Doctors may order an ELISA test on a patient’s sample to identify if the patient is suffering from an on-going infection in an effort to diagnose disease. ELISA can also help identify if the patient has developed protection against a pathogen resulting from a prior exposure to the pathogen. Another use for an ELISA is to determine antibody titer (concentration) after vaccination. ELISA is also useful to test for the presence of inflammatory markers in blood, blood drug screening, and the presence of known food allergens in food preparations.

One type of ELISA test directly looks for the presence of an antigen, a non-host specific protein like bacterial or viral proteins, in a patient’s sample. Since this type of ELISA directly looks for the antigen, it is referred to as the direct ELISA test (Figure 10-1). Direct ELISA only uses one antibody, called the primary antibody, to detect the presence of the specific antigen that it recognizes. This primary antibody will have an enzyme conjugated (attached) to it. Binding of the enzyme conjugated primary antibody to the antigen results in a color change as soon as you add the chromogen to the mixture. This color change indicates that the antigen is present in the sample. Since this type of ELISA requires that the antigen be present in the patient sample for the antibody-antigen interaction and the color change to occur, the direct ELISA indicates that the infectious agent was present in the patient at the time of sampling. Assaying the patient’s sample with a well-characterized primary antibody with an established antigen specificity helps in the disease diagnosis. Patient samples that do not have the antigen will not produce color because the unbound enzyme conjugated primary antibody will be lost in the washing steps performed during the procedure.

The other type of ELISA test looks for the presence of an antibody produced by the patient as part of the immune response produced by the host in an effort to eliminate the pathogen. The antibody produced by the patient may be in the patient sample due to an on-going infection or a previous infection. Therefore, this ELISA test indirectly looks for an antigen by assaying for the host’s immune response to the pathogen. This type of ELISA is the indirect ELISA test (Figure 10-1). The host’s antibody found in the patient’s sample is the primary antibody. The indirect ELISA kit will have a species-specific secondary antibody that recognizes the host’s primary antibody, if present in the patient’s sample. This secondary antibody will have an enzyme linked to it. Binding of the secondary antibody to the host’s primary antibody will result in a color change upon the addition of the chromogen. The change in color indicates that the host
produced the primary antibody due to exposure to the antigen. In the case of the indirect ELISA test, the test will not be able to distinguish if the patient was suffering from the infection at the time of sampling or not. The indirect ELISA only looks for the host’s response to the antigen (pathogen), since it assays for the host’s antibody that is antigen specific. The results from the indirect test only confirms that the patient may be suffering from the pathogen at the time of sampling or may have been exposed to the pathogen at a time prior to the sampling. Patient samples that do not have the host’s antibody (response) will not produce color because the unbound enzyme conjugated secondary antibody will be lost during the washing steps performed during the procedure.

**Lab Procedure**

**Instructions to Students**-The goal today is to do an ELISA test to *simulate* the testing of several patients expected to have HIV. This is ONLY A SIMULATION. The samples you are real patient samples and do not have the virus.


**Methods**-Note: This procedure does have any washing steps.

1. Place the microtiter plate flat on the table and add three drops of antigen into wells A1 all the way to B12 using a narrow plastic pipette that was supplied to your group.

2. Add three drops of “Positive Control” to wells A1, A2, and A3 using the narrow plastic pipette located next to the positive control.

3. Add three drops of “Negative Control” to wells A4, A5, and A6 using the narrow plastic pipette located next to the negative control.

4. Add three drops of “Patient A” sample to wells A7, A8, and A9 using the narrow plastic pipette located next to the patient A sample.

5. Add three drops of “Patient B” sample to wells A10, A11, and A12 using the narrow plastic pipette located next to the patient B sample.

6. Add three drops of “Patient C” sample to wells B1, B2, and B3 using the narrow plastic pipette located next to the patient C sample.

7. Add three drops of “Patient D” sample to wells B4, B5, and B6 using the narrow plastic pipette located next to the patient D sample.

8. Add three drops of “Patient E” sample to wells B7, B8, and B9 using the narrow plastic pipette located next to the patient E sample.

9. Add three drops of “Patient F” sample to wells B10, B11, and B12 using the narrow plastic pipette located next to the patient F sample.
10. Add three drops of antibody into wells A1 all the way to B12 using a narrow plastic pipette that was supplied to your group.
11. Add three drops of chromogen into wells A1 all the way to B12 using a narrow plastic pipette that was supplied to your group.

12. Place the microtiter plate on top of a white paper towel to follow the purple color development.

**Exercise**-Record your data in the table below.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Purple Color (Yes or No)</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient F</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Lab Exercise 10-2 (Epidemiology Simulation)**

**Background**-Epidemiology is the field of study that deals with the spread of disease within the population. It deals with a very important concern, public health. Epidemiologists identify risk-factors and means of prevention and establish policy in an effort to control the spread of disease. Epidemiologists calculate factors such as prevalence (commonality of disease), incidence (morbidity-the number of new cases in a given population), and incidence rates (incidence/time), and determine the index case (the first documented patient identified during an epidemiological investigation).

**Lab Exercise:**

In this exercise, the group will consist of two students. The epidemiology simulation lab kit consists of 1 tube containing a SIMULATED body fluid per group, 1 transfer pipette per group, 1 microtiter plate per group, and phenol red.

Step 1: You and your lab partner will use the transfer pipette to mix the simulated body fluid and then transfer 3 drops to the H1 well. **RECORD THE NUMBER FOUND ON YOUR BODY FLUID TUBE>>> ____________________.** This will be your group number.

Give a chance for all the other groups to finish.
Step 2: Choose another group at random and add 1 ml of your group’s body fluid sample into theirs. After mixing, take 3 drops of that sample and place it in your H2 well. RECORD THEIR NUMBER FOUND ON THEIR BODY FLUID TUBE>>> ____________________. That same group will then place 1 ml of the mixed body fluid back into your tube and sample it by adding 3 drops into their H2 well and recording your body fluid tube number.

Give a chance for all the other groups to finish.

Step 3: Repeat step 2 again with a new group that you did not sample before and add the 3 drop sample into well H3. RECORD THEIR NUMBER FOUND ON THEIR BODY FLUID TUBE>>> ____________________.

Give a chance for all the other groups to finish.

Step 4: Repeat step 2 again with a new group that you did not sample before and add the 3 drop sample into well H4. RECORD THEIR NUMBER FOUND ON THEIR BODY FLUID TUBE>>> ____________________.

Step 5: Add 1 drop of phenol red into each well (H1, H2, H3, and H4). Record their colors (Orange = Sample Not Infected; Pink = Infected Sample).

Record your group’s results on the board for everyone to copy into the following table:

<table>
<thead>
<tr>
<th>Group #</th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
<th>H4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Which group is considered to be the index case?
2. What is the prevalence of the disease? (Prevalence = number of cases / number of individuals) X 100. A group with at least one infected sample is considered to be a case. The number of individuals is the same as the total number of groups doing the experiment.

3. Based on the prevalence calculate in question #2, how many individuals are expected to be infected if the population was made up of 100,000 individuals?

4. Calculate this simulated disease’s incidence. (Incidence = number of NEW cases / number of susceptible individuals). A group that is not infected at the start of the experiment (H1 = Orange), but infected in any of the other 3 samples (H2, H3, or H4 = Pink) will be considered to be a new case. The number of susceptible individuals is the same as the number of groups.

5. Calculate the incidence rate of this simulated disease assuming a year passed by and 100,000 individuals were involved. (Assume that the calculated incidence that you obtained in question #4 is for a one month period of time.)

Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):

1. Upon exposure to an antigen, the immune system will produce an ______________ to help fight the pathogen.

2. The direct ELISA assays for the presence of ______________ in the sample.

3. The indirect ELISA assays for the presence of ______________ produced by the immune system once it is exposed to an ______________.
**LAB #11: Differential Blood Cell Count**

**Lab Objectives:**

1. To learn about the different type of blood cells (characteristics and functions).
2. To be able to differentiate between all the different types of leukocytes using the microscope.
3. To recognize changes in the abundance of one type of white blood cells compared to its normal expected abundance.

**Pre-Lab Exercise (Complete Prior to Lab, Sample Quiz/Exam Questions)**

1. What is the most abundant type of white blood cell in a healthy individual?

2. Which white blood cells have granules in their cytoplasm?

**Background**

Blood is made up of liquid and cells. The liquid portion of blood, which contains various components like clotting factors, antibodies, ions, waste, and nutrients, is called plasma. The cellular component of blood includes three types: erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). These cells are produced in the bone marrow through a process called hematopoiesis (Figure 11-1).

Each cell type has its own characteristics and function (Figure 11-2). With respect to fighting pathogens, the main responsibility lies with the
white blood cells and their ability to phagocytose bacteria, release antimicrobials, and produce antibodies. Some of these white blood cells contain granules (Figure 11-3) in their cytoplasm (granulocytes), whereas some do not have granules (agranulocytes).

In a healthy individual, the relative abundance of each type of white blood cell is predictable. The order of abundance is as follows: Neutrophil > lymphocyte > monocyte > eosinophil > basophil. However, an infected individual will exhibit fluctuations in the abundance of one or more types of white blood cell that gives clues regarding the type of infection. A blood smear slide can be prepared to analyze a blood sample for the presence of all different types of blood cells and the percentages that they are found in. A deviation from expected values can indicate some type of infection or even cancer and other disorders.
Lab Exercise:

You will be viewing a blood smear slide to find and classify the 50 white blood cells. You will keep count of the different types of white blood cells to determine their relative abundance. Using a light microscope, focus onto a blood smear slide using the high-power dry objective (40X). Start with the field of view at upper left hand corner of the slide and move through the slide in a sequential manner so that you do not count the same white blood cell more than once. Follow this pattern of movement as you go through the slide:

Figure 11-4 illustrates what each of these white blood cells should look like to help you in your identification as you look for them on your slide.

White Blood Cells
(Leukocytes)

- Neutrophils: Pink Granules in Cytoplasm; Lobed Nucleus (3-5 lobes)
- Lymphocytes: Spherical Nucleus; No Granules
- Monocytes: Bean Shaped Nucleus, No Granules
- Eosinophils: Red or Reddish -Purple Granules in Cytoplasm; Lobed Nucleus (2 lobes)
- Basophils: Deep Blue Granules in Cytoplasm

Figure 11-4: Key characteristics to help you identify the different types of white blood cells.

White Blood Cell Count:

Neutrophils: 
Lymphocytes: 
Monocytes: 
Eosinophils: 
Basophils: 
1. From your results, which blood cell type was the most abundant, give your result as a % of total?

2. Did you find a basophil? If yes, what was its abundance in % of total? If no, why do you think you could not find one?

Post-Lab Exercise (Complete During or After Lab, Sample Quiz/Exam Questions):

1. Which type of white blood cell will increase in abundance during a fungal infection?

2. Which type of white blood cell secretes antibodies into the blood?

3. Which type of white blood cell or cells kills virally infected host cells?

4. What is the key cellular characteristic that helps in identifying a lymphocyte, while using a microscope?
Appendix A: Unknown Identification Guide (Guides obtained from Microbiology: Laboratory Theory & Application by Leboffe and Pierce)

**Step #1:** Gather your IMVC code and compare it to the IMVC code guide to guide you to the appropriate figure that you will be using to identify your unknown.

<table>
<thead>
<tr>
<th>(Indole)</th>
<th>(MR)</th>
<th>(VP)</th>
<th>(Citrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>(-)</td>
<td>(+/-)</td>
<td>(+/-)</td>
</tr>
<tr>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+/-)</td>
</tr>
<tr>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+/-)</td>
</tr>
<tr>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+/-)</td>
</tr>
</tbody>
</table>

**Step #2:** Use the figure that corresponds to your unknown’s IMVC code to identify your unknown. You sequentially go through your chosen figure with your unknown’s data in hand following the pathway that corresponds to your unknown until you get to your unknown’s name at the end of the pathway.

**Figure: Appendix A-1**

![Diagram of IMVC pathway showing Klebsiella pneumoniae, Hafnia alvei, Serratia marcescens, Enterobacter cloacae, and Enterobacter aerogenes.]
Step #3: Record the name of your unknown in your lab manual.
Appendix B: Zone of Inhibition Interpretation Table (Guide obtained from Microbiology: Laboratory Theory & Application by Leboffe and Pierce)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>&lt; or = 12</td>
<td>13-17</td>
<td>= or &gt; 18</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&lt; or = 15</td>
<td>16-20</td>
<td>= or &gt; 18</td>
</tr>
<tr>
<td>Penicillin</td>
<td>&lt; or = 28</td>
<td></td>
<td>= or &gt; 29</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>&lt; or = 10</td>
<td>11-15</td>
<td>= or &gt; 16</td>
</tr>
</tbody>
</table>

Appendix C: pH Indicator Guide

**Bromocresol Purple**
pH less than 5.2 indicator turns YELLOW.
pH greater than 6.8 indicator turns VIOLET (PURPLE).

Reference:
https://en.wikipedia.org/wiki/Bromocresol_purple

**Bromothymol Blue**
pH less than 6 indicator turns YELLOW.
pH between 6 and 7.6 turns the indicator GREEN.
pH greater than 7.6 indicator turns BLUE.

Reference:
http://en.wikipedia.org/wiki/PH_indicator

**Neutral Red**
pH less than 6.8 indicator turns RED.
pH greater than 8 indicator turns YELLOW.

Reference:
https://en.wikipedia.org/wiki/Neutral_red

**Phenol Red**
pH below 6.4 indicator is YELLOW
pH in the range of 6.4 to 8.0 is ORANGE
pH above 8.0 indicator is RED

Reference:
http://en.wikipedia.org/wiki/PH_indicator
1. Fill in the blanks:

**Parts of the Light Microscope**

A. **EYEPIECE**
   Contains the lens

B. **TUBE**
   Holds the HIGH- and LOW- power objective LENSES; can be rotated to change MAGNIFICATION.

C. **OBJECTIVE LENSES**
   Magnification ranges from 10X to 40X

D. **STAGE CLIPS**
   HOLD the slide in place

E. **STAGE**
   Supports the SLIDE being viewed

F. **LIGHT SOURCE**
   Projects light UPWARDS through the diaphragm, the SPECIMEN, and the LENSES

G. **FOOTTELLS**
   Supports the MICROSCOPE

H. **DIAPHRAGM**
   Regulates the amount of LIGHT on the specimen

I. **FINE ADJUSTMENT KNOB**
   Moves the stage slightly to SHARPEN the image

J. **COARSE ADJUSTMENT KNOB**
   Moves the stage up and down for...

K. **ARM**
   Used to SUPPORT the microscope when carried

2. What happens to the size of the field of view as you go from the 4X objective to the 40X objective?

3. What is the TOTAL magnification of the low-power objective of the microscope you used in the lab?
4. The resolving power of the high-power (Dry) lens is 273.68 nm (the high-power numerical aperture is 0.65; the Condenser numerical aperture is 1.25; and the wavelength is 520 nm). Write out the calculation or equation that when solved would give you the 273.68 nm resolving power as the answer. Use the numbers given above.

5. Using your answer in #4, which of the following will you be able to see clearly (mark all that apply):

___ Two objects 300 nm apart
___ Two objects 274 nm apart
___ Two objects 250 nm apart
___ Two objects 272 nm apart
___ Two objects 1 x 10⁻³ mm apart
___ Two objects 273.68 nm apart
___ Two objects 290 nm apart
___ Two objects 0.35 μm apart

6. Convert the following:
   100 mm (millimeters) into ______________ micrometers (μm).
   100 cm (centimeters) into ______________ millimeters (mm).
   100 nm (nanometers) into ______________ millimeters (mm).

7. Give an example of a microscopic prokaryote. ________________________.

8. Give an example of a microscopic unicellular eukaryote. __________________________.

9. After you inoculate media, what do you have to do to get the bacteria to grow?

10. What is another name for liquid media? ________________________________.

11. What do you have to add to liquid media to get it to solidify?

12. Which technique do you have to follow to prevent cross-contamination of the sterile tubes?
    ________________________________.
13. Which technique or procedure did you use in the lab to isolate a mixed culture?

14. Bacteria appears as mounds called _________________ when grown on an agar plate.

15. How do you improve contrast when using a microscope? Give two ways

16. Why does the cationic stain stick to the cell?

17. The cell remains colorless, when using a negative stain. Why?

18. If you place different species of bacteria on the same slide and use crystal violet (a simple stain) to stain them, both types of bacteria will be the same color. What would be one way that you might still be able to distinguish between them?

19. What was the name of the stain that we used in negative staining? __________________________.

20. Did we heat fix the positive or negative stain slide? ____________________________________.

21. Why do we heat fix? ________________________________________________________________.

22. For the staining labs, we had to use the 100X objective. When using this objective you must add ___________ to be able to see the specimen clearly.

23. The resolving power of the 100X objective is the highest compared to the resolving power of all the other objective lenses. However, to take advantage of the 100X objective lens numerical aperture, you have to prevent the loss of ______________ by using a substance (answer for #22) with a high _________________________________.

24. In negative staining, what will happen if you by mistake wash the slide? And Why?
25. If you de-stain your slide for too long during positive staining, you will not be able to see anything when you view your slide. What did the prolonged de-staining decrease that resulted in you not being able to see anything?

26. Name one type of differential staining procedure. _________________________________.
27. Name another type of differential staining procedure. _________________________________.
28. Name a third type of differential staining procedure. _________________________________.
29. The Gram-negative bacterium is ___________ in color.
30. The Gram-positive bacterium is ___________ in color.
31. Give the scientific name for the three common shapes of bacteria. Next to the scientific name, give the actual shape that corresponds to the scientific name.

<table>
<thead>
<tr>
<th>Scientific Name</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. ____________</td>
<td>___________</td>
</tr>
<tr>
<td>b. ____________</td>
<td>___________</td>
</tr>
<tr>
<td>c. ____________</td>
<td>___________</td>
</tr>
</tbody>
</table>

32. What shape is the bacterium that is found in the tetrad arrangement? ___________. How many cells makes up a tetrad arrangement? _____________________________.

33. What does strepto- mean?

34. The name of the arrangement that corresponds to “cluster” is called _________________.

35. What is the main difference between Gram-positive bacteria and Gram-negative bacteria, besides color and shape? You may describe and/or diagram/label. Be clear in your answer and thorough.

36. When using a blood agar plate, how do you confirm that the bacterial species growing on the plate is β-hemolytic?
37. What is the selective agent (if any) and differential agent (if any) in the following:

Blood Agar: Selective Agent _______________ : Differential Agent: _______________

Phenylethyl Alcohol Agar: Selective Agent _______________ : Differential Agent: _______________

Salt Mannitol Agar: Selective Agent _______________ : Differential Agent: _______________

38. We differentiate bacteria growing on MacConkey agar based on their ability to ______________ or not.

39. Which hydrolysis test requires the addition of iodine prior to the examination of the plate to obtain results?

40. Give the location where each of the following types of bacteria will grow inside a fluid thioglycollate tube:

Obligate anaerobe: ___________________________________

Obligate aerobe: _____________________________________

Facultative anaerobe: _________________________________
Appendix E: Example Lab Exam #2

1. Write the chemical reaction that catalase catalyzes. ___________________________________.

2. The oxidase test distinguishes between bacteria that have cytochrome C oxidase and those that do not. What is cytochrome C oxidase a component of? ____________________________________.

3. Which media did we use to do the coagulase test? ____________________________________.

4. What color will the KIA media be if the inoculated bacteria was only metabolizing protein? ______.

5. What color will the KIA media be if the inoculated media is reducing sulfur? __________________.

6. Bacteria that metabolize protein grow in the slant region of a KIA tube. Why?

7. Draw a typical amino acid before and after deamination.

   BEFORE  
   AFTER

8. What does it mean when a nitrate reduction tube inoculated with bacteria turns red after the addition of nitrate reduction reagents A and B?

9. What does it mean when a nitrate reduction tube inoculated with bacteria does not change color even after the addition of the nitrate reduction reagents A/B and zinc?

10. What is the name of the process that converts nitrate all the way to nitrogen gas?
11. Bacteria found outside of the zone of inhibition multiply using binary fission. Why are bacteria no longer multiplying within the zone of inhibition?

12. Bacteria with a zone of inhibition of zero for penicillin is ________________ to the antibiotic.

13. In the unknown identification experiment, what are we testing for in the phenol red tubes?

14. In bacteria, where is Citrate Permease located? ________________________________.

15. What are we testing for in the methyl red experiment? ________________________________.

16. Draw a typical amino acid before and after decarboxylation.

BEFORE

AFTER

17. Why do we layer oil on top of the Lysine and Ornithine decarboxylation media?

18. Why does a positive urea hydrolysis test appear pink in color?

19. The SIM test is three test in one. What is a positive Indole test indicating about your unknown bacteria? _______________________________________________________________________.

107
20. What color is a positive Voges-Proskauer test? ________________________________.

21. Identify these two unknowns (UNK #1 and UNK #2):

<table>
<thead>
<tr>
<th>Unknown #1</th>
<th>Unknown #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simmons’ Citrate</td>
<td></td>
</tr>
<tr>
<td>Urea Hydrolysis Test</td>
<td></td>
</tr>
<tr>
<td>Lysine Decarboxylase</td>
<td></td>
</tr>
<tr>
<td>Ornithine Decarboxylase</td>
<td></td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td></td>
</tr>
<tr>
<td>Methyl Red</td>
<td></td>
</tr>
<tr>
<td>Indole Test</td>
<td></td>
</tr>
<tr>
<td>Xylose Fermentation</td>
<td></td>
</tr>
<tr>
<td>Lactose Fermentation</td>
<td></td>
</tr>
<tr>
<td>Arabinose Fermentation</td>
<td></td>
</tr>
</tbody>
</table>

Fill Table with either “+” for positive test or “-“ for negative test for each unknown:

22. Use the following plate and data to determine if the bacteria inoculated on the plate is susceptible, resistant, or intermediate for each of the antibiotics tested.
Size of Zone of Inhibition Data:

<table>
<thead>
<tr>
<th></th>
<th>Chloramphenicol</th>
<th>Ciprofloxacin</th>
<th>Penicillin</th>
<th>Streptomycin</th>
<th>Trimethoprim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria 1</td>
<td>5</td>
<td>24</td>
<td>2</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>Bacteria 2</td>
<td>30</td>
<td>26</td>
<td>48</td>
<td>40</td>
<td>13</td>
</tr>
</tbody>
</table>

Reference Table:

<table>
<thead>
<tr>
<th></th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>&lt; or = 12</td>
<td>13-17</td>
<td>= or &gt; 18</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&lt; or = 15</td>
<td>16-20</td>
<td>= or &gt; 18</td>
</tr>
<tr>
<td>Penicillin</td>
<td>&lt; or = 28</td>
<td></td>
<td>= or &gt; 29</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>&lt; or = 11</td>
<td>12-14</td>
<td>= or &gt; 15</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>&lt; or = 10</td>
<td>11-15</td>
<td>= or &gt; 16</td>
</tr>
</tbody>
</table>

Fill in the table with either R for resistant, “I” for intermediate, or “S” for susceptible for bacteria 1 and bacteria 2:

<table>
<thead>
<tr>
<th></th>
<th>Chloramphenicol</th>
<th>Ciprofloxacin</th>
<th>Penicillin</th>
<th>Streptomycin</th>
<th>Trimethoprim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Which bacteria (bacteria 1 or bacteria 2) is Gram-negative for sure? ________________________.

Explain Why:

23. To perform the Kirby-Bauer test, we always have to use the Müller-Hinton agar. Why?
24. In bacteria, what activates the decarboxylase gene?

25. Matching (use the following word bank, words can be used more than once and there might be multiple answers, give all that apply):

**GIVE THE LETTER THAT CORRESPONDS TO YOUR CHOICE OR ANSWER** (more than one answer is possible. Multiple use of the choices given below is possible)

<table>
<thead>
<tr>
<th>A. Voges-Proskauer</th>
<th>B. Urea</th>
<th>C. Phenol Red</th>
<th>D. Durham Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. Methyl Red</td>
<td>F. Urease</td>
<td>G. Citrate</td>
<td>H. Indole</td>
</tr>
<tr>
<td>I. Bromothymol Blue</td>
<td>J. Green</td>
<td>K. Red</td>
<td>L. Yellow</td>
</tr>
<tr>
<td>M. Purple</td>
<td>N. Pink</td>
<td>O. Black</td>
<td>P. Sulfur Reduction</td>
</tr>
<tr>
<td>Q. Citrate Permease</td>
<td>R. Gelatinase</td>
<td>S. Blue</td>
<td>T. Bomcresol</td>
</tr>
<tr>
<td>U. Tryptophanase</td>
<td>V. Ammonia</td>
<td>W. Motility</td>
<td>X. Simmons Citrate Test</td>
</tr>
</tbody>
</table>

A hydrolytic enzyme that results in the production of ammonia.

A product of tryptophan breakdown, which reacts with Kovac’s reagent.

Color of a Simmons Citrate positive test.

Indicator used in the urea hydrolysis test.

Color of a positive Voges-Proskauer test.

A basic molecule that increases the pH in the urea hydrolysis test.

Confirms fermentation by trapping the gas produced.

Color produced resulting from the decarboxylation of lysine.
These three tests are commonly tested together using a single culture tube.

Besides a change in color, this gives a visual indication of fermentation.

Results of these tests produces a code used to orient you towards identifying the unknown.

Consists of two ammonia molecules attached to each other.

26. What effect does UV have on DNA?

27. Give two repair mechanisms used by bacteria to fix UV damaged DNA.

28. Give the names of the enzymes used in the repair mechanisms that you gave in question #27.

29. Which macromolecule did we transform into bacteria during the transformation lab?

30. Using the pGLO system, how did we induce the expression of the green fluorescent protein?

31. Give all the proteins that need to bind to the pBAD promoter for transcription to initiate.

32. Which type of ELISA requires the use of a secondary antibody?

33. What is the difference between an antigen and an antibody?
References
