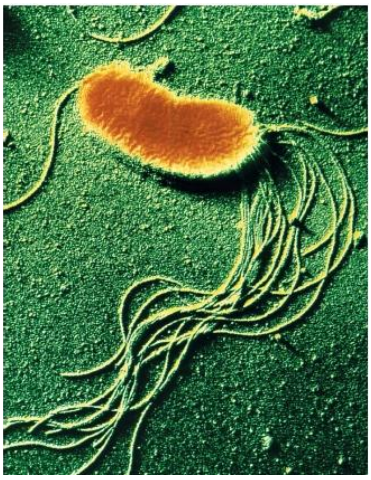
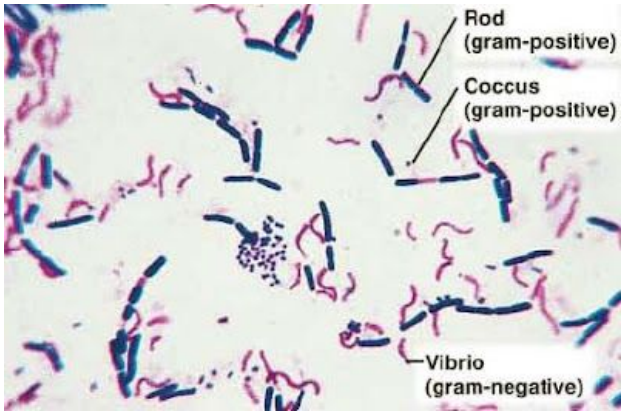
San Bernardino Community College District

San Bernardino Valley College

MICROBIOLOGY





LAB MANUAL

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Experiment 1 Introduction to the Microscope

Microscope Structure

The microscopes used in this course are called compound, bright-field microscopes. Figure 1.1 demonstrates some components of a bright-field microscope.

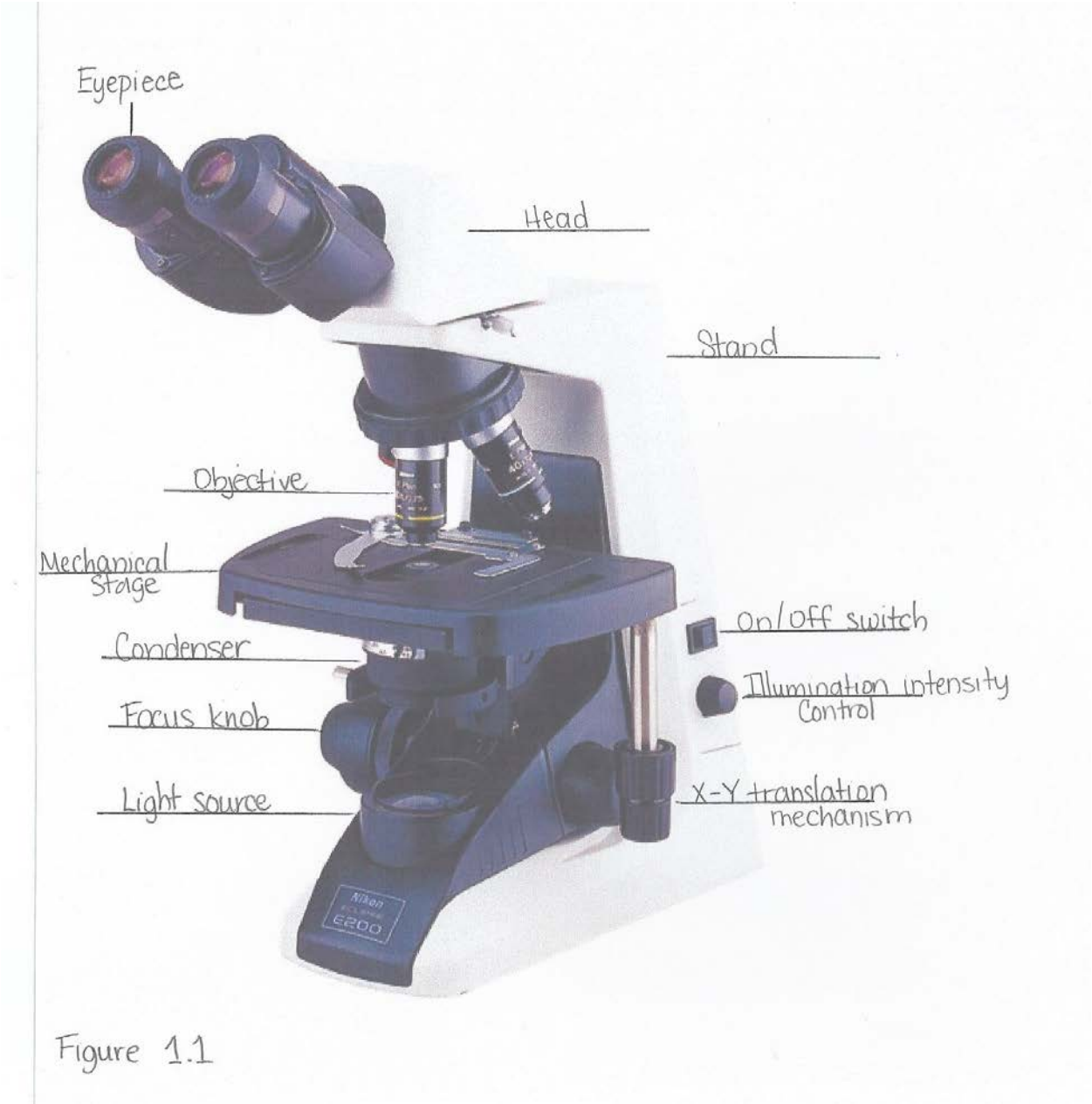


Figure 1.1

Components of the Microscope

Stage: This mechanism supports the microscope slide. There is also the adjustment knob that moves the stage around to better place the slide for viewing.

Light source: Illuminates the slide. The light intensity control varies the intensity of the lamp in the base.

Lenses:

Eyepieces or **oculars** are located at the top of the instrument for viewing the slide. These usually have a magnification of 10X.

Objectives are attached to the rotatable nosepiece. They are used to increase the magnification at which the slide is viewed at. Our microscopes have a 4X, a 10X, a 40X, and a 100X. These lenses are known as scanning, low-power, high-dry, and oil immersion, respectively. Total magnification is determined by multiplying the power of the ocular by the power of the objective lens being used. Ocular x objective = total i.e.: 10 x 40 = 400

The **condenser** is located under the stage. It collects and directs the light from the lamp to the slide. The amount of light that enters the condenser can be controlled by the **iris diaphragm** that is located as a slider on the condenser.

Focusing knobs: The **coarse focus knob** is used with the 4x and 10x objectives to bring the specimen on the slide into focus. Using the **fine focus knob** is used with the 40x and the 100x objectives and will sharpen the image of the specimen.

How to Use the Microscope

- Your instructor will go over these steps as a demo before you move onto the letter 'e' worksheet for practice.
- Always remove the microscope from the cabinet and carry the microscope with two hands. **Never carry the microscope to another location.**
- It is not necessary to use full intensity on the light when viewing slides. Use the iris diaphragm (aperture iris) to adjust the light coming through the condenser. Use only the amount of light necessary to view the object on the slide. **Rule of Thumb:** as you go up in magnification, open the diaphragm.
- To adjust the **interpupillary distance**, look through the oculars and adjust the distance between the eyepieces until you see only one circle of light. Make note of the number of the position since others will be using the same microscope.
- Place the slide in the clamping device on the mechanical stage. Raise the stage to its highest position using the coarse adjustment knob while viewing from the side. This will prevent you from accidently running into the slide should you be working with a microscope without an automatic stop.
- Using the **mechanical stage knobs** center the specimen under the 4X objective (shortest lens). Always begin with the 4X objective.
- Begin with the stage at the highest position. Using the coarse adjustment knob slowly turn the knob to lower the stage until the specimen comes into focus while viewing through the eyepiece. Use the fine adjustment knob to fine tune the clarity.

- **Diopter adjustments:**
    - Swing the 40X objective in the optical path. Rotate the coarse adjustment knob the bring the specimen into focus.
    - Switch back to the 10X objective. While looking into the right eyepiece with your right eye, focus on the specimen by rotating the right diopter ring and not using the coarse adjustment knob.
    - While looking into the left eyepiece with your left eye, focus on the specimen by rotating the left diopter ring and not using the coarse adjustment knob.
- To increase the magnification, rotate the nosepiece clockwise until the 10X objective clicks into place. Using the fine adjustment knob clarify the specimen. **Never rotate the fine adjustment knob more than 3 turns in either direction. Always rotate the nosepiece of the microscope clockwise to avoid getting oil onto the objectives.**
- To increase magnification further follow the step above for the 40X objective. The next lens after the 40X is the 100X which utilizes oil to view the specimen. Follow the next set of directions for the 100X lens.
- **Oil immersion / 100X lens:**
    - When using the 100X lens it is necessary to use oil. This bends the light, increasing the resolution. Before rotating the 100X lens into place position the nosepiece so the space between the 40X and the 100X is above the specimen. Place a small drop of oil onto the slide and rotate the 100X lens into place.
    - Use the fine focus knob to bring the specimen into focus. **Never use the coarse adjustment knob to focus as this will damage the lens or the slide.**
    - **When working with oil be careful not to get any oil on the other lenses as this will damage them**. If you do get oil on the other lenses clean it off with lens cleaner and lens paper. **Do Not Use Kimwipes on any of the lenses.**
- Once done with the microscope clean lenses with lens cleaner and lens paper. Put the microscope away according to the microscope checklist in the safety packet or on the door to the microscope cabinet.

Practice your skill with the letter ‘e’ worksheet that follows.

Microbiology **Name:**_____

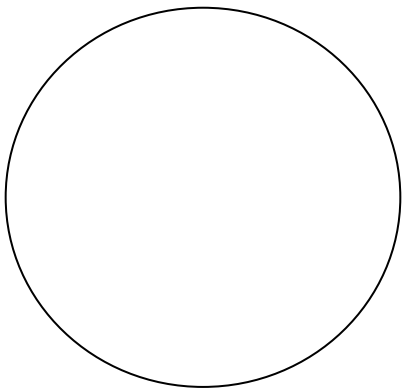
Lab Hour:_____

Letter 'e' Worksheet

1. Draw the letter 'e' slide, **life size**, as it looks to your **naked eye**, in the position it will be placed on the stage of the microscope.
    a. total magnification of the letter 'e' slide = _____
    b. diameter of the 'e' (top to bottom) in **millimeters** = _____

2. Rotate nosepiece clockwise to position scanning objective
    a. what is the total magnification of the letter “e” slide=_____
    b. what is the diameter of the field of view in **micrometers**_____

3. Draw the letter 'e' at **low power**, as it appears **through** the microscope. The circle represents the field of view; approximate the proportions of the letter 'e' within the field of view as you see it at low power.
    a. total magnification = _____
    b. based on the size of the field of view, what is the diameter of the letter 'e' (top to bottom) in **micrometers** = _____



4. Note the orientation of the letter 'e' as is sits on the slide compared to its orientation as viewed under the microscope. What happened?

5. Move the stage to the left. Quickly view the letter 'e' under the microscope as you move the stage. Which direction is the letter moving? _____

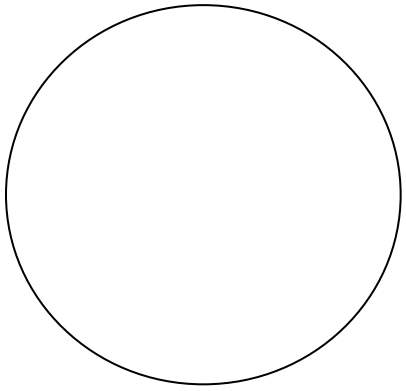
6. Move the stage toward you. Again, quickly view the letter 'e' under the microscope as you move the stage. Which direction is the letter moving?_____

7. What is this phenomenon called? _____

8. Draw the letter 'e' at **high power**, as it appears through the microscope. Illustrate the approximate proportions as seen within the field of view.

   a. total magnification = _____

   b. diameter (width) of the ink line in micrometers.



9. Open and close the iris diaphragm. What happens_____?

10. When you go up in magnification, what happens to the field of view? Why?

11. Notice that when you go up in magnification that you do not completely lose sight of the letter 'e'. Clarity of the specimen may require only a turn of the fine adjustment knob. The ability to go from one objective to another and **not** lose sight of the specimen is called _____.

12. Rotate clockwise half way between the next lens and place a drop of oil on the slide. Rotate oil immersion lens into place. Use fine focus to adjust the focus.

Draw the letter 'e' at **oil immersion** as it appears **through** the microscope. The circle represents the field of view; approximate the proportions of the letter 'e' within the field of view as you see it at low power.

a. total magnification = _____

b. based on the size of the field of view, what is the diameter of the letter 'e' (top to bottom) in **micrometers** = _____

Experiment 2 Microscope work with Helminthes, Protozoa, Fungi

To better familiarize yourself with the workings of the microscope at the various objective lens magnifications, this experiment will go through various sizes of specimens.

Each student should draw each of the following commercial slides. For the lab final students should know classification and anatomical structures. In addition, you will be expected to know life cycles and pathology when asked by your lab instructor at the end of the semester.

Kingdom Animalia

Sub-kingdom Invertebrata

I. **Phylum Platyhelminthes** - flatworms

A. **Class Cestoda** -tapeworms

Taenia pisiform's. - head (scolex) and segments (proglottids) may be on separate slides

Label: scolex, suckers, hooks if present, proglottid, testes, ovaries

II. **Phylum Nematoda** - roundworms

Enterobius vermicularis female - pinworm

Label: mouth, pharynx

Trichinella spiralis- threadworm encysted in muscle

Label: cyst, muscle, larva

IV. **Phylum Arthropoda**

Class Arachnia

Dermatophagoides spp.- dust mite

Label: thorax, legs, abdomen

Kingdom Fungi

A**. Division: Zygomycota**

Rhizopus stolonifer – Black bread mold. zygote (sexual spore).

label: zygote and hyphae

Rhizopus stolonifer - sporangiospores (asexual spore)

label: sporangium, sporangiophore, and rhizoids

B. **Division: Ascomycota -** sac fungi.

Penicillium notatum - ascospores (sexual spore)

label: cleistothecium, hyphae, ascus, and ascospore

Penicillium notatum - phialospore (a type of conidiospore) (asexual spore)

label: conidia, septa, conidiophore, and hyphae

Saccaromyces cerevisiae – yeast - (asexual spore)

label: parent cell, blastospore

Kingdom Protista

Subkingdom Protozoa - cyst (dormant) and trophozoite (vegetative) stages

I. **Phylum Sarcomastigophora -** amoebas and flagellates

A. **Subphylum Sarcodina** - amoebas

Entamoeba histolytica (intestinal parasite)

label: nucleus, food vacuoles Identify whether cyst (4 nuclei) or trophozoite (1 nucleus)

B. **Subphylum Mastigophora** - flagellates

Giardia lamblia - intestinal parasite

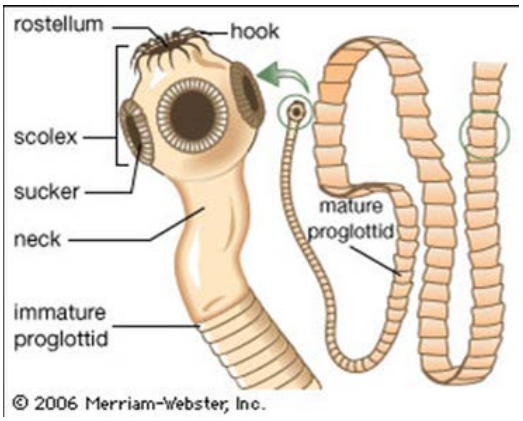
label: nuclei and flagella if seen, Identify whether cyst (4 nuclei) or trophozoite (2 nuclei)

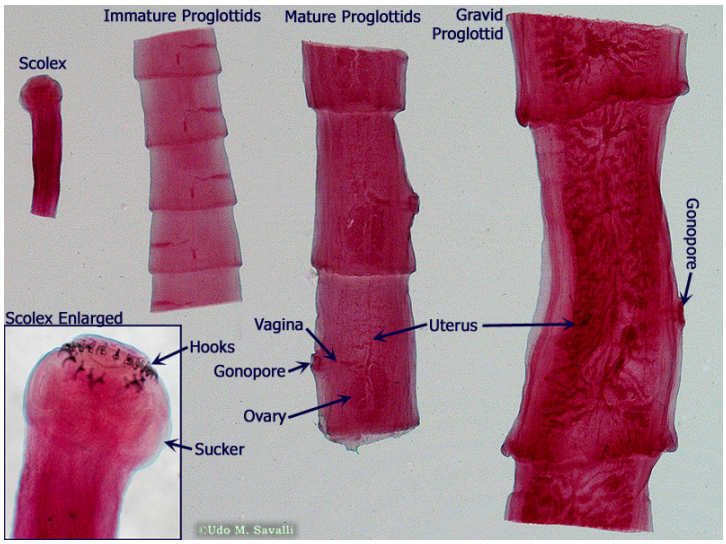
Trichomonas vaginalis - urogenital parasite

label: nucleus and flagella if seen, no cyst stage

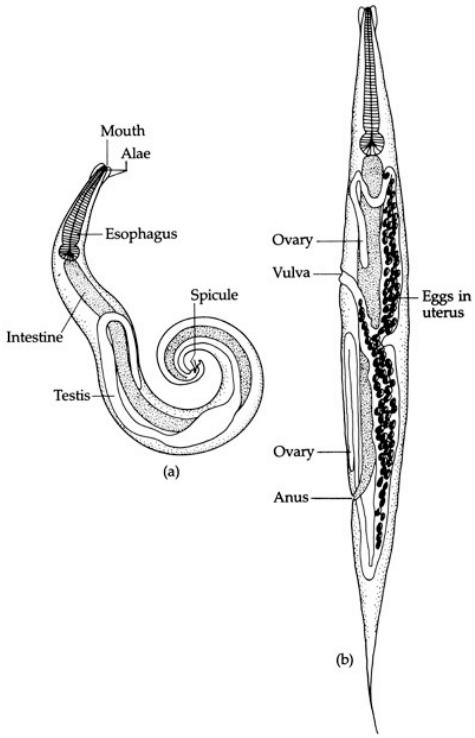
Reference photos for help with identifying the specimen on the slide.

Taenia

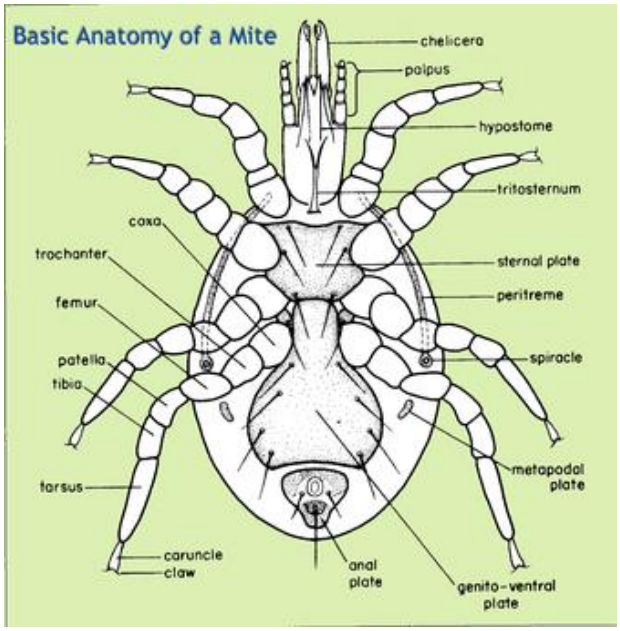




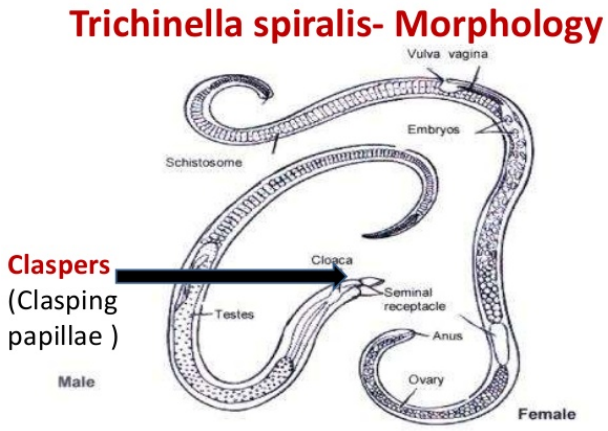
Enterobius



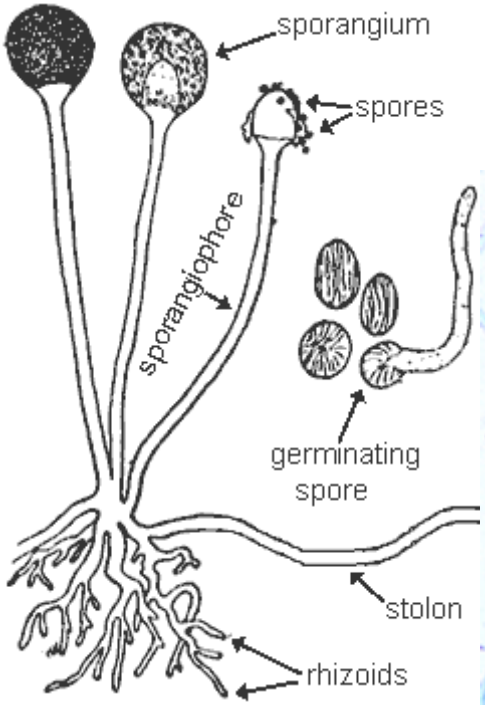
Dermatophagoides

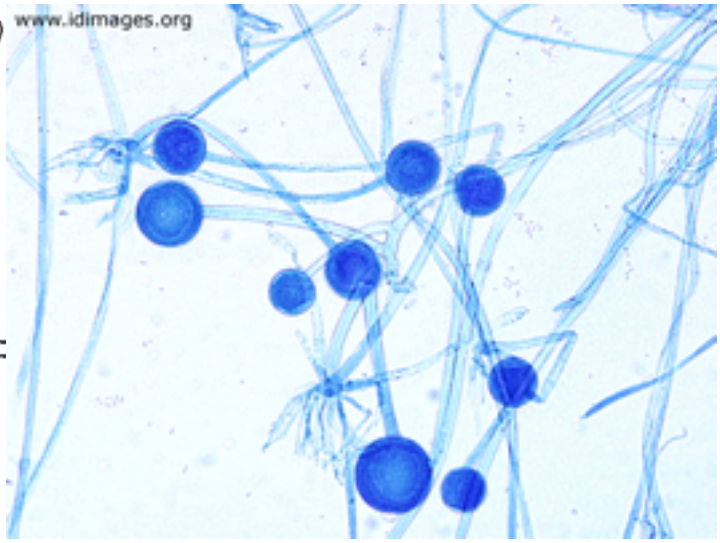


Trichinella



Rhizopus





Penicillium

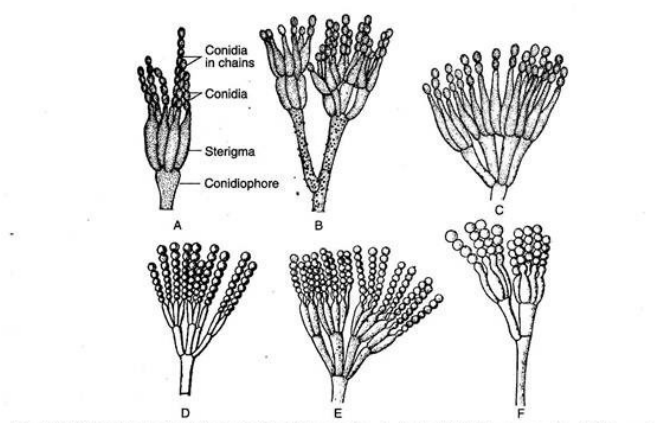
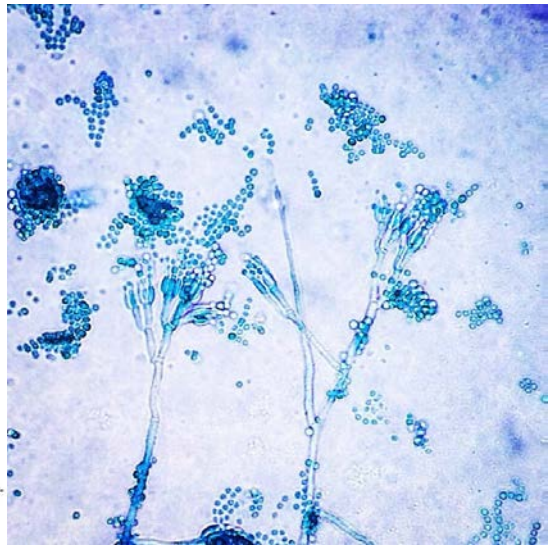


Fig. 4.43 : Different types of conidiophore of *Penicillium* species : A. *P. thomii*, B. *P. lanoso-caerulium*, C. *P. vermiculatum*, D. *P. glaucum*, E. *P. herquei* and F. *P. chrysogenum*



Saccharomyces

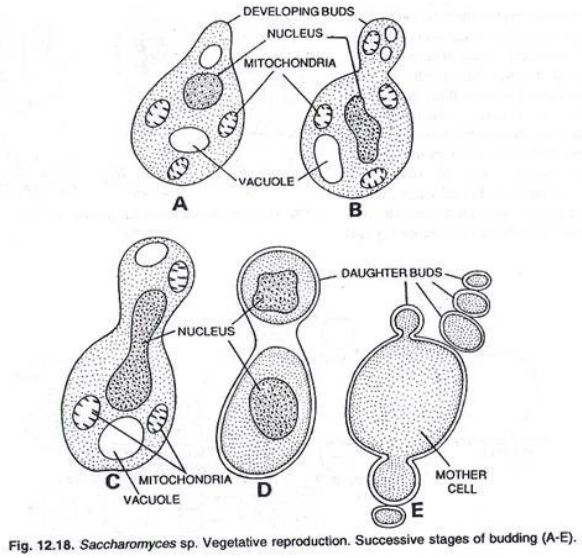
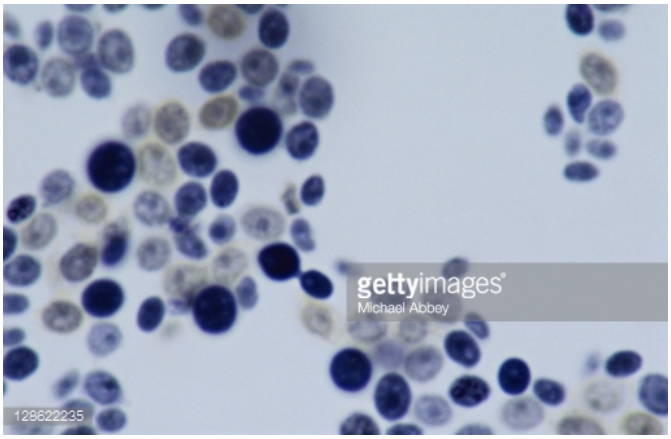
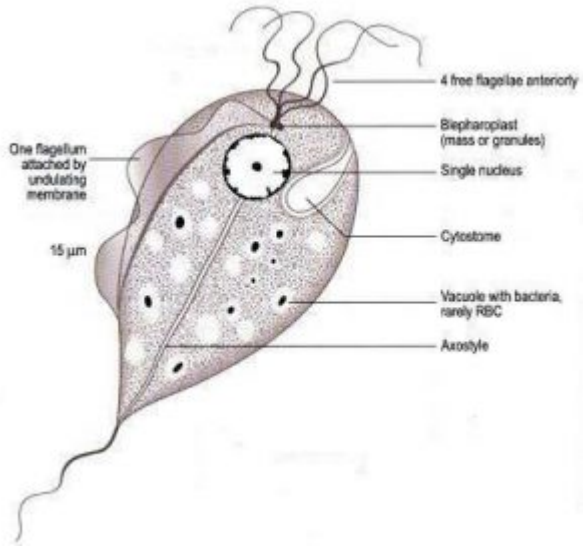
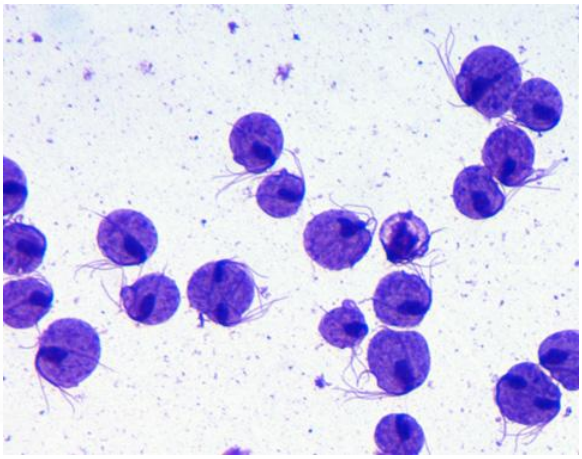


Fig. 12.18. *Saccharomyces* sp. Vegetative reproduction. Successive stages of budding (A-E).

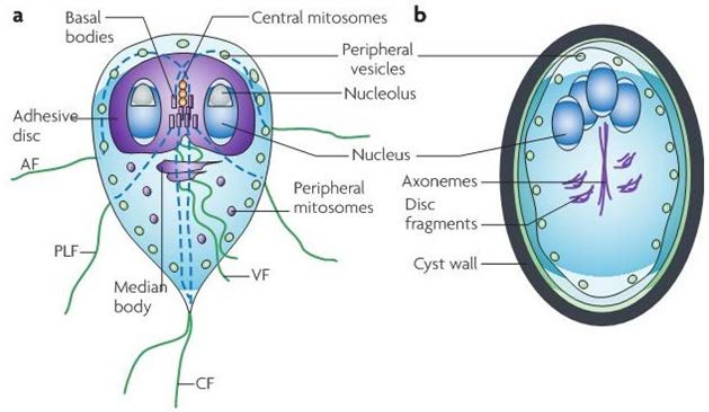


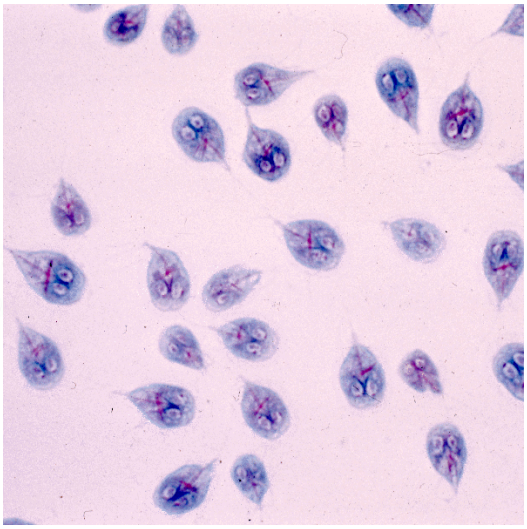
Trichomonas





Giardia





Entamoeba

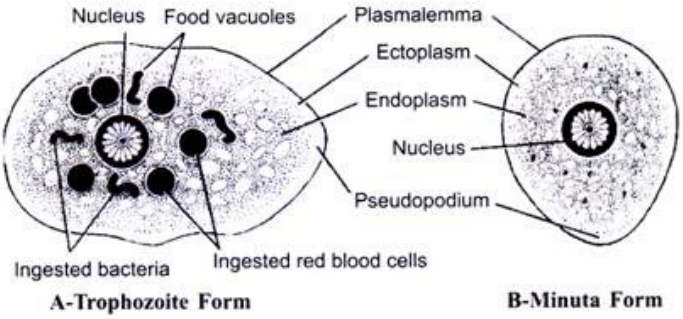
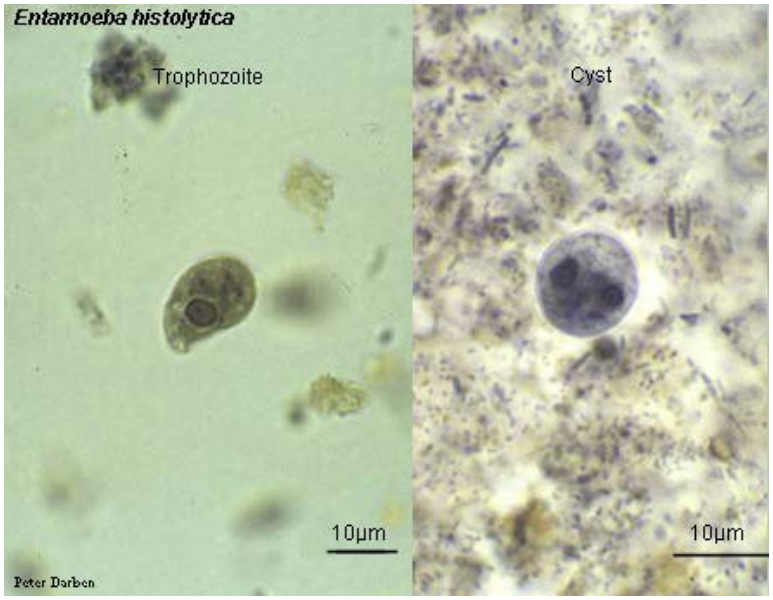


Fig. 9.1 A & B *E. histolytica*



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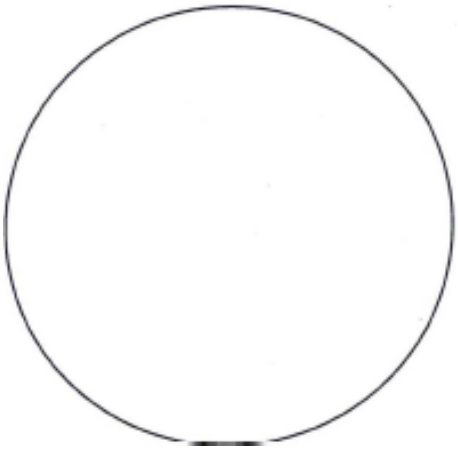

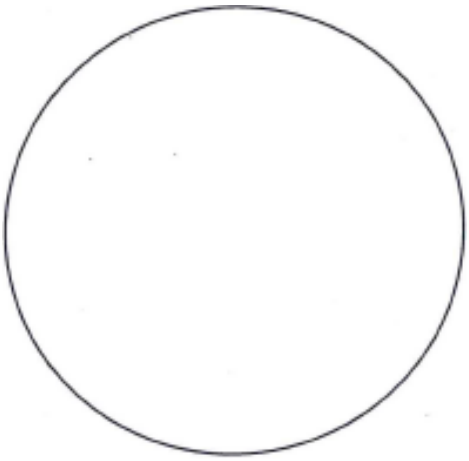

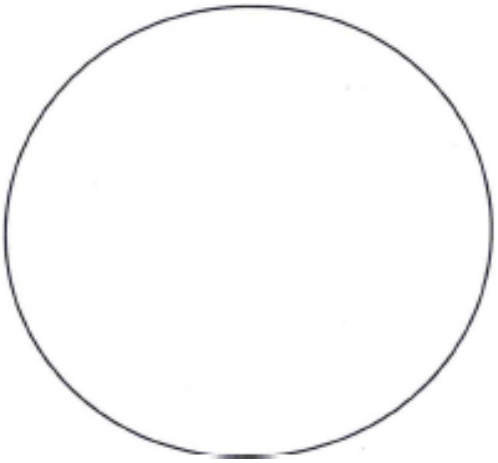

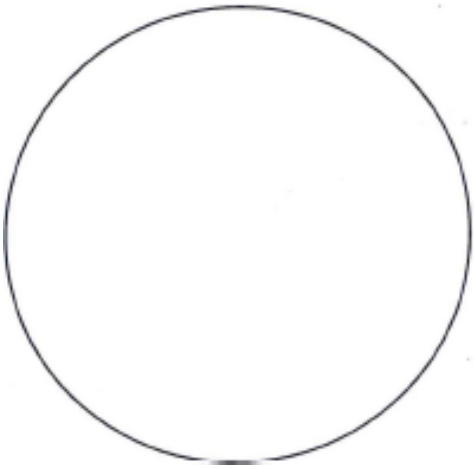

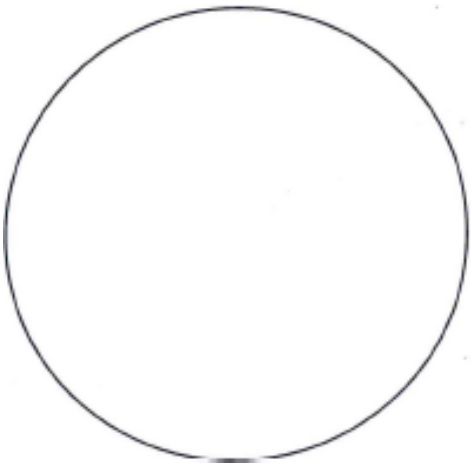

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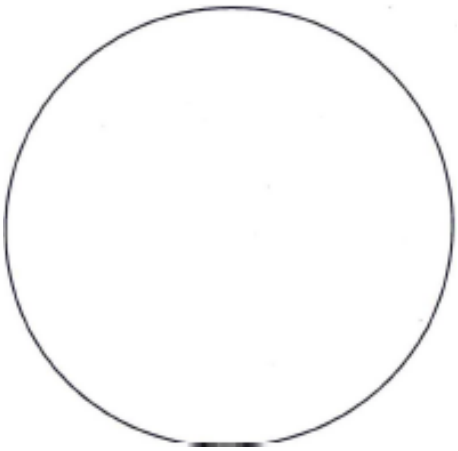

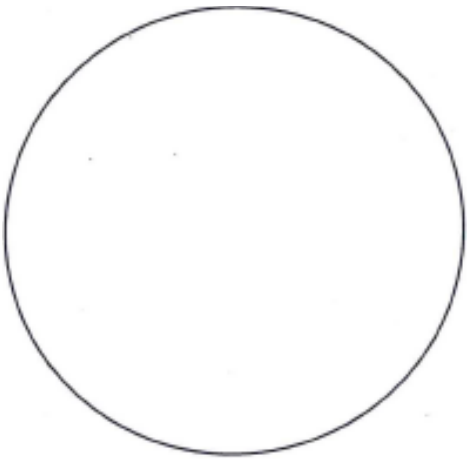

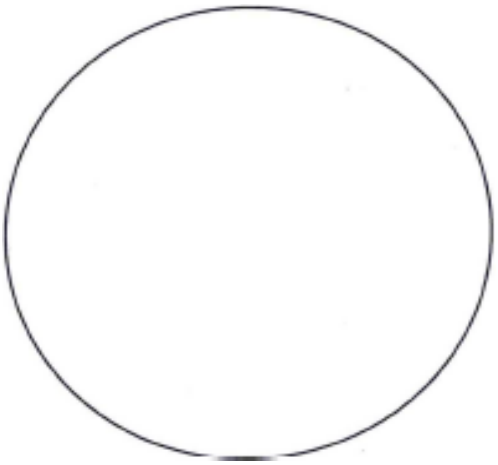

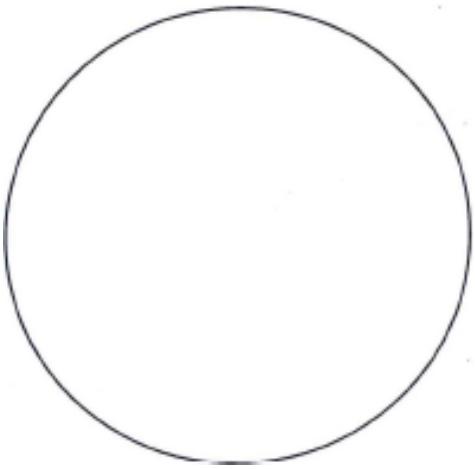

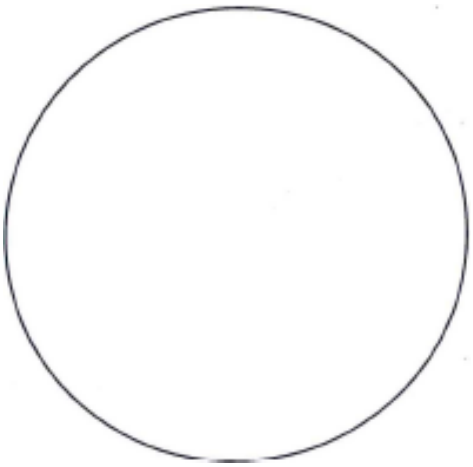

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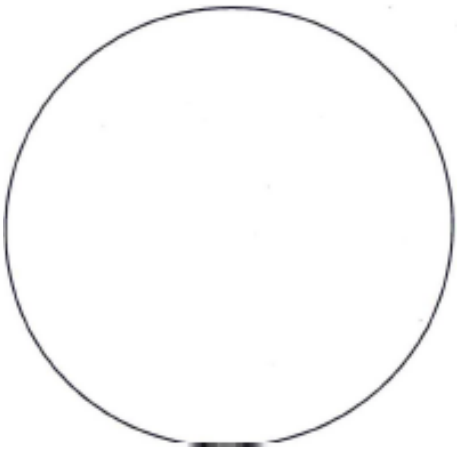
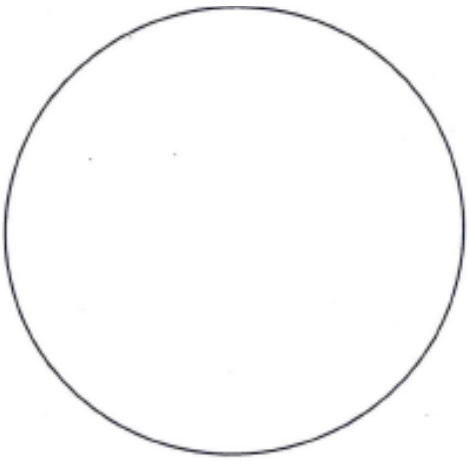
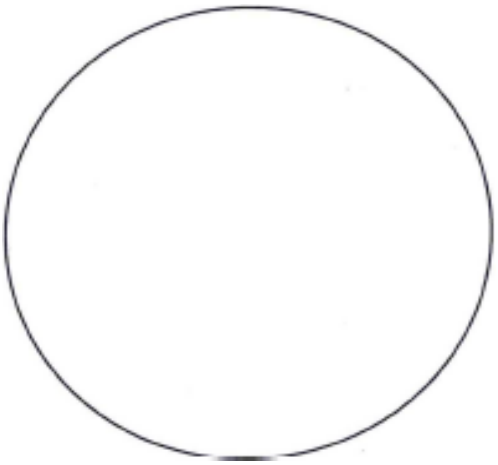
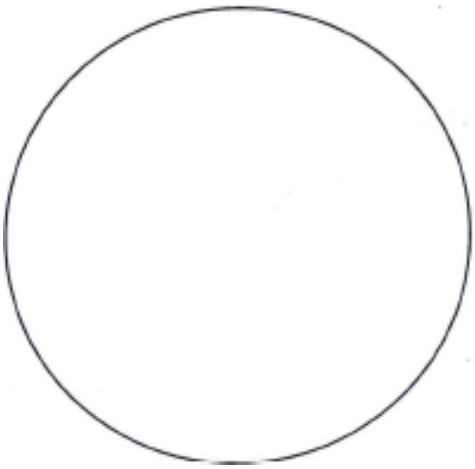
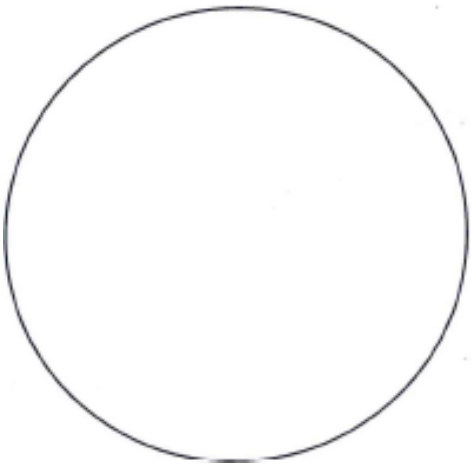










Experiment 3 Aseptic Technique

An introduction to the concepts of Aseptic Technique:

Aseptic Technique insures that there is no contamination of microorganisms introduced into the work space, into the cultures you are working with, or to others that may be present.

Key items to be aware of:

- Disinfect your bench top surface before beginning work. You should already be doing this before you start lab and after you are done with the lab.
- Loops and needles need to be sterilized in the Bunsen burner flame till red hot. Allow these implements to cool before doing any inoculations to prevent killing the organism.
- The opening of sterile media tubes and the tube of microorganism that will be used to inoculate those sterile media tubes need to be flamed after opening and before closing.
- Flame the loop or needle after each inoculation and before a new inoculation is done. This kills any leftover organism that is still on the instrument.
- Loops are used to transfer **from** liquid media to liquid media or petri plates. Needles are used to transfer **from** solid media to other solid media or petri plates.
- Hold the cap of the tube with your little finger. **NEVER** place the cap on the bench top.

Demonstration:

Your lab instructor will demonstrate how to do the aseptic technique procedure for one or two of the different transfers.

Media used:

TSA – Tryptic soy agar

Agar: from red algae, melting point = 100°C, solidifies ~45°C, not a nutrient

TSB – Tryptic soy broth

Organism used:

Escherichia coli – Gram (-) rod

Procedure:

Label all sterile media with your name, media type, organism used, and lab hour. Label plates on the bottom not the lid. **DO NOT label the white part of the tube.**

Transfer of *E.coli* from a plate to a sterile slant

1. Flame the inoculating needle until it is red hot, allow to cool.

2. Raise the lid of the petri plate just enough to put the needle inside and remove some of the organism on the plate. Close the lid. DO NOT gouge the agar.
3. Pick up your sterile slant with your free hand and remove the cap with your little finger that is holding the needle. Flame the opening of the tube before applying the organism on the needle to the surface of the slant. DO NOT gouge or stab the slant.
4. Remove the needle from the tube and flame the opening before capping the tube. Flame the needle as well.

Transfer of *E.coli* from a slant to a sterile broth

1. Flame the inoculating needle until it is red hot, allow to cool.
2. Remove cap from the slant and flame opening. Hold cap with little finger that is holding the needle. Use the needle to pick up the organism from the slant.
3. Flame opening of the slant before capping.
4. Pick up your sterile broth with your free hand and remove the cap with your little finger that is holding the needle. Flame the opening of the tube before inserting the needle.
5. Move the needle in a side to side motion to mix the organism into the broth.
6. Remove the needle from the tube and flame the opening before capping the tube. Flame the needle.

Transfer of *E.coli* from a broth to a sterile plate

1. Flame the inoculating loop or needle (your preference) until it is red hot, allow to cool.
2. Remove the cap from the broth and flame the opening. Hold the cap with your little finger that is holding the loop/needle. Use the loop/needle to pick up organism from the broth.
3. Flame the opening of the broth before closing.
4. Pick up your sterile plate. Open the lid just enough to stick the loop/needle inside and glide the instrument over the surface of the agar. DO NOT gouge the agar.
5. Cover the plate back up and flame the loop/needle.

Put slants, broths, and plates into the plate rack or the tube rack at the back of the class that is labeled with the experiment name.

Next lab period

Results

Obtain both tubes and your plates. Examine them for growth and uniformity of the cultures. Ask yourself these questions.

1. Were the three transfers you did successful?

2. How can you tell that the transfers were successful?

3. If the transfers were not successful, what may have happened?

4. Was there any evidence of another organism (i.e. Fungus) in any of your culture tubes or on your plate? If so what may have happened?

Experiment 4 Smear Preparation

Smear preparation is done to prepare the organism for staining. Smear preparations are used to determine cell shape, arrangement of the cells, and internal structures. Most of the stains we will be doing utilize a smear preparation to place organisms on the slide. However, pay attention to the instructions in the procedure or the instructions on the white board to make sure that a smear preparation is needed.

The key to a successful smear preparation is to make sure that the smear is not too thick otherwise there may be too many cells on the slide to distinguish shape or arrangement of the cells . Organisms growing on solid media tend to stick together in clumps and need to be dispersed into a few loopfuls of water. When taking from a solid media you do not need to have a bunch of cells on the needle; remember less is better.

Your instructor will demonstrate how to make a smear preparation.

Procedure:

1. If taking from a broth:
    a. Using aseptic technique, transfer three loopfuls of organism to the middle of the slide. Spread the organisms around on the slide to make a dime-sized circle on the center of the slide.
    b. Allow the smear to air dry.
    c. Heat-fix the slide by passing the slide through the Bunsen burner flame five times. This step kills the organisms and adheres them to the slide so they are not washed off during the staining procedure. DO NOT heat the slide too much as this will crack the slide.
2. If taking from a solid media:
    a. Place three loopfuls of water onto the center of the slide. Make sure to flame the loop before using.
    b. Flame the needle before use. Allow to cool. If using a solid media in a tube remove the cap from the tube and flame opening before use. If using a solid media from a plate, just lift the lid enough to slide the needle in. DO NOT take too many cells; you do not need a big clump of organisms on the needle.
    c. Spread the organisms around in the water to make a dime-sized circle on the center of the slide. Only use one needle full of organisms.
    d. Allow the smear to air dry.
    e. Heat-fix the slide by passing the slide through the Bunsen burner flame five times.

Questions

1. What is the purpose of heat fixing the slide?

2. Why is it important to limit the amount of organism used in the smear preparation?

Experiment 5 Simple Staining

The simple staining procedure is used to make bacterial cells easier to visualize on the slide, it also utilizes one dye to color the bacterial cell. There are two types of dyes used for staining.

Basic dye

- Positively charged chromophore
- Works well with bacterial cells because they have a negative charge
- Stains the inside of the cell

Acidic dye

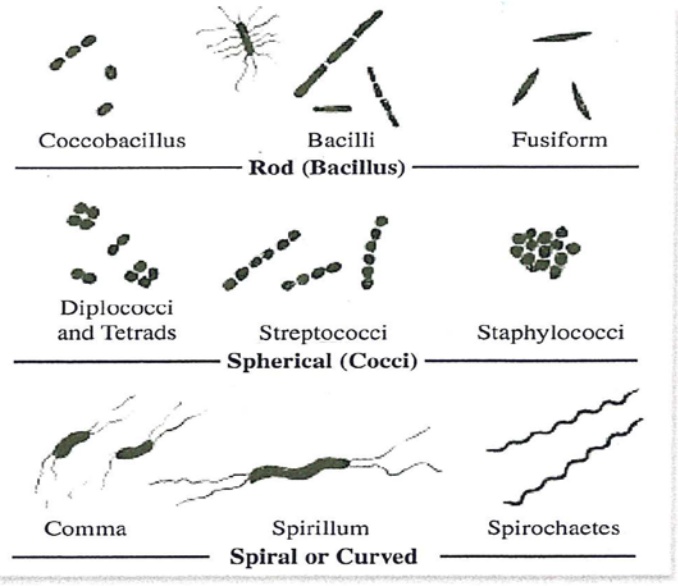
- Negatively charged chromophore
- Repelled by the negative charge of the bacterial cell
- stains the outside of the cell

Simple stains are used for determining bacterial cell morphology (cell shape). Figure shows some common shapes of bacteria. There are three main types:

Rods (bacilli) can have flat, rounded, or tapered ends

Cocci (spherical)

Spiral or Curved



Organisms used:

- *Bacillus cereus* - Gram (+) rod
- *Staphylococcus aureus* – Gram (+) cocci
- *Escherichia coli* - Gram (-) rod

Dyes used:

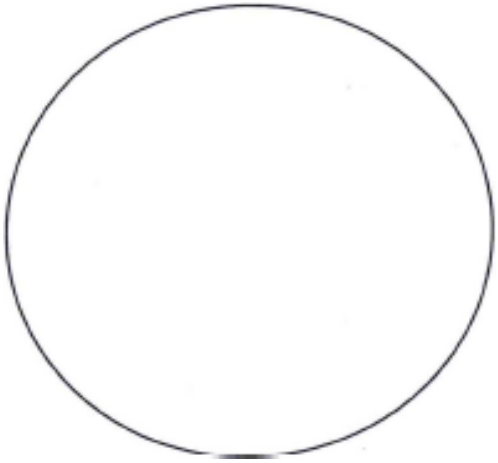
- Methylene blue

Procedure

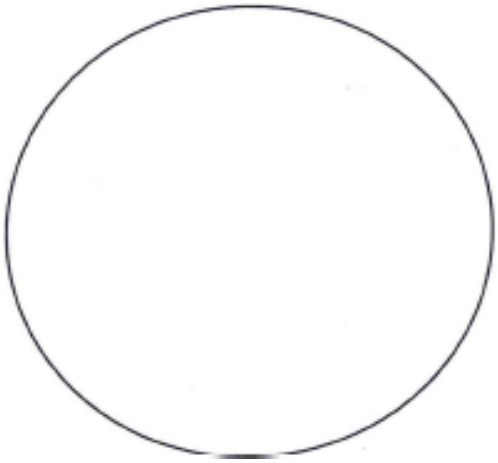
- Using aseptic technique, make a mixed smear preparation of *B. cereus* and *E.coli.* If one organism is on a TSA slant the organism in the TSB is used first as the liquid that goes down on the slide, then one needle on the organism on the TSA slant is used and mixed around the slide.
- Air dry the slide and then heat-fix the slide.
- Apply methylene blue to the heat-fixed smear. Let sit for 1 minute.
- Rinse the slide briefly. Blot dry with bibulous paper and view under oil immersion.
- View the prepared slide for comparison and to see what it is you should be looking for.
- Prepare the next mixed slide of *E. coli* and *S. aureus* using the technique you used for the first slide.
- Clean your slides once you are done viewing them and have drawn what you saw.

Results and Questions

1. Draw your observations of the slides that you made.



Escherichia coli and *Staphylococcus aureus*



Bacillus cereus and *Escherichia coli*

2. Can you see the different cell morphologies of both organisms? What are the morphologies?

3. Explain the difference between acidic dyes and basic dyes.

Experiment 6 Negative Staining

Negative staining is a staining procedure that demonstrates the size and shape of bacteria. Negative stains are acidic dyes and repelled by the bacterial cell. These dyes will stain the background of the bacterial cell. Bacterial cells will appear transparent against a dark background.

Your instructor will demonstrate the procedure of how to spread the India ink across the slide.

Organisms used:

- *Escherichia coli* - Gram (-) rod
- *Staphylococcus aureus* – Gram (+) cocci

Dyes used:

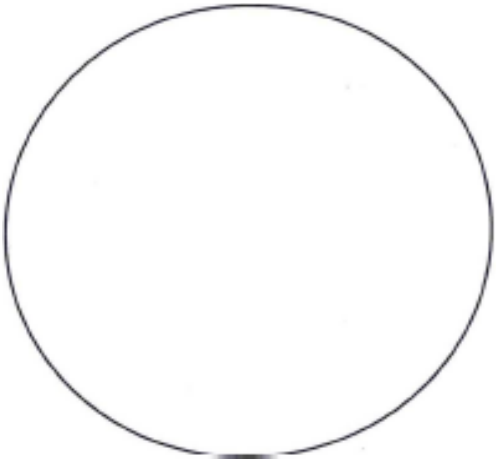
- Nigrosin or India Ink

Procedure

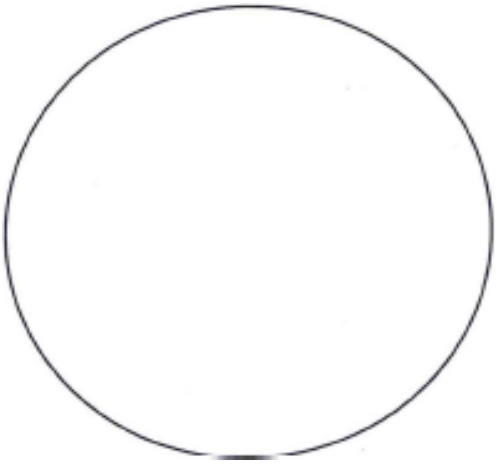
- Add one drop of nigrosin (India ink) near the end of the slide. Flame needle or loop depending on media used. Aseptically transfer *E. coli* or *S. aureus* into the drop of India ink. Remember to flame between taking another loopful otherwise you will add India ink to the broth.
- With another slide place the edge of the new slide against the drop of India ink against the surface of the other slide. This is the spreader slide.
- Push the spreader slide to the left without breaking contact with the other slide. This will drag the India ink across the bottom slide.
- Allow the slide to air dry, then view under oil immersion.
- View the prepared slide for comparison.
- Make another slide with the other organism you did not make the first slide with.
- **Make sure that you disinfect the slide for 15 min before washing the slides you made. This is due to the fact that we did not heat fix the slide so these are live organisms.**

Results

Draw what you saw for the slide with *Escherichia coli.*



Draw what you saw for the slide with *Staphylococcus aureus.*



Experiment 7 Capsule Staining

Some bacterial cells are surrounded by a gel-like layer outside the cell wall. This layer can be of two distinct types.

Capsule

- A distinct gelatinous layer
- Attached tightly to the bacteria
- Most capsules are made up of polysaccharides. Known as glycocalyx

Slime layer or biofilm

- A diffuse and irregular layer of extracellular matrix
- Loosely associated with the bacteria and can be washed off

These structures preform important functions in a cell. They are protective structures which help the cells from becoming engulfed or destroyed. Another function is attachment to solid surfaces in the environment.

The staining procedure for capsule staining differs from the traditional smear preparation. We utilize the Negative staining procedure for the first part of the step. There is no heat fixing step in this procedure for the capsule stain, all slides are air dried. Heat fixing the smear would destroy or shrink the capsule and this structure would not be seen when viewed under the microscope. However, there are capsule staining techniques that do use heat fixing.

Under oil immersion capsules will appear as halos around pink/red cells with a dark background.

Organisms used:

- *Klebsiella pneumoniae* - Gram (-) rod

Dyes used:

- Congo red – acidic dye, stains the background
- Maneval's – basic dye, stains the inside of the cell

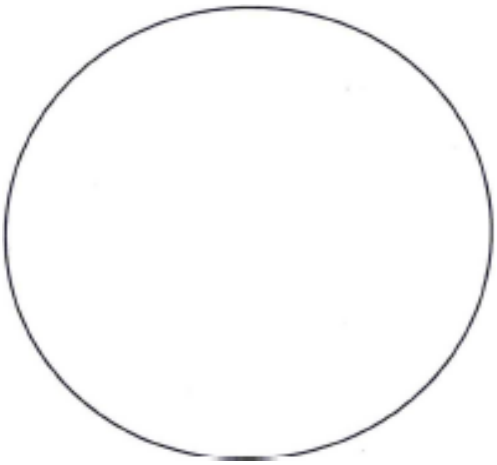
Procedure

- Add one drop of congo red near the end of the slide. Flame needle or loop depending on media used. Aseptically transfer *K. pneumoniae* into the drop of congo red. Remember to flame between taking another loopful otherwise you will add congo red to the broth.
- With another slide, place the edge of the new slide against the drop of congo red and against the surface of the other slide. This is the spreader slide.
- Push the spreader slide to the left without breaking contact with the other slide. This will drag the congo red across the bottom slide.

- Allow to air dry.
- Add Maneval's, as the counter stain, to cover the congo red. Leave the Maneval's on for 1 minute.
- Gently rinse. Do Not rinse all the dye off the slide otherwise there will be nothing to view.
- Air dry the slide and view under oil immersion.
- View the prepared slide for comparison.
- **Make sure that you disinfect the slide for 15 min before washing the slides you made. This is due to the fact that we did not heat fix the slide so these are live organisms.**

Results and Questions

- Draw your slide of *Klebsiella pneumoniae.*



- What is the expected outcome if you heat fix the slide?

Experiment 8 Gram Staining

Hans Christian Gram – Danish physician in 1884 developed a staining technique that would differentiate bacterial cells from eukaryotic nuclei.

Gram staining is a very important diagnostic tool used to provide presumptive identification of unknown bacteria. This staining technique is an example of a differential stain. Differential stains take advantage of the fact that cells or cell structures can be distinguished from others due to dissimilar staining.

Gram positive and Gram negative cells are differentiated with the Gram stain based on the composition and structure of their cell wall. Cell wall features in a Gram positive cell is 90% peptidoglycan plus 10% teichoic acids. Cell wall features in a Gram negative cell is 10% peptidoglycan plus 90% outer membrane. These characteristic differences between the two cells give them two distinct color variations. Gram positive cells will show purple and Gram negative cells will be pink or red; this is due to the thickness of the peptidoglycan layer.

Steps of the Gram stain:

- Crystal violet – this is the primary stain that will give cells a purple color.
- Gram's Iodine – this is a mordant. It combines an insoluble complex with the crystal violet in Gram positive cells.
- 95% Ethanol – used as a decolorizing agent. This will remove the dye-mordant complex from the Gram negative cells.
- Safranin – counterstain for Gram negative cells so that they may be seen as pink or red.

Reagent	**Gram positive**	**Gram negative**
None – heat-fixed	Clear	Clear
Crystal violet	Purple	Purple
Gram's iodine	Purple	Purple
95% Ethanol	Purple	Clear
Safranin	Purple	Pink/red

Color changes at each step in the Gram stain.

Organisms

- *Bacillus cereus* – Gram (+) rod
- *Staphylococcus aureus* – Gram (+) cocci
- *Escherichia coli* – Gram (-) rod

Dyes and Reagents used

- Crystal violet
- Gram's iodine
- 95% Ethanol

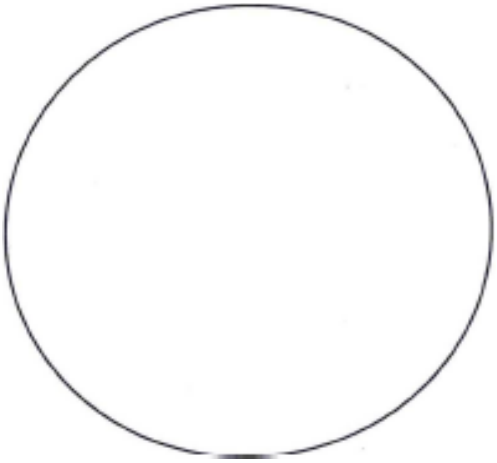
- Safranin

Procedure

- Make a mixed smear preparation of *B. cereus* and *E. coli*. Make sure to heat-fix the slide.
- Cover the smear preparation with crystal violet for 30 seconds.
- Briefly wash off the stain with water.
- Cover the smear with Gram's Iodine for 1 minute
- Pour off the Gram's Iodine.
- Decolorize with 95% ethanol. No more than 10 to 15 seconds.
- Stop the decolorization process with a water rinse.
- Counterstain with Safranin for 1 minute.
- Rinse with water, blot dry, view under oil
- View the prepared slides for comparison.
- Make a new mixed smear preparation of *E. coli* and *S. aureus.* Complete the Gram stain steps above for this new slide.

Results and Questions

1. Draw what you see in your mixed stain of *B. cereus* and *E.coli.*



2. Draw what you see in your mixed strain of *E. coli* and *S. aureus.*

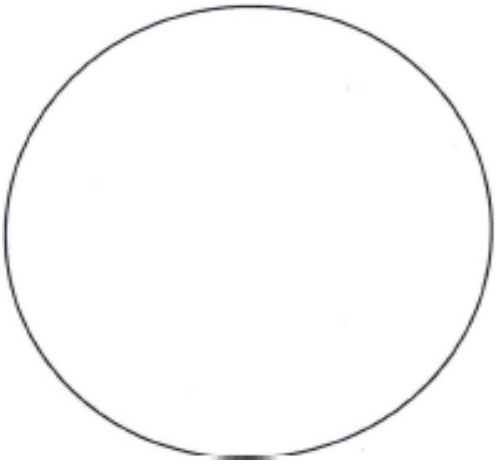


Table of results of slides

Bacterial Species	**Gram reaction**	**Cell shape**
Staphylococcus aureus		
Bacillus cereus		
Escherichia coli		

3. Using the table below, fill in what would happen for each cell type if you forgot to do one of the steps in the Gram stain procedure.

Reagent	**Gram positive**	**Gram negative**
None – heat-fixed		
Crystal violet		
Gram's iodine		
95% Ethanol		
Safranin		

Experiment 9 Acid-Fast Staining

Acid-fast staining is used to identify *Mycobacterium* and *Nocardia* species of bacteria. These bacteria have a component of the cell wall lipids that is a waxy material called mycolic acid. This layer makes certain staining procedures ineffective at permeating the mycolic acid in the cell wall.

Acid-fast bacteria are stained pink to red by the dye basic fuchsin. Non-acid-fast bacteria will be a blue color with the counterstain of methylene blue.

Dyes and Reagents used

- Carbolfuchsin
- Acid alcohol
- Methylene blue – counter stain

Organisms

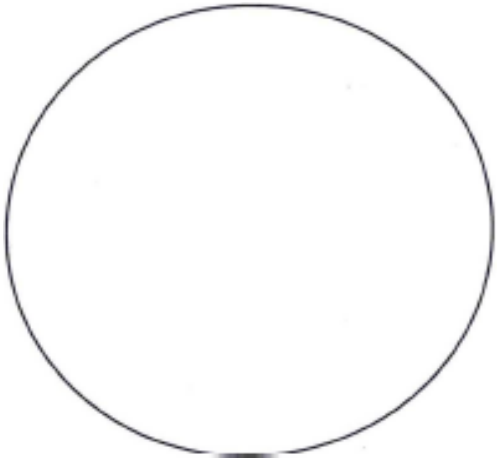
- *Mycobacterium smegmatis* – rod
- *Staphylococcus aureus* – cocci

Procedure

- Prepare a mixed smear preparation of *Mycobacterium smegmatis* and *Staphylococcus aureus.*
- Cover the smear with carbolfuschin for 5 minutes. (Your instructor might instruct you to use heat to help drive the carbolfuchsin into the waxy layer of the cell)
- Gently rinse with water
- Decolorize with acid alcohol for 1 minute
- Rinse with water
- Counterstain with methylene blue for 30 seconds
- Rinse briefly, blot dry and view under oil
- View the prepared slide for a comparison

Results and Questions

1. Draw the cells from your mixed stain. Use colors to distinguish between the acid-fast and the non-acid fast bacteria.



M.smegmatis and *S. aureus*

2. What color is *M.smegmatis* supposed to be? What color is *S. aureus* supposed to be?

3. Why combine an acid fast organism and a non-acid fast organism when doing the stain?

Experiment 10 Endospore Staining

Certain species of Gram positive bacteria form endospores when they are stressed. Endospores are utilized as resting stages to allow bacteria to survive non-favorable environmental conditions. Once environmental conditions become favorable endospores can go through the germination process and form vegetative cells. Two species of medically important bacteria that form endospores are *Bacillus* and *Clostridia.*

Endospores are resistant to heat, radiation, acids, and many chemicals. This resistant is due in part to the protein coat that forms a protective barrier around the spore. Conditions that define sterility are based on the conditions necessary to destroy an endospore. To destroy an endospore by heat they must be steamed under pressure for 15 to 20 minutes at a temperature of 121°C.

Endospores are also not easily penetrated by staining procedures. Heat needs to be applied to help the stain enter the endospore. This heating process acts as the mordant. In this staining procedure, the endospore will be green due to the malachite green and the vegetative cell will be red due to the safranin.

Dyes used

- Malachite green
- Safranin – Counter stain

Organism

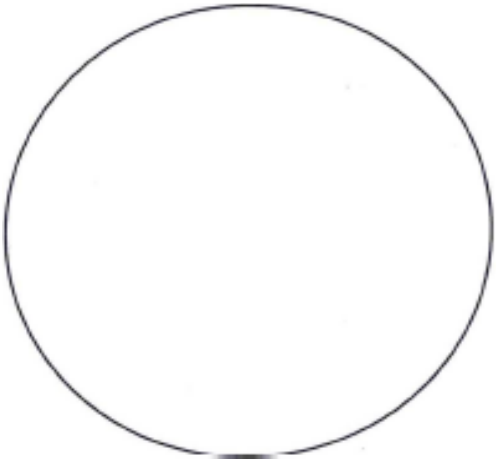
- *Bacillus stearothermophilus*

Procedure

- Prepare a smear preparation of *Bacillus stearothermophilus.*
- Cover the smear preparation with malachite green. Steam for 5 minutes. (Your instructor may have you pass the slide through the Bunsen burner flame for the heating method). Replenish the malachite green as needed. Do not let it dry or boil.
- Allow the slide to cool, rinse with water.
- Counter stain with safranin for 1 minute
- Rinse, blot dry, and view under oil
- View the prepared slide for comparison

Results and Questions

1. Draw the cells from your spore stain. Differentiate the spore from the vegetative cell.



2. What is the color of the vegetative cell in a spore stain? What is the color of the endospore in the spore stain?

3. Why is it necessary to use high heat for a long period of time to kill an endospore?

Experiment 11 Motility

Motility is most often found in both spirochetes and rod shaped bacteria. Flagella is the major organelle used for motility. Flagella are used primarily as a way to move away from harmful substances or towards nutrients in the environment. Since flagella are very thin structures they must be stained in order to be seen under light microscopy.

Motility can be observed by several different methods. Those methods are semisolid media, wet mount, or hanging drop.

When looking at organisms on a slide there may be movement of the cells that is not true motility. This movement is called Brownian motion. This is movement of the cells by currents under the cover glass that makes cells appear to move or by molecules that are bombarding the cells. If you see only vibrational movement or movement of the cells in a straight line in one direction then the cells are not motile.

Organisms

- *Proteus vulgaris*
- *Staphylococcus aureus*
- *Bacillus megaterium*

Procedure

1. **Wet mount**
    - **a.** Prepare wet mount slides of both *S. aureus* and *P. vulgaris* using 5 loopfuls of each organism in the center of a slide
    - **b.** Cover the organism with a cover slip.
    - **c.** View under 400X magnification immediately, otherwise the culture will dry and you will not see anything move. Do not go to oil, you may crack the coverslip.
2. **Semisolid agar**
    - **a.** This media is known as motility agar. This motility agar has 2,3,5-Triphenyltetrazolium Chloride or TTC in it. TTC is a colorless dye that when bacteria reduce it, it becomes red.
    - **b.** Inoculate one tube of motility media with your needle by stabbing into the agar for *S. aureus* and one tube of motility media for *P. vulgaris.*
    - **c.** Incubate this media at 25°C.
    - **d.** Results: Motile organisms will move away from the stab line and give a fuzzy appearance to the tube. If the motile organism can reduce the TTC then the fuzzy areas will be red. Non-motile organisms will not move away from the stab line and the rest of the media will be clear. If the non-motile organism can reduce the TTC the red color will be in the stab line only.

3. **Hanging drop**
    - **a.** Check out a depression slide from your instructor or the technician. The organism you will use for the hanging drop will be *B. megaterium.*
    - **b.** Obtain a cover slip. Place a small amount of Vaseline on each corner of the cover slip with a tooth pick.
    - **c.** Add two loopfuls of organism to the center of the cover slip.
    - **d.** With the depression slide inverted so the depression in the slide is facing the cover slip press it against the cover slip and quickly invert it (turn the slide up right so the coverslip is not against the bench top).
    - **e.** View the slide right away under 400X magnification.

Results and Questions

1. Which of the bacteria viewed under the slide exhibited motility?

2. Draw the appearance of the motility agar tubes. Which organism was motile? Which organism was not motile?

Experiment 12 Pure Culture Technique

Pure culture technique is a useful tool that helps to obtain a single kind of organism from a mixed culture. Robert Koch was one of the first to utilize this technique to isolate a specific agent from a mixture that contained other unwanted agents.

The two commonly used methods of pure culture are the pour plate and the streak plate. Both of these methods result in individual colonies that are isolated from a mixed culture. In this exercise you will do both. The organisms used can be differentiated by the color of the colony in addition to the different Gram stain results. This exercise will be done over a period of three lab meetings.

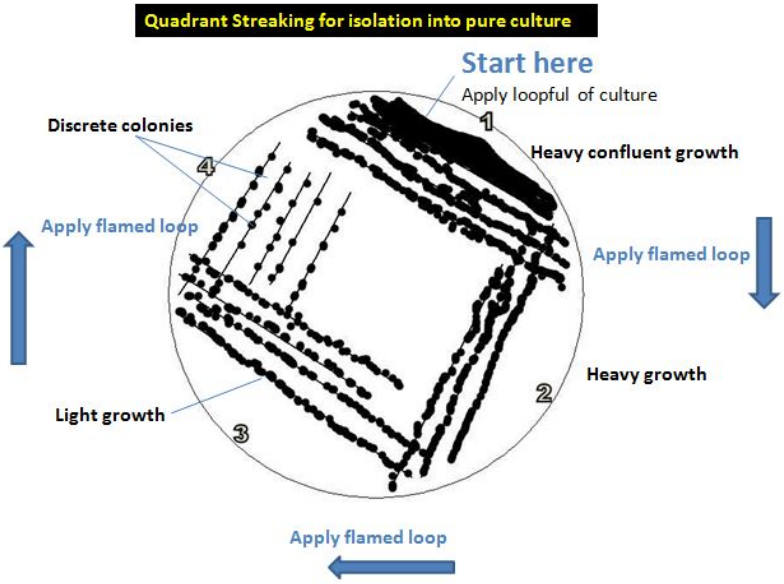
Meeting One

Organisms

- Mixed culture of
    - *Staphylococcus aureus* – Gram (+) cocci, cream/yellow colonies
    - *Chromobacterium violaceum* – Gram (-) rod, blue/purple colonies
    - *Serratia marcescens* – Gram (-) rod, red colonies

Procedure

- **Streak plate method**
    - Obtain one TSA plate and streak for isolation using either a quadrant streak or a T streak of the mixed culture.
    - The figure below will demonstrate how to do the streak for isolation. Your instructor will also demonstrate this on the white board.



- **Pour plate method**
    - **CAUTION:** the melted TSA tubes in the water bath will solidify fast, so only obtain the tubes when you are ready. Also make sure to pour the tubes right away into the sterile plates as it will solidify in the tube. There is no way to go back to a liquid once it has solidified without heating it in the autoclave which would kill the organisms in the tube.
    - Obtain three sterile empty petri plates. Label them 1, 2, and 3 along with all the necessary information the goes onto the bottom of the plate.
    - After you have labeled the plates go to the water bath and obtain one melted TSA tube. Aseptically transfer one loopful of the mixed culture broth to this tube, mix. DO NOT pour this into the plate just yet.
    - Have your partner obtain another melted TSA tube and transfer one loopful from tube 1 to this new tube (tube 2), mix. Pour the contents of tube 1 into the petri plate labeled 1. DO NOT pour tube 2 into plate 2 yet.
    - Have your partner obtain another melted TSA tube and transfer one loopful from tube 2 into this new tube (tube 3), mix. Pour the contents of tube 2 into plate 2. Pour the contents of tube 3 into plate 3.
    - Spread out the media in each plate as best as you can by gently rotating the plate.
    - Once the agar in the plates has solidified, invert them so the lid is facing down and place them in the plate rack.

Meeting two

Procedure

- Look at the pour plates and the streak plates. You should hopefully have isolation of each of the three colonies.
- Obtain three TSA slants for transferring one colony of each color (red, purple, white) to. If you only have two colors as isolated colonies then just obtain two.
- Aseptically transfer a well isolated colony of either color to the TSA slant. Do this step for each color that you have well isolated colonies of. It does not matter which plate you take the one colony from as long as you have clear isolation of either the with, red, or purple colony.
- Incubate the slants at 25°C.

Meeting three

Procedure

- Inspect your TSA slants. They should hopefully be the color of the organism that you pulled the colony from.

- Make a separate smear preparation of each organism from each slant. Next Gram stain each smear preparation to determine if you have a pure culture on the slant that it came from.

Results and Questions

1. Which method, either pour plate or streak plate, achieved the best separation?

2. Do each of your slants contain pure cultures?

Experiment 13 Ultraviolet Light Effects

Ultraviolet (UV) light falls between 4 nm and 400 nm and is known as nonionizing short wavelength radiation. In general the shorter the wavelength the more damaging it is to cells. UV light in general is used to sterilize surfaces as most bacteria are killed by the effect of UV light.

One of the primary lethal effects of ultraviolet light is its mutagenic properties. At 260nm UV light is the most germicidal because at this wavelength DNA absorbs the UV light and bonds are broken at its maximum. Pyrimidine dimers are formed when DNA absorbs UV light.

Pyrimidine dimers are covalent bonds that form between two adjacent thiamine or cytosine molecules of DNA. This then causes the shape of DNA to become deformed and the DNA polymerase cannot replicate the DNA strand past this point.

The SOS system of DNA repair removes these dimers and inserts the correct pyrimidine molecules. However if the damage to the DNA strand is too great the SOS system may make errors and start inserting incorrect bases where the damaged bases are.

Time of exposure and presence of materials that block radiation are factors that influence the killing effect of UV light.

Organism

- *Bacillus subtilis* – Gram (+) rod, soil microbe that produces endospores
- *Staphylococcus aureus* – Gram (+) cocci, non-spore former

Procedure

- Your instructor will have you sign up for an exposure time and organism with your lab partner.
- Once you have been assigned an exposure time and an organism. Obtain a TSA plate and a sterile swab. With the sterile swab, swab the plate all over for confluent growth of your organism. Your instructor will demonstrate confluent growth on the board. (Also your instructor may have you wait to swab the TSA plate until they are ready to place it into the UV light). If you have one of the control exposure times leave the lid on.
- When your instructor has called for your exposure time place the plate into the biosafety cabinet with the lid placed near it. Take an index card and cover half the plate. (Make sure to label the side that was covered with the index card of the bottom or agar side of the plate.)
- Time your exposure and take your plate out when the time is up. (Your instructor will demonstrate or give directions on the use of the UV light in the biosafety cabinet). Recover the plate with the lid and place it into the plate rack, lid side down.

Next Lab period

- Examine your plate. Count the number of colonies on the half of the plate that was exposed to the UV light (not the half covered with the index card). Place your number of colonies or if you had confluent growth (a lawn of bacteria on the plate) into the chart on the board.

Results and Questions

1. Record the information for each exposure time from the board into the table.

Organisms	**Exposure Times**							
	10 sec	20 sec	40 sec	80 sec	2.5 min	5 min	10 min	10 min control
S. aureus								
	1 min	2 min	4 min	8 min	15 min	30 min	60 min	60 min control
B. subtilis								

2. Why was half the plate covered? What was the purpose of leaving the cover on one set of plates for each organism?

3. Which organism is more resistant? How many times more resistant is that organism than the other? Why is the organism that is more resistant, resistant?

Experiment 14 Enumeration of Bacteria : Standard Plate Count

Enumeration or the number of viable and non-viable microbes in a sample is important in many different situations. Ensuring safety of food or water utilizes some form of enumeration of the bacterial count in a sample. The bacterial count in a urine sample is used in a hospital setting to determine infection. There are several different methods for determining the number of bacteria in a sample. Here are some of the direct methods used to determine bacterial count in a sample: Microscopic count, Most probable number, and Standard plate count.

If you only want to know if a sample has bacteria in it, you could use an indirect method. Turbidity would be an indirect method since it uses the absorption of light to determine the amount of colloidal material in suspension. Turbidity is measured with a machine called a spectrophotometer where %Transmittance or Absorbance is obtained. These numbers are then used to make a standard curve to determine bacterial numbers in relation to the standard plate count method. However there is a disadvantage to utilizing this method, culture turbidity is the contribution of both dead and living cells.

We will be using both a regular 5 ml pipet, a 1ml micropipette, and a 0.1ml micropipette in the experiment. Your instructor will demonstrate how to use the pipet aid for the 5ml pipet and how to use the micropipette and the tips that go with them. The micropipettes are already set to the proper volume and color coded with colored tape for the specific volume.

Organism

- *Escherichia coli*

Procedure Day 1

- **Standard plate count**
    - We will be using a modified pour plate method for the standard plate count. Remember that the cooled melted agar solidifies fast, so do not take it out of the water bath until you are ready to use it.
    - This part of the experiment will be done by one set of pairs in the row. All other pairs in that row will just do the plating step.
    - Each pair will obtain 4 sterile petri plates. Label these plats as follows:
        - Plate 1: (B) 1ml 1:10000
        - Plate 2: (B) 0.1ml 1:100000
        - Plate 3: (C) 1ml 1:1000000
        - Plate 4: (C) 0.1ml 1:10000000
    - **One set of pairs in the row will do the following 100 fold serial dilution:**
        - Take one of the 99ml water bottles at the end of the bench. Label this bottle with the letter A and 1:100. Next take the culture flask of TSB

with*E. coli*, mix it up before use. Obtain a 1ml micropipette from the rack and a box of tips. Transfer 1ml of the *E. coli* to bottle A. Mix the water in the bottle.

- Take the second bottle of 99ml water from the end of the bench and label this one with the letter B and 1:10000. Take bottle A (not the flask of *E. coli*) and transfer 1ml of the water with the micropipette from bottle A into bottle B. Mix.
- Take the third bottle of 99ml water from the end of the bench and label it with a letter C and 1:100000. Take bottle B and transfer 1ml of water with the micropipette from bottle B to bottle C. Mix.

- **Plating technique done by each pair in the row after the serial dilution has been done only once:**
  - Take bottle B (mix) and transfer 1ml of water from bottle B with the micropipette into the bottom of plate 1. Have your partner obtain the melted TSA tube from the water bath. Pour the melted agar into the plate over the 1ml of liquid. Spread the agar out over the plate.
  - With bottle B transfer 0.1ml of water with the micropipette from bottle B into the bottom of plate 2. Have your partner obtain the melted TSA tube from the water bath. Pour the melted agar into the plate over the 0.1ml of liquid. Spread the agar out over the plate.
  - Take bottle C (mix) and transfer 1ml of water from bottle C with the micropipette into the bottom of plate 3. Have your partner obtain the melted TSA tube from the water bath. Pour the melted agar into the plate over the 1ml of liquid. Spread the agar out over the plate.
  - With bottle C transfer 0.1ml of water with the micropipette from bottle C into the bottom of plate 4. Have your partner obtain the melted TSA tube from the water bath. Pour the melted agar into the plate over the 0.1ml of liquid. Spread the agar out over the plate.
  - Cover all the plates with the lids after you have poured the melted agar. Wait for them to solidify before inverting them and placing them into the plate rack for incubation.

- **Turbidity readings**
  - Turbidity is measured using a spectrophotometer. Spectrophotometers read % Transmittance of the sample. In this part of the experiment you will obtain % transmittance readings from the spectrophotometer and calculate optical density (O.D.).
  - O.D. is calculated using the following formula and the use of a log table at the end of the lab manual.
    - O.D.= 2-log(%T). %T is the transmittance value from the spectrophotometer. Log = character + mantissa. Character = number of

whole places in the original number – 1. Mantissa is read off the log table.
        - Your instructor will go over how to calculate the O.D. for sample readings.
    - Procedure for taking spectrophotometer readings is as follows:
        - This is a 2 fold serial dilution.
        - Obtain 4, 4ml TSB tubes with silver caps. Label them 1:2, 1:4, 1:8, and 1:16.
        - With a 5 ml pipet and pipet aid transfer 4 ml of liquid from the culture flask with *E. coli* in it into the 1:2 tube. (mix)
        - With the same or a different 5 ml pipet transfer 4 ml from the 1:2 tube into the 1:4 tube. Mix
        - Next transfer 4 ml from the 1:4 tube into the 1:8 tube, mix.
        - Next transfer 4 ml from the 1:8 tube into the 1:16 tube, mix.
        - After the transfers have been done take all tubes over to the spectrophotometer to obtain % Transmittance readings for each tube.
        - Make sure to transfer the contents of each tube into a **cuvette, do not put the larger tubes into the machine.**
        - Make sure to obtain a reading of 4 ml of the *E. coli* in the culture flask, this is your 1:1.
        - Follow the directions of your instructor and the directions found in this manual for using the spectrophotometer.
        - Calculate the O.D. values.
        - Make sure to rinse any tubes into the waste beaker by the machines. **NOTHING GOES DOWN THE DRAIN.**

The Spectrophotometer

A spectrophotometer is an instrument that measures the transmission (or absorption) of light through a substance at various wavelengths of light. A schematic diagram of the essential components of the spectrophotometer is given in figure 1.

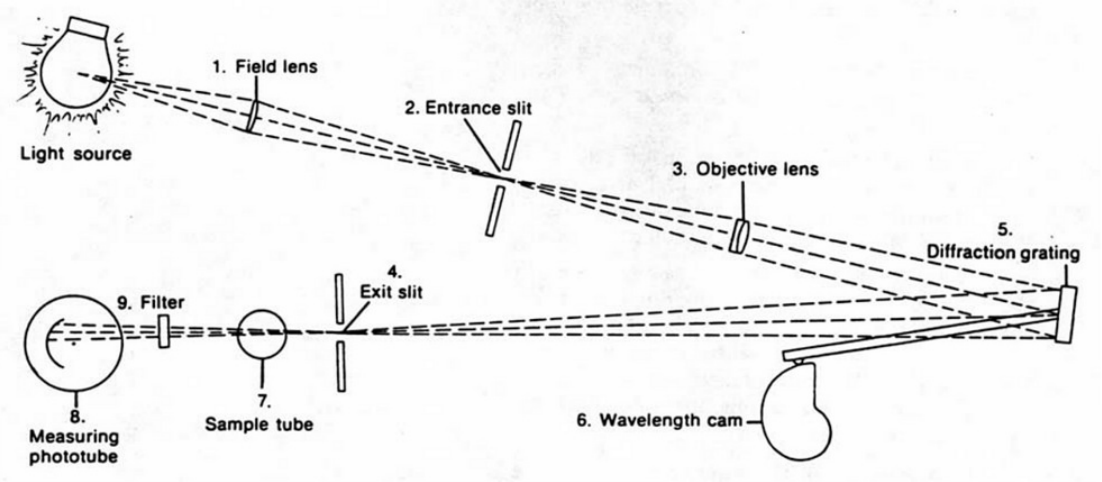


FIG. B-1. Optical system of the Spectronic 20 colorimeter.

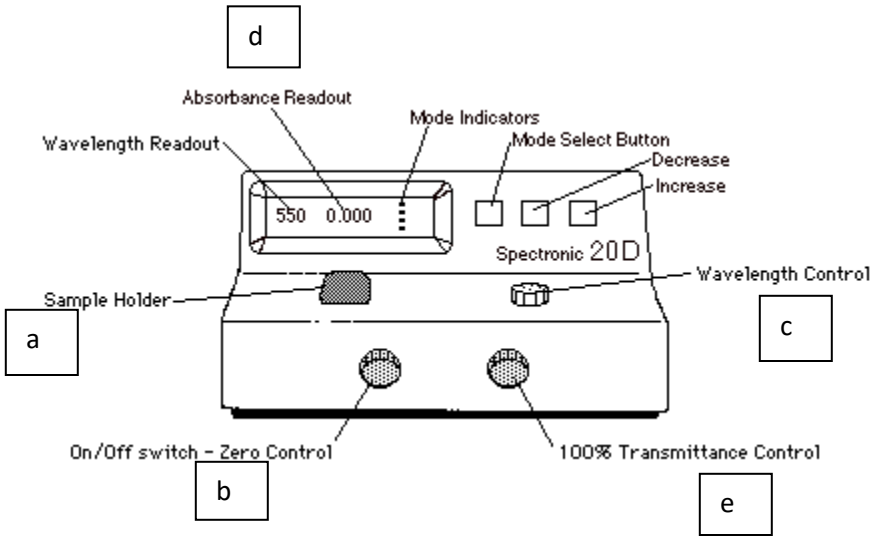
Figure 1: Schematic diagram of the spectrophotometer.

A narrow beam of light from the lamp is defined by the slits and lenses. The movable mirror equipped with a grating that disperses the light into a spectrum of different wavelengths. The slit passes only a narrow range of wavelengths, allowing essentially monochromatic light to pass through the sample and strike the phototube (detector). This generates an electric current that is proportional to the intensity of the light, and this current strikes a meter. The light control (100% transmission control) permits variation of the intensity of light striking the sample. A wavelength scale linked to the movable dispersing mirror is calibrated to indicate the wavelength of light that illuminates the sample and phototube in the instruments, including the Spectronic 20D. The meter is able to read both percent transmittance and absorbance. The relationships between these two measurements and the light intensity is

$$\%T = I_t/I_o * 100 \qquad A = \log I_o/I_t = \log 100/\%T$$

where I_o equals the intensity of light before passing through the sample, and I_t equals the amount of light passing through the sample.

A Description



GENERAL OPERATING INSTRUCTIONS

1. Turn the power switch (zero control knob (b)) to the on position. Allow at least 15 minutes for the instrument to warm up. This will be done for you.

2. Turn the WAVELENGTH control knob (c) to the desired setting (590nm). Set the power switch, zero control (b) so that the absorb/transmittance display (d) reads zero with no cuvette (sample tube) in the sample compartment (a). *Be certain that the sample compartment is closed during this and all other measurements.* This will be done for you.

3. Carefully wipe the exterior of the blank cuvette (one with only TSB, no growth) with kimwipes. Insert the blank into the sample compartment (a), aligning the marker on the cuvette with the mark on the sample compartment holder. Close the compartment cover. Adjust the absorbance control knob (e) until the meter (d) reads 100% transmittance (zero absorbance). You will now have compensated for any absorption due to the TSB so that reading your sample must be due to solute absorption. *This procedure must be repeated each time the wavelength is changed.* It is best to repeat steps 2 & 3 until the 1 $\Rightarrow$ 100 readings stabilize.

4. Rinse a clean cuvette twice with 1-2mL of water use the waste beaker to discard the water into. Fill the cuvette about 2/3 full with your sample and wipe the exterior with a kimwipe. Insert the sample cuvette in place of the blank cuvette in the sample holder (a), again aligning with the markers on the cuvette and sample compartment . Close the cover and read the % transmittance from the meter (d).

5. When you measurements are completed, remove your sample from the sample holder (a). Dispose of the sample and rinse the cuvettes with water and pour the waste into the waste

beaker next to the spec. Take care not to scratch them on any hard surface. Never use an abrasive cleaner on them. Do not turn the specs off. Please leave the area clean for the next person. **DO NOT pour any of the liquid down the drain**.

Procedure Day 2

- Collect your plates from the standard plate count. Count the colonies on one plate that has between 30-300 colonies on it. Calculate the concentration.
- Concentration = # of colonies x dilution factor of the plate used (units are in organisms/ml)
- Calculate the concentration of your dilution tubes from your turbidity readings.
    - 1:1
    - 1:2
    - 1:4
    - 1:8
    - 1:16
- Plot O.D. vs concentration for the standard curve.

Results and Questions

1. Record your plate count data in the table:

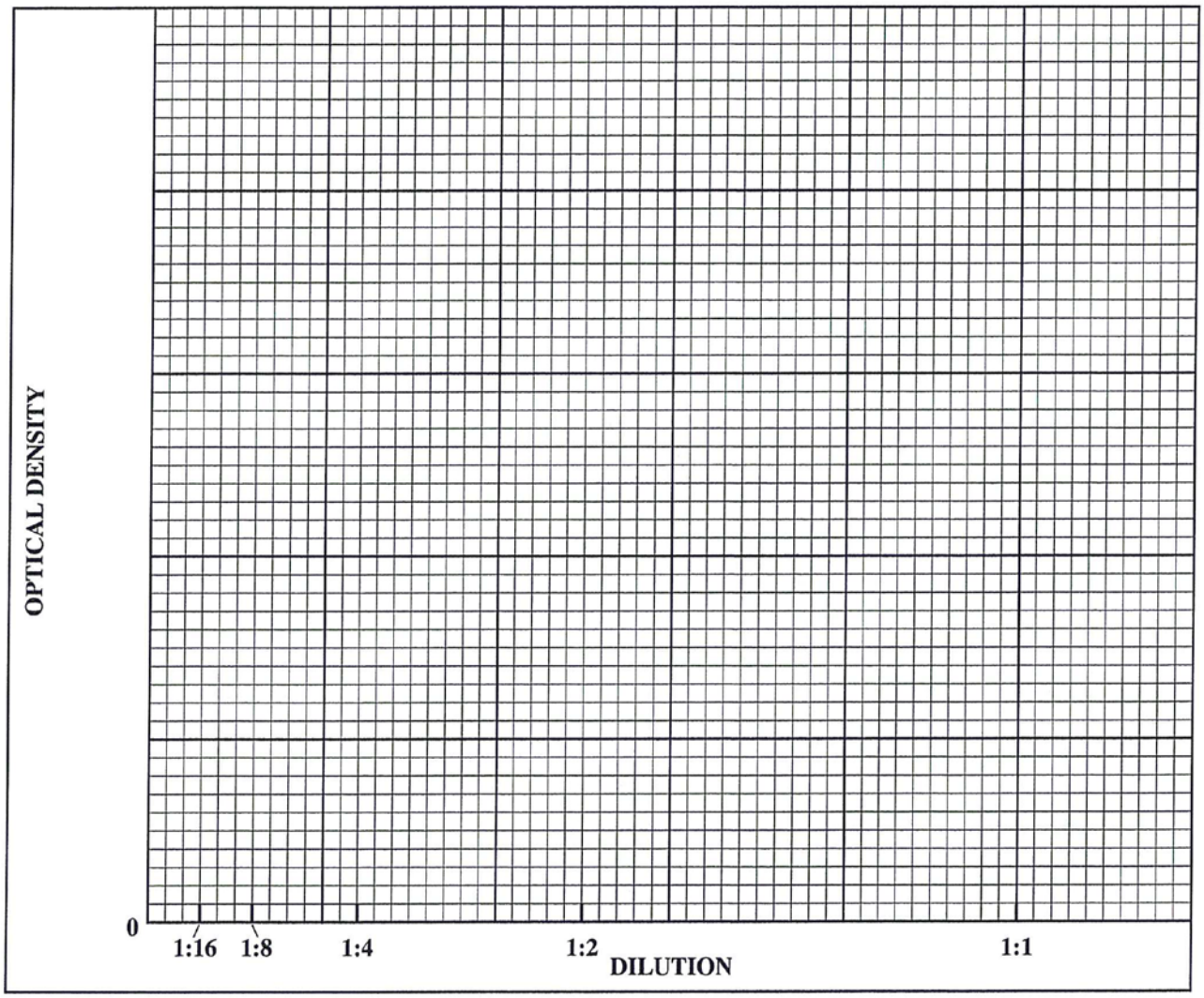
Dilution bottle	ml plated	Dilution	Dilution factor	Number of colonies
B (1:10000)	1.0	1:10000	10^4	
B (1:10000)	0.1	1:100000	10^5	
C (1:1000000)	1.0	1:1000000	10^6	
C (1:1000000)	0.1	1:10000000	10^7	

2. What is the concentration of the undiluted culture? What is the concentration of the rest of the dilutions, 1:2, 1:4, 1:8, and 1:16?

3. Optical density table:

Dilution	Optical Density
1:1	
1:2	
1:4	
1:8	
1:16	

4. Plot O.D. versus the concentration.



5. What is the relationship between O.D. and % Transmittance?

Experiment 15 Effects of Temperature on Growth

Microorganisms can grow in a broad array of temperatures from below 0°C to over 100°C. Optimum temperature growth requirements can place microorganisms into four different groups.

Psychrophiles: grow between -5°C and 20°C. These organisms can be found in the waters of the Antarctic and Arctic.

Mesophiles: grow between 20°C and 50°C. Most of the organisms fall into this temperature range, with many pathogens growing between 35°C and 40°C.

Thermophiles: grow between 50°C and 80°C. These organisms occur in soils or compost piles.

Hyperthermophiles: grow above 80°C. These organisms occur in thermal vents or environments where volcanic activity is found.

Temperature affects many metabolic factors in a cell. Enzyme function or activity is directly affected by temperature. The cell membrane and transport can be affected by temperature as well. Ribosomes can also be affected by temperature.

In this experiment we will look at the effects of temperature on growth of several organisms and how it effects pigment production of one of them.

Organism

Deck 1: *Staphylococcus aureus*

Deck 2: *Serratia marcescens*

Deck 3: *Bacillus stearothermophilus*

All pairs: *Serratia marcescens*

Procedure Day 1

- Obtain 6 TSB tubes per pair. Label each tube 5°C, 25°C, 30°C, 38°C, 40°C, and 60°C.
- Inoculate each TSB tube with your assigned organism.
- Next obtain 2 TSA slants per pair. Label one slant with 25°C and the other slant with 38°C. Inoculate each slant with *Serratia marcescens.*
- Place all tubes in their correct temperature racks at the back of the room.

Procedure Day 2

- Obtain your TSB tubes. Measure % Transmittance of each temperature tube.
- Determine O.D. for each temperature for your organism. Write this on the board.
- Note the pigment production of the TSA slant for *Serratia marcescens.*

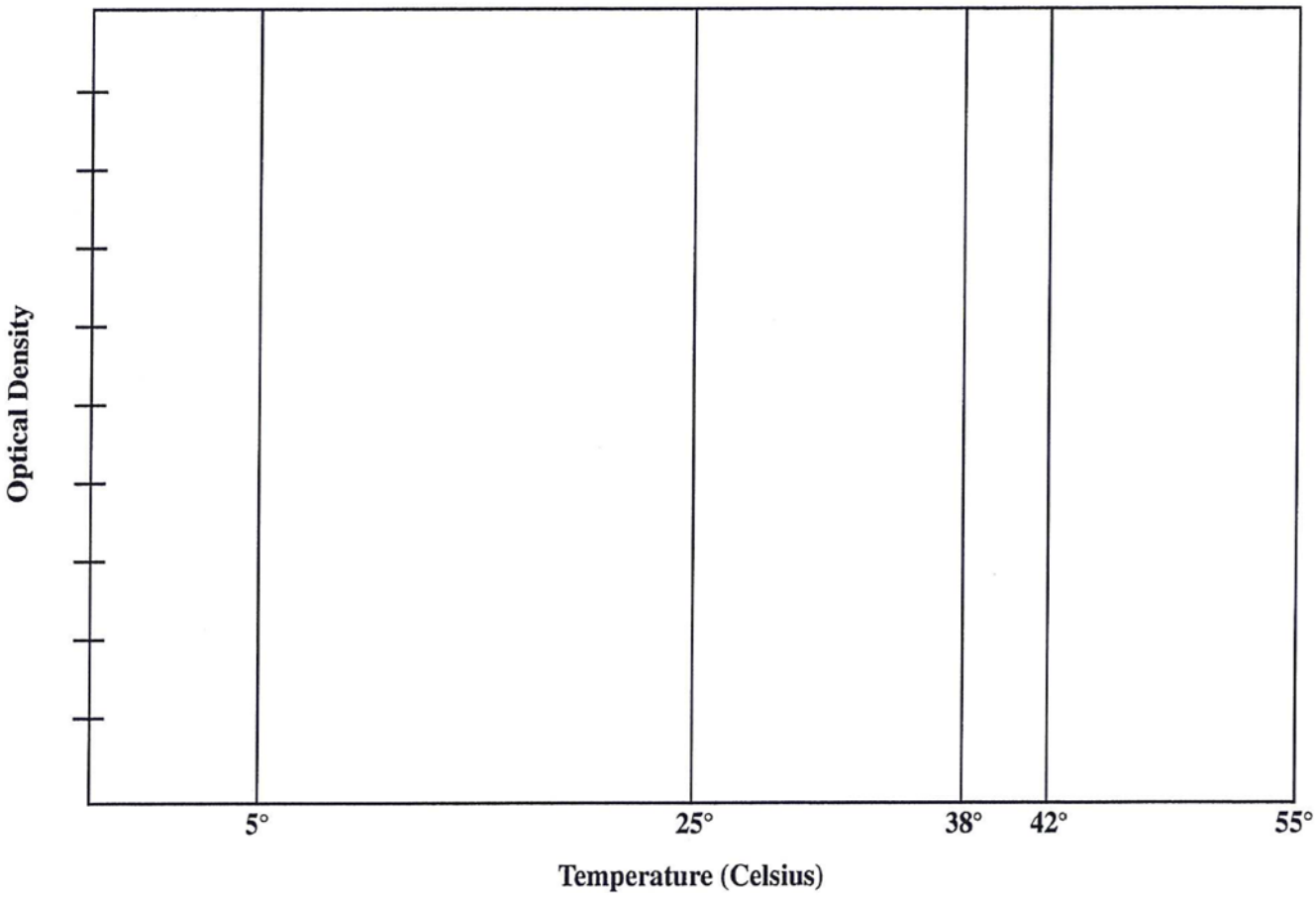
Results and Questions

1. What is the optimum temperature for pigment production in *Serratia marcescens*? What color is each slant? What could cause pigment production at the temperature where there is no pigment production?

2. Use the spectrophotometer and the board to fill in the table.

	S. aureus		***S. marcescens***		***B. stearothermophilus***	
Temperature	**% Transmittance**	**O.D.**	**% Transmittance**	**O.D.**	**% Transmittance**	**O.D.**
5 °C						
25°C						
30°C						
38°C						
40°C						
60°C						

3. Plot O.D. vs Temperature for each organism on the graph on the next page. Use a different color for each organism or label each line with the organism name.



4. What is the optimum temperature for each organism based on the graph above? What group does each organism belong to based on the data i.e. Mesophile, thermophile?

Experiment 16 The Effectiveness of Hand washing

Ignaz Semmelweis is accredited with the observation that hand washing is important in preventing the spread of diseases. He noted that there was a high patient death rate from childbed fever due to medical students and physicians not washing their hands after exiting autopsy rooms or dissections. He also noted that the nurses or midwifes that were not allowed in autopsy rooms had a lower patient death rate. He instituted a hand washing policy after visiting the autopsy room and before visiting patients.

Today it is routine to wash hands before examining a patient or before surgery. Day care workers and food preparers also follow hand washing practices. When these practices are not utilized then there is in increase in infections to individuals.

There is a diverse group of microorganisms that inhabit the human skin. There are three main groups that the normal flora can be placed into. They are:

Diphtheroids : These are represented by *Corynebacterium* and *Propionibacterium.* These bacteria prefer the oily regions of the skin. They degrade the fatty secretions in the hair follicles.

Staphylococci : These organisms tend to be coagulase-negative, salt tolerant, and grow in dry areas. They are thought to be beneficial since they produce antimicrobial compounds. There is a small percentage of the population that has *Staphylococcus aureus* as part of their natural flora.

Fungi: These are represented by *Malassezia furfur*. This organism is a nonpathogenic yeast that utilizes fatty substances. Most often this organism is found on the face as flaky skin around the nose.

Nosocomial infections are infections that are hospital acquired infections. One nosocomial infection of note is methicillin-resistant *S. aureus* (MRSA). In addition to the normal flora on the skin there are transient organisms that are picked up in our daily activities. These organisms are easily washed from the skin whereas the resident or normal flora are harder to wash off.

Procedure

- Your instructor will pick or ask for volunteers for the hand washing part of the experiment. All other students will be assigned a basin in groups and do a modified pour plate.

Hand washing procedure

- One student will be the person that is getting their hands washed. The other student will assist them and make sure they do not touch anything other than the sterile brushes. Do Not scrub hard.

- Step 1: Wash each hand for a total of 30 seconds with a sterile brush into Basin A. No soap is used in this step, just the water from Basin A.
- Step 2: Wash each hand for a total of 1 minute with the same brush as step 1. Use the green soap and do this step in the sink. Rinse with water from the sink.
- Step 3: Wash each hand for a total of 30 seconds with a new sterile brush into Basin B. No soap is used in this step, just the water from Basin B.
- Step 4: Wash each hand for a total of 1 minute with the same brush as step 3. Use the green soap and do this step in the sink. Rinse with water from the sink.
- Step 5: Wash each hand for a total of 30 seconds with a new sterile brush into Basin C. No soap is used in this step, just the water from Basin C.
- Step 6: Wash each hand for a total of 1 minute with the same brush as step 5. Use the green soap and do this step in the sink. Rinse with water from the sink.
- Step 7: Wash each hand for a total of 30 seconds with a new sterile brush into Basin D. No soap is used in this step, just the water from Basin D.
- Step 8: Wash each hand for a total of 1 minute with the same brush as step 7. Use the green soap and do this step in the sink. Rinse with water from the sink.
- Step 9: Wash each hand for a total of 30 seconds with a new sterile brush into Basin E. No soap is used in this step, just the water from Basin E.
- Dry off hands and apply lotion.

Modified pour plate procedure (Groups)

- While the hand washing is going obtain 6 sterile petri plates. Label two of them with 0.1 ml, label two of them with 0.2 ml, and the other two with 0.4 ml.
- When your basin is ready stir the water with the pipet. Dispense 0.1 ml with the micropipette into the 0.1 ml plates. Dispense 0.2 ml with the micropipette into the 0.2 ml plates. Dispense 0.4 ml with the micropipette into the 0.4 ml plate.
- Once you are done dispensing the volume into the plates obtain a melted Brain heart infusion (BHI) agar tube from the water bath. Remember that this will solidify fast so only obtain one when you need it. Pour the melted BHI into one of the plates.
- Obtain 5 other melted BHI agars to pour into the other 5 sterile petri plates. Let the agar solidify and cool before placing them upside down in the plate rack.

Next lab Period

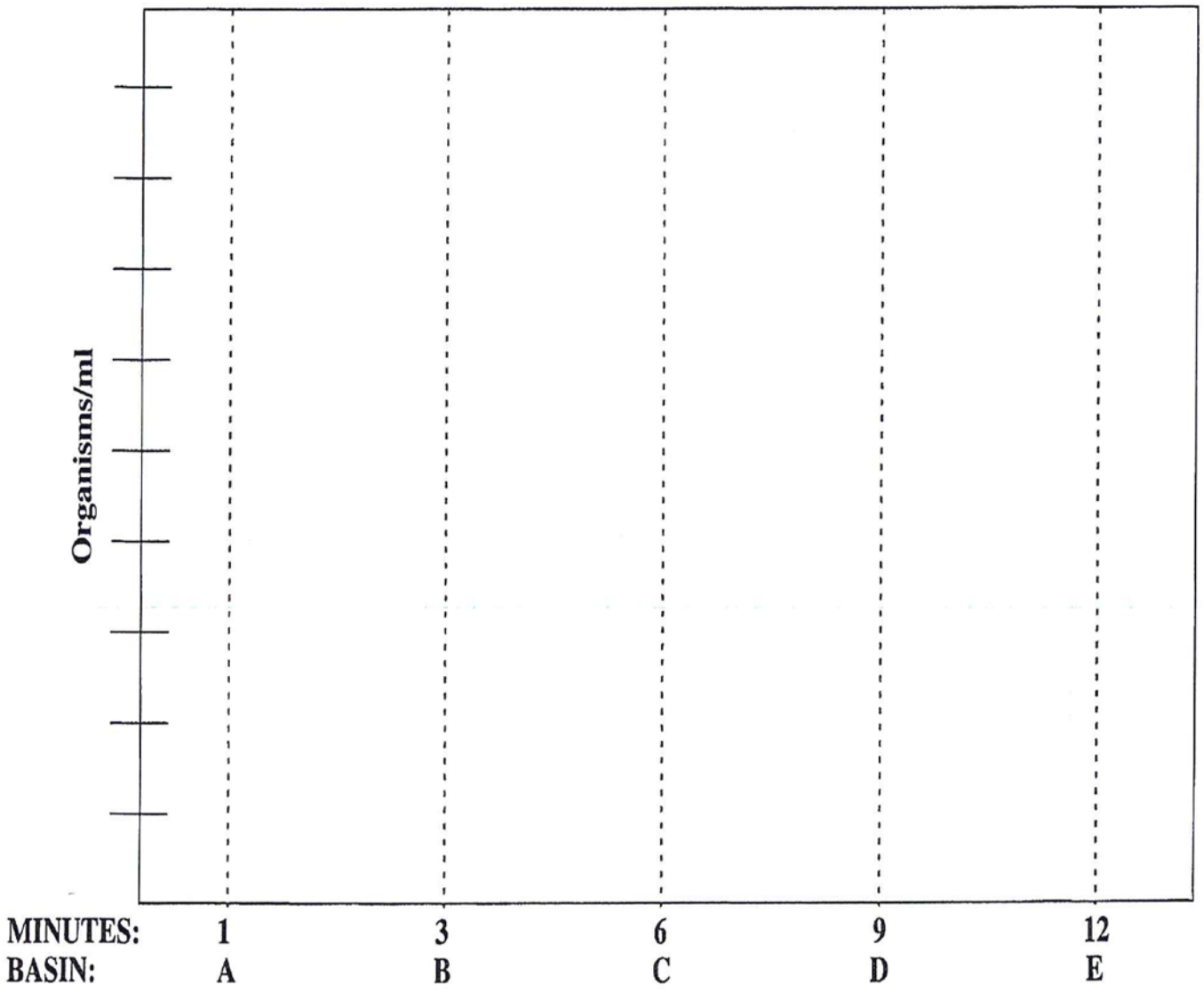
- Obtain your plates. Count one plate that has between 30 and 300 colonies. Record that data on the board.

Results and Questions

1. Record the data for each basin in the table. Calculate the concentration for each plate. Dilution factors are: 0.1 ml = 10, 0.2 ml = 5, and 0.4 ml = 2.5.

Basin	Count of plate used	Dilution factor of plate used	Concentration
A			
B			
C			
D			
E			

2. Graph the concentration vs the minutes for each basin.



3. What is your conclusion of the results of the graph?

Experiment 17 pH and Microbial Growth

Each organism has an optimum pH at which it grows the best. Microorganisms can be subdivided into groups based on their ability to grow at different pH levels.

Neutrophiles: grow at or near neutral pH. Most bacteria are neutrophiles. Although many can grow over a range of 2-3 pH units.

Acidophiles : grow at an acidic pH. An example of an acidophile is yeast, which prefers a pH of 4 to 6.

Alkaliphiles : grows at an alkaline pH. Many of these organisms belong to the genus *Bacillus*.

The bacteria that grow at the extremes of the pH scale maintain their cytoplasm at a neutral pH to help prevent damage. pH is utilized in food production of yogurt, pickles, and some cheeses to inhibit the growth of some microorganisms.

Organism

- Deck 1: *Alcaligenes faecalis*
- Deck 2: *Lactobacillus plantarum*
- Deck 3: *Staphylococcus aureus*

Procedure Day 1

- Obtain one of each pH broth (pH 2, 4, 7, 10, and 12). Label each tube with the pH.
- Inoculate each pH tube with your assigned organism. Note the color of the media, this will be important when you take your spectrophotometer readings.
- If you have *Lactobacillus plantarum* incubate it in the 30°C rack. The other two organisms can go into the 38°C rack.

Procedure Day 2

- Measure the % Transmittance for each pH tube with the spectrophotometer. Calculate the O.D. Put your data on the board.
- As you are taking the %Transmittance measurements, note the color and the pH of the blanks. Use the correct blank for each of the pH tubes.

Results and Questions

1. Place the data on the board in the table.

A. faecalis

pH	% Transmission	O.D.
2		
4		
7		
10		
12		

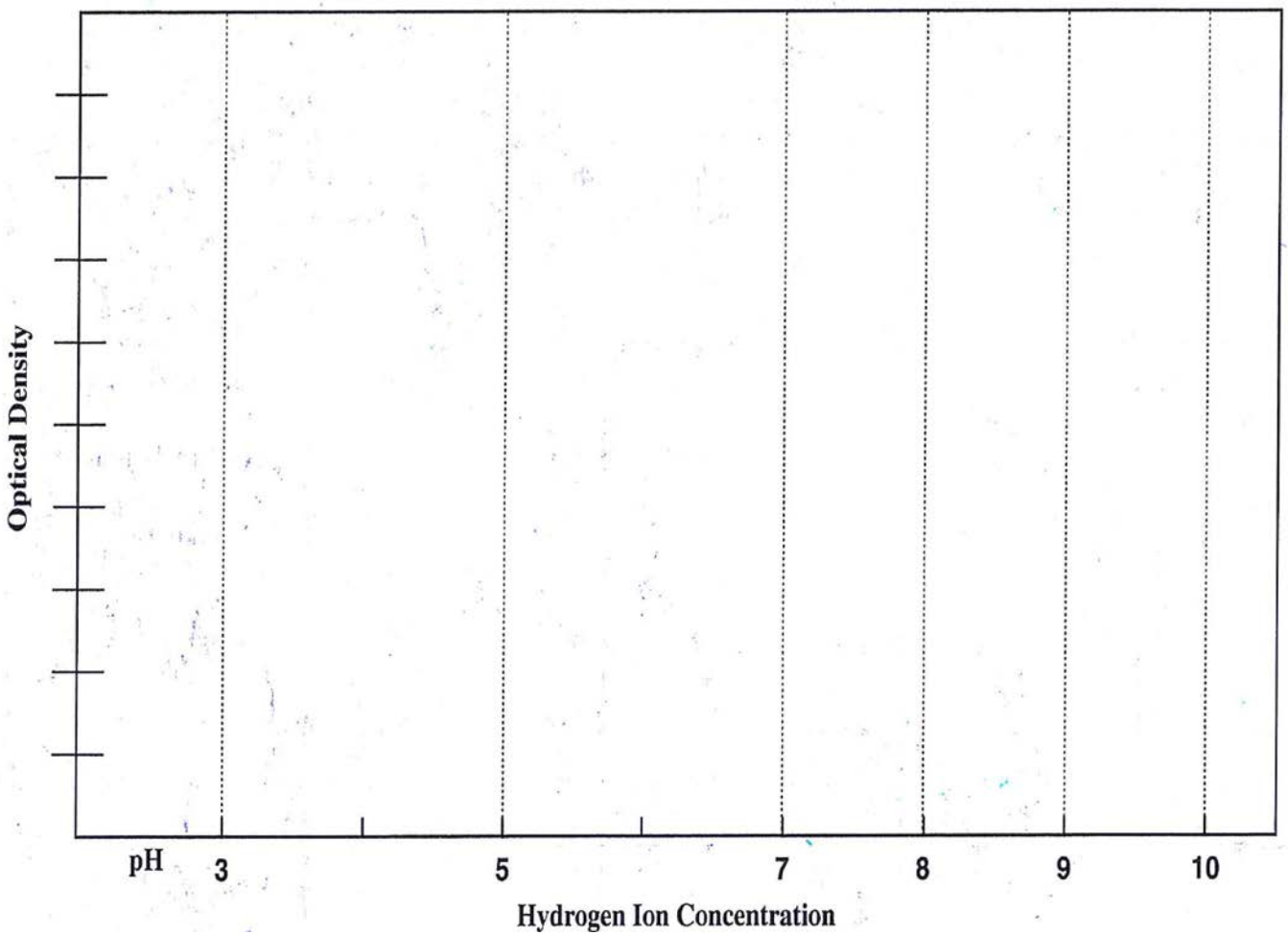
S. aureus

pH	% Transmission	O.D.
2		
4		
7		
10		
12		

L. plantarum

pH	% Transmission	O.D.
2		
4		
7		
10		
12		

2. Graph O.D. vs pH values. Use different colors or label each organism.



3. Which organism grows best at an acidic pH?

4. Which organism grows best at an alkaline pH?

5. Which organisms seem to tolerate a broad range in pH?

Experiment 18 Evaluation of Antiseptics

Utilization of many different chemical agents to kill or control bacteria is used every day by a variety of people and institutions. Chemical agents come in two different forms either antiseptics or disinfectants.

Antiseptics : substances that kill or inhibit microbial growth and are gentle enough to be applied to living tissue.

Disinfectants : Substances that are applied to inanimate objects to kill or reduce microorganisms. They are harsh and damaging to living tissue.

Sanitizers are substances that do not completely eliminate all bacteria but reduce them to a safe number. Those agents that inhibit the growth of bacteria only are called bacteriostatic. However those agents that kill bacteria are known as bactericidal.

Organism

- Deck 1 : *Enterococcus faecalis*
- Deck 2 : *Staphylococcus aureus*
- Deck 3 : *Pseudomonas aeruginosa*
- Mouth organisms

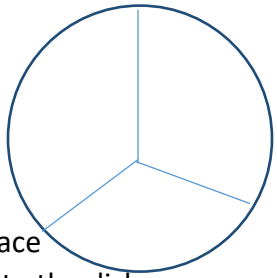
Procedure Day 1

- Each student will obtain a TSB tube and a sterile swab.
- Swab the inside of your mouth with the swab. You can wet the swab with some of the sterile TSB.
- Place the swab into the TSB, break off the end of the swab poking out of the tube, flame the opening and incubate.

Procedure Day 2

Mouth organism isolate:

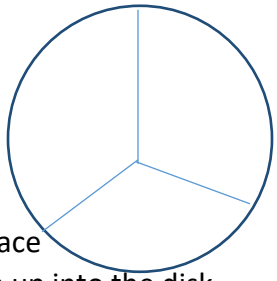
- For your mouth organism obtain a TSA plate and divide it into 3 different quadrants on the bottom of the plate. As shown:



- Label each quadrant with a different oral antiseptic:
- Scope, Listerine, and Cepacol
- Obtain a new sterile swab and streak for confluent growth.
- Obtain a dish with sterile disks.
- Using sterile forceps collect a sterile disk and touch it to the surface of the oral antiseptic. Allow the oral antiseptic to be drawn up into the disk.
- Place the disk in the center of its labeled section and gently tap to make it stick.
- Do this for each oral antiseptic.

Pairs: assigned organism

- For your assigned organism obtain a TSA plate and divide it into 3 different quadrants on the bottom of the plate. As shown:



- Label each quadrant with a different topical antiseptic:
- 70% Isopropanol, Bactine, and Povidone iodine
- Obtain a sterile swab and streak for confluent growth.
- Obtain a dish with sterile disks.
- Using sterile forceps collect a sterile disk and touch it to the surface of the topical antiseptic. Allow the topical antiseptic to be drawn up into the disk.
- Place the disk in the center of its labeled section and gently tap to make it stick.
- Do this for each topical antiseptic.

Results and Questions

1. Measure the zone of inhibition from the edge of the disk to the edge of the growth for each oral antiseptic or topical antiseptic. Record the data on the board and in the chart.

Mouth organisms

Oral antiseptic	**Zone of inhibition**
Scope	
Listerine	
Cepacol	

	Zone of inhibition	Zone of inhibition	Zone of inhibition
Topical antiseptic	*S. aureus*	*E. faecalis*	*Ps. aeruginosa*
70% isopropanol			
Bactine			
povidone iodine			

2. Which is the most effective antiseptic? For both the mouth organisms and the assigned organism

3. Which is the least effective antiseptic? For both the mouth organisms and the assigned organism.

4. Which is the most susceptible organism from the assigned organisms?

5. Which is the least susceptible organism from the assigned organisms?

Experiment 19 Antibiotic Sensitivity : Kirby-Bauer Method

Antimicrobials : are agents that kill or inhibit microorganisms.

Antibiotics : are usually low molecular weight antimicrobials that are produced by microorganisms that inhibit or kill other bacteria.

These are our first line of defense that have been used to battle diseases. Alexander Fleming's observation of a *Penicillium* species inhibiting growth of *Staphylococcus aureus* led to the purification of penicillin by Howard Florey, Norman Heatley, and Ernest Chain. This led to penicillin being the first antibiotic used in clinical treatment.

While we have a vast many compounds to treat bacterial infections there has been an increasing problem of antibiotic resistant microorganism strains. Antimicrobials vary in their effectiveness against bacteria. Some are narrow spectrum and more effective against Gram positive or Gram negative bacteria not both of them. Some are broad spectrum and are effective against both Gram negative and Gram positive bacteria.

Some antimicrobials are protein synthesis inhibitors, cell wall inhibitors, DNA or RNA synthesis inhibitors, or vitamin synthesis inhibitors. The Kirby-Bauer method is a standardized test that is used to determine the sensitivity or resistance of a bacteria to an antimicrobial agent. This test is performed with Mueller-Hinton agar plates and disks that have specific antibiotics and concentrations.

Once the disks have been placed on the plate a zone of inhibition around the disk may form and this is what is used to determine if a bacteria is resistant or sensitive to a certain antibiotic.

Organism

- *Staphylococcus aureus* (Deck 2)
- *Enterococcus faecalis* (Deck 1)
- *Escherichia coli* (Assigned by instructor)
- *Pseudomonas aeruginosa* (Deck 3)

Procedure

- Obtain a Mueller-Hinton plate. Obtain a sterile swab.
- Swab the whole plate for confluence with your assigned organism.
- After you have swabbed the plate place it on the tray either up front or at the window for either the technician or your instructor to place the antibiotic disks on the plate.

Second Lab Period Procedure

- Obtain your plates. Measure the zone of inhibition for each antibiotic disk on the plate. Place your data on the board. Your instructor will demonstrate how to read the zone of inhibition.

- Use the tables that follow to determine if the bacteria is sensitive, resistant, or intermediate to the antibiotic.

Table 31.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Amikacin	AN-30	30 µg			
Enterobacteriaceae					
P. aeruginosa, Acinetobactor					
staphylococci			≤14	15–16	≥17
Amoxicillin/Clavulinic acid	AmC-30	20/10 µg			
Enterobacteriaceae			≤13	14–17	≥18
Staphylococcus spp.			≤19	—	≥20
Haemophilus spp.			≤19	—	≥20
Ampicillin	AM-10	10 µg			
Enterobacteriaceae			≤13	14–16	≥17
Staphylococcus spp.			≤28	—	≥29
Enterococcus spp.			≤16	—	≥17
Listeria monocytogenes			≤19	—	≥20
Haemophilus spp.			≤18	19–21	≥22
β-hemolytic streptococci			—	—	≥24
Aziocillin	AZ-75	75 µg			
P. aeruginosa			≤17	—	≥18
Bacitracin	B-10	10 units	≤8	9–12	≥13

Table 31.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing (continued)

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Carbenicillin	CB-100	100 μg			
Enterobacteriaceae and *Acinetobacter*			≤19	20–22	≥22
P. aeruginosa			≤13	14–16	≥17
Cefaclor	CEC-30	30 μg			
Enterobacteriaceae and staphylococci			≤14	15–17	≥18
Haemophilus spp.			≤16	17–19	≥20
Cefazolin	CZ-30	30 μg			
Enterobacteriaceae and staphylococci			≤14	15–17	≥18
Cephalothin	CF-30	30 μg			
Enterobacteriaceae and staphylococci			≤14	15–17	≥18
Chloramphenicol	C-30	30 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobactor*, staphylococci, enterococci, and *V. cholerae*			≤12	13–17	≥18
Haemophilus spp.			≤25	26–28	≥29
S. pneumoniae			≤20	—	≥21
Streptococci			≤17	18–20	≥21
Ciprofloxacin	CIP-5	5 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, staphylococci, and enterococci			≤15	16–20	≥21
Haemophilus spp.			—	—	≥21
N. gonorrhoeae			≤27	28–40	≥41
Clarithromycin	CLR-15	15 μg			
Staphylococcus spp.			≤13	14–17	≥18
Haemophilus spp.			≤10	11–12	≥13
S. pneumoniae and other streptococci			≤16	17–20	≥21
Clindamycin	CC-2	2 μg			
Staphylococcus spp.			≤14	15–20	≥21
S. pneumoniae and other streptococci			≤15	16–18	≥19

Table 31.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing (continued)

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Doxycycline	D-30	30 μg			
Enterobacteriaceae, P. aeruginosa, Acinetobactor, staphylococci, and enterococci			≤12	13–15	≥16
Erythromycin	E-15	15 μg			
Staphylococcus spp. and enterococci			≤13	14–22	≥23
S. pneumoniae and other streptococci			≤15	16–20	≥21
Gentamicin	GM-120	120 μg			
Enterobacteriaceae, P. aeruginosa, Acinetobacter, and staphylococci			≤12	13–14	≥15
Imipenem	IPM-10	10 μg			
Enterobacteriaceae, P. aeruginosa, Acinetobacter, and staphylococci			≤13	14–15	≥16
Haemophilus spp.			—	—	≥16
Kanamycin	K-30	30 μm			
Enterobacteriaceae and staphylococci			≤13	13–17	≥18
Lomefloxacin	LOM-10	10 μg			
Enterobacteriaceae, P. aeruginosa, Acinetobacter, and staphylococci			≤18	19–21	≥22
Haemophilus spp.			—	—	≥22
N. gonorrhoeae			≤26	27–37	≥38
Loracarbef	LOR-30	30 μg			
Enterobacteriaceae and staphylococci			≤14	15–17	≥18
Haemophilus spp.			≤15	16–18	≥19
Meziocillin	MZ-75	75 μg			
Enterobacteriaceae and *Acinetobacter*			≤17	18–20	≥21
P. aeruginosa			≤15	—	≥16

Table 31.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing (continued)

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Minocycline	MI-30	30 μg			
Enterobacteriaceae,					
P. aeruginosa, Acinetobacter,					
staphylococci, and enterococci			≤14	15–18	≥19
Moxalactam	MOX-30	30 μg			
Enterobacteriaceae,					
P. aeruginosa, Acinetobacter,					
and staphylococci			≤14	15–22	≥23
Nafcillin	NF-1	1 μg			
Staphylococcus aureus			≤10	11–12	≥13
Nalidixic Acid	NA-30	30 μg			
Enterobacteriaceae			≤13	14–18	≥19
Neomycin	N-30	30 μg	≤12	13–16	≥17
Netilmicin	NET-30	30 μg			
Enterobacteriaceae,					
P. aeruginosa, Acinetobacter,					
and staphylococci			≤12	13–14	≥15
Norfloxacin	NCR-10	10 μg			
Enterobacteriaceae,					
P. aeruginosa, Acinetobacter,					
staphylococci, and entercocci			≤12	13–16	≥17
Novobiocin	NB-30	30 μg	≤17	18–21	≥22
Oxacillin	OX-1	1 μg			
Staphylococcus aureus			≤10	11–12	≥13
staphylococcus (coagulase negative)			≤17	—	≥18
Penicillin	P-10	10 units			
Staphylococcus spp.			≤28	—	≥29
Enterococcus spp.			≤14	—	≥15
L. monocytogenes			≤19	20–27	≥28
N. gonorrhoeae			≤26	27–46	≥47
β-hemolytic streptococci			—	—	≥24
Piperacillin	PIP-100	100 μg			
Enterobacteriaceae, and					
Acinetobacter			≤17	18–20	≥21
P. aeruginosa			≤17	—	≥18

Table 31.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing (continued)

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Polymyxin B	PB-300	300 U	≤8	9–11	≥12
Rifampin	RA-5	5 μg			
Staphylococcus spp.					
Enterococcus spp. and *Haemophilis* spp.			≤16	17–19	≥20
S. pneumoniae			≤16	17–18	≥19
Spectinomycin	SPT-100	100 μg			
N. gonorrhoeae			≤14	15–17	≥18
Streptomycin	S-300	300 μg			
Enterobacteriaceae			≤11	12–14	≥15
Sulfisoxazole	G-25	250 μg			
Enterobacteriaceae,					
P. aeruginosa, Acinetobacter,					
V. cholerae, and staphylococci			≤12	13–16	≥17
Tetracycline	Te-30	30 μm			
Enterobacteriaceae,					
P. aeruginosa, Acinetobacter,					
V. cholerae, staphylococci, and					
enterococci			≤14	15–18	≥19
Haemophilus spp.			≤25	26–28	≥29
N. gonorrhoeae			≤30	31–37	≥38
S. pneumoniae and other streptococci			≤18	19–22	≥23
Tobramycin	NN-10	10 μg			
Enterobacteriaceae,					
P. aeruginosa, Acinetobacter,					
and staphylococci			≤12	13–14	≥15
Trimethoprim	TMP-5	5 μg			
Enterobacteriaceae and					
staphylococci			≤10	11–15	≥16
Vancomycin	Va-30	30 μg			
Staphylococcus spp.			—	—	≥15
Enterococcus spp.			≤14	15–16	≥17
S. pneumoniae and other streptococci			—	—	≥17

Results and Questions

1. Record the results in this table for each organism.

S. aureus

Antibiotic	Zone of inhibition	Rating (R,I,S)
Penicillin		
Erythromycin		
Ampicillin		
Chloramphenicol		
Amikacin		
Tetracycline		
Lomefloxacin		
Kanamycin		
Vancomycin		
Gentamicin		
Rifampin		
Clindamycin		

E. faecalis

Antibiotic	Zone of inhibition	Rating (R,I,S)
Penicillin		
Erythromycin		
Ampicillin		
Chloramphenicol		
Amikacin		
Tetracycline		
Lomefloxacin		
Kanamycin		
Vancomycin		
Gentamicin		
Rifampin		
Clindamycin		

E. coli

Antibiotic	Zone of inhibition	Rating (R,I,S)
Chloramphenicol		
Meziocillin		
Nalidixic acid		
Ampicillin		
Streptomycin		
Amikacin		
Tetracycline		
Kanamycin		
Gentamicin		
Carbenicillin		
Lomefloxacin		
Piperacillin		

Ps. Aeruginosa

Antibiotic	Zone of inhibition	Rating (R,I,S)
Chloramphenicol		
Meziocillin		
Nalidixic acid		
Ampicillin		
Streptomycin		
Amikacin		
Tetracycline		
Kanamycin		
Gentamicin		
Carbenicillin		
Lomefloxacin		
Piperacillin		

2. Which antibiotics would be suitable to be used to control each organism?

Experiment 20 Morphological Study of An Unknown Bacteria

You will be assigned an unknown bacteria that you will identify with the help of the flow charts provided in class, this manual, and Bergey's Manual. You will also write a paper that goes along with the unknown.

- The first day you will obtain a lab journal and a descriptive chart. You are expected to keep a record of your investigative results in a laboratory journal. The journal typically requires you to make colored sketches and written analyses describing the outcome of each test you do, whether macroscopic or microscopic. Only one journal will be made available to you and any written notes will be made with pen. This is to prevent the natural instinct to edit. When a test must be repeated or errors corrected, they are added to the already noted information and differentiated by date and/or by crossing out the information that is being corrected.
- As you accumulate test results, you will utilize the flow charts provided in class and in your lab manual to identify the classification group and then the genus of your unknown bacteria. The species of the organism will require careful study of Bergey's Manual.
- Your instructor will check your progress periodically by asking you to submit a summary of your test results on an official Descriptive Chart. On the back of each Descriptive Chart, you should include a preliminary flow chart. The flow chart should begin with the number of your unknown.
- Your final report will be submitted as a formal written lab report. Refer to the "Unknown Written Report" below for specific instructions and suggestions.
- You will sign up for an unknown broth at the front of the class. This unknown broth will be returned to the front with your name on it after you are done with the first days Gram stain and inoculations.
- It will be your job to keep your culture alive and uncontaminated for the duration of this exercise. Be sure to put your unknown number, as well as standard labeling information, on all tubes and plates you inoculate.
- Every day you will do a Gram stain to check for purity of your culture. Your instructor will verify your Gram stain this first day and give you a Gram stain slip to fill out to be graded.
- Once your instructor has confirmed purity you can begin inoculating the media for the day.
- Unknown stock cultures:
    - Your reserve stock - This is your back-up. After you have inoculated a reserve slant, incubate it 24-48 hours to achieve good growth. It will then be stored in the refrigerator up to 10 days to keep it viable. You will use your reserve stock to make inoculations only if your working stock is contaminated; Gram stain your reserve stock to ensure that it is pure before using.

        - If your reserve stock is also contaminated you will need to do a streak for isolation on a selective agar plate. You can arrange to come in to a different lab period in order to avoid falling too far behind. From a portion of an isolated colony, Gram stain it to determine if it is your organism. Then use material from the same colony to streak new working and reserve stocks.
        - If your reserve stock is pure, you can inoculate and incubate a new reserve and a new working stock from the existing reserve stock.
    - Your working stock - Every lab period, right at the beginning, before you do any other inoculations, you perform a Gram stain to check for purity of the working stock you inoculated and incubated at the last lab period.
        - If you detect contamination, discard that working stock and turn to your reserve stock as described above
- Every day you will do a Gram stain of your new working slant you made the day before to check for purity. If it is still pure then you can do inoculations after the instructor has introduced the lab.
- Every day after you have checked for purity you will then make a new working TSA slant. This insures that you have a fresh culture to work with for all inoculations. It is important to practice aseptic techniques during all inoculations.
- Pay attention to what is placed on the board for what you are doing that day as the following experiments are listed by morphological characteristics of the unknown, cultural characteristics of the unknown, and physiological characteristics of the unknown.
- **Never** begin inoculations or data collection before your instructor has given an introduction to the material.
- All controls will be inoculated by the technician and will be available to compare your results to on the day you are reading the test. Make sure to write and draw each control in your lab journal.

UNKNOWN WRITTEN REPORT

Each student must write his or her own report in their own words. Reports must be typed and in scientific format. Proofread &/or spell check for correct grammar and spelling. Always use scientific notation with the names of organisms. Clearly title each section as illustrated below.

I. Purpose

In this section express what you expect to gain from this experience and why this exercise is important for you to have performed. It does not have to be long but needs to express the importance of the exercise for microbiology students and how it may impact specifically to your field of interest.

II. Materials and Methods

The purpose of this area is to provide all the necessary information that may be necessary for someone to repeat your study. In the science field a study must be reproducible in order to be valid. Though this exercise is not true research this report gives students the opportunity to become acquainted with the practice for making known the details of your work. In order to expedite the report however we are going to ask you to separate this section into two parts, **A. Materials** and **B. Methods**. This is not normally done in a scientific article but it allows students to begin to "see" the steps they took in determining the identity of their unknown organism. In the materials section, make a **list** of the materials by category. For example:

Media:	Equipment:
TSA	microscope
TSB	spectrophotometer
Blood agar	loops & needles
Simmon's citrate agar	

Include lists of

1. cultures
2. stains
3. media
4. chemical reagents
5. equipment.

The **methods** section is written in complete sentences and describes the procedure used in each test. The procedures need to be detailed enough for someone to repeat the tests as you performed them. In science, repeatability is essential to validate a study. The test procedures can be referenced if performed exactly as described in the reference; if changes were provided in class or a different procedure was followed, then you must outline the procedure used. Include the interpretations and the controls. DO NOT ADDRESS THE RESULTS OF YOUR TESTS. Describe methods in subsections, not by exercise number. Include sections on

1. maintaining viable and uncontaminated stocks
2. determining morphological attributes
3. determining cultural traits

4. determining physiological characteristics; include purpose of test, any

end product(s); reagents utilized, interpretations and controls

Example of a method:

Simmon's citrate agar is utilized to determine the ability of an organism to use citrate as a sole carbon source. The procedure is detailed in Brown, Microbiological Applications p. 241. The pH indicator bromphenol blue and ammonium salts are incorporated into the media. If the organism cannot use citrate as a carbon source it will not grow. If the organism can use the citrate, it will grow and ammonia will be released because the organism is using the ammonium salts as a source of nitrogen causing the media to turn alkaline. The media will change to the color change to blue. The positive control in this test is *Enterobacter aerogenes.*

Standard procedures such as sterile technique do not need to be explained in detail; to indicate that sterile technique was applied to all procedures is adequate.

Likewise for incubations temperatures; state that all incubations were at a certain temperature unless otherwise indicated.

Since the information for the materials and methods sections is acquired as you work through the unknown exercise, it can be written as you proceed through the exercise. Hence to avoid a "last minute writing frenzy" **and** insure a **better** score on the report, it is recommended that you begin to write this section from the onset of the unknown exercise.

III. Results

This section consists of your journal, a complete descriptive chart and the flow chart. The descriptive chart and the flow chart should identify the name of your unknown. The flow chart should start with the number of your unknown and proceed through to the group, the genus and then to the species. The grader should be able to follow the process of the elimination of options and reasons for the choices.

IV. Conclusion and Discussion

It is in this section that you make reference to your purpose; enlarge on that purpose. Did you accomplish it?

Expand on the flow chart by discussing your approach to identifying your unknown bacterium; why you took the various pathways that led to its identification. If you found that you needed a special test to differentiate species or could have used a special procedure to assist you, this is where you can address these types of issues.

It is in this section that you can elaborate on the experience, express your ideas and concerns about the exercise. What kinds of problems did you run into and how did you resolve them?

Include a discussion on the background of your organism. What is its habitat? What kinds of problems, if any, can this organism cause?

Common questions:

1. What if I'm already pretty sure what I have? Do I need to keep testing?

Some students have a pretty good guess as to what their organism is right at the start, but unless that guess is backed up by the test results, it isn't worth anything. Complete all the tests.

2. What do I do if my unknown bacteria dies?

You should always have at least two stocks (reserve and working), plus possibly test inoculations so this is extremely unlikely.

3. What do I do if my culture gets contaminated?

You should be gram-staining your working stock at the beginning of each class to avoid using a contaminated culture, because you don't want to get erroneous result. If your working stock does get contaminated, hopefully your reserve stock will provide a pure culture.

4. What if my reserve stock is contaminated, too?

You need to delay further tests (and possibly plan to repeat any questionable tests already performed) until you get a pure culture to work with. Usually, this entails doing a streak for isolation and subculturing new reserve and working stocks.

5. What do I do if I'm absent?

You'll need to arrange to come in at another time to get caught up. Only you can carry out the test on your unknown.

6. What if I misinterpret a test result?

If you keep a good laboratory journal, you'll have a clear record of actual results. This will allow you to re-check any questionable interpretation.

7. What if I mis-identify my unknown bacteria, after all that work?

Don't worry. You are being evaluated on the process much more than on the outcome. If your identification is logical, based on your test results, you will not be penalized.

PLEASE LET YOUR INSTRUCTOR OR THE TECHNICIAN KNOW ABOUT ANY DIFFICULTIES YOU MAY HAVE DURING THE UNKNOWN INVESTSIGATION, DON'T STRUGGLE ALONE, WE'RE HERE TO HELP YOU!.

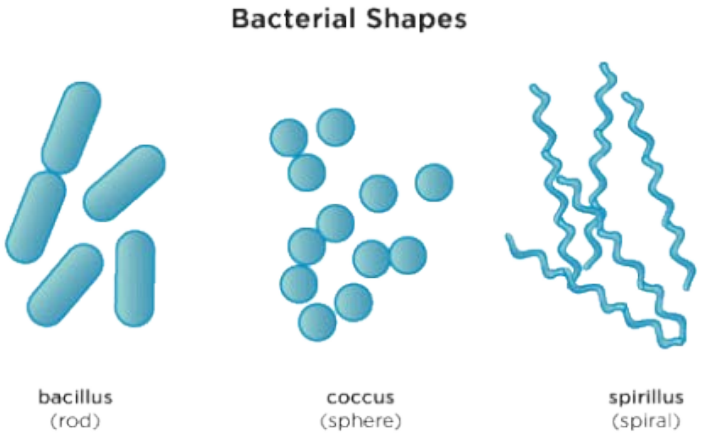
Experiment 21 Morphological Characteristics of An Unknown Bacteria

Cell shape, arrangement, size, motility, and staining fall under morphological characteristics.

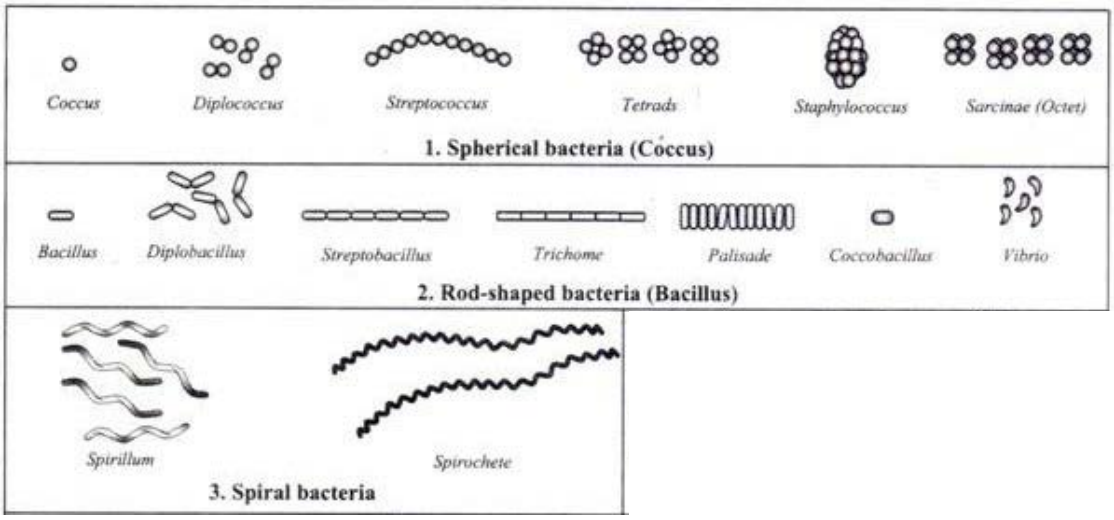
Cell shape, and arrangement can be determined from the Gram stain done on your organism. However if you are unsure you can always do a negative stain to confirm your Gram stain results.

Cell shape:

- Rod (bacillus)
- Spherical (cocci)
- Spiral or curved



Cell arrangement:



Cell size:

- Do a mixed Gram stain with your organism and an organism of known size.
- Here are the known organism you will use.
    - *Staphylococcus aureus* Gram positive cocci
        - 0.5µm – 1.5µm
    - *Bacillus cereus* Gram positive rod
        - 1-1.2µm (wide) x 3-5µm (long)
    - *Escherichia coli* Gram negative rod
        - 1-1.5µm (wide) x 2-6µm (long)

Motility:

- Obtain a motility agar tube. Stab the agar with your unknown. Incubate at 25°C.
- If your organism reduced the 2,3,5-triphenyltetrazolium chloride and is motile you should see a red color an movement away from the stab line. This might look like hazy areas with red color in the tube.
- If your organism did not reduce the 2,3,5-triphenyltetrazolium chloride and is motile you should see a haziness or cloudiness to the media.
- If your organism reduced the 2,3,5-triphenyltetrazolium chloride and is non-motile you should see a red color at the stab line and nowhere else.
- If you are unsure of your results after comparing your tube to the controls up front, do a wet mount.

Staining:

- All students will do a capsule stain.
- If your organism is a Gram positive you will need to do a spore stain. However you will need to have an older culture for spores to appear. This is achieved by keeping one of your working TSA slants and incubating it for at least 5 days.
- If your organism is a Gram positive and a non-spore forming rod you will need to do an acid fast stain.

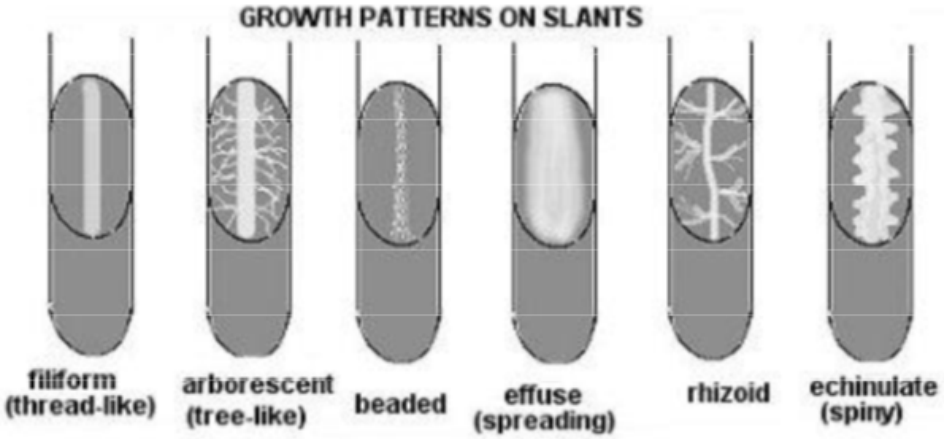
Record all results in your lab journal and in your descriptive chart. You can find a descriptive chart at the end of this lab manual.

Experiment 22 Cultural Characteristics of An Unknown Bacteria

Pattern of growth, colony morphology, optimum temperature, oxygen requirement, and hemolysis are the items we will be looking at in this section.

Tryptic soy agar slant:

- The first day you will inoculate two of these. One for your reserve and the other for your new working for the following day. One of these you will inoculate with a straight line streak to read pattern of growth, color, and opacity.
- **Pattern of growth:**
    - Filiform – uniform growth along the line of inoculation
    - Echinulate – toothed appearance along margin of growth
    - Beaded – separate colonies along the line of inoculation
    - Effuse – thin growth, unusually spreading
    - Arborescent – branched growth
    - Rhizoid – root like growth

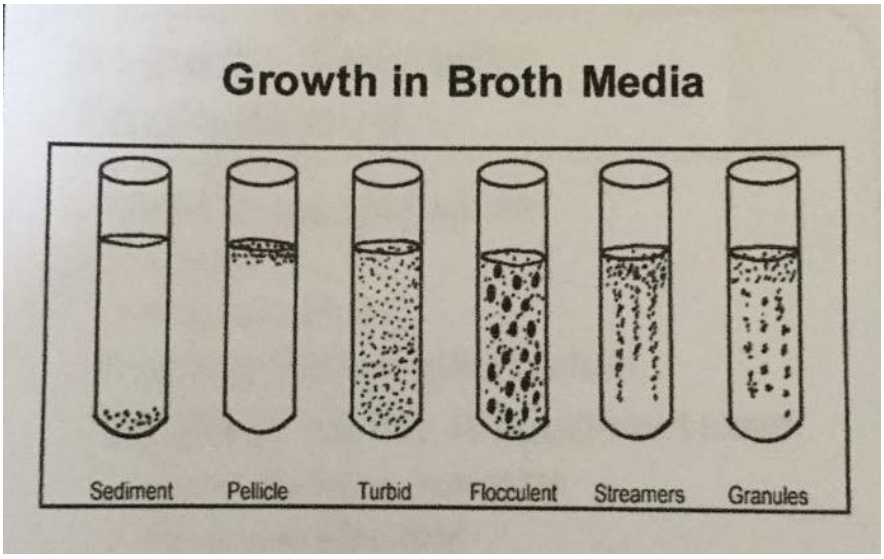


- **Opacity:**
    - Opaque
    - Transparent
    - Translucent – partially transparent

Tryptic soy broth

- The first day you will inoculate three of these. One will be placed at 25°C, one at 30°C, and the other at 38°C. You will be reading for pattern of growth and obtaining % Transmission readings with the spectrophotometer to determine the optimum temperature of your organism and where to place all your inoculated media at.

- **Pattern of growth:**
    - Pellicle – thick growth at top of tube
    - Ring – ring of growth at top of tube
    - Flocculent – small particles floating around in turbid media
    - Membranous – thin growth at the top of the tube
    - Granular – small floating mases in the media
    - Flaky – large particles floating in the tube
    - Turbid – all of the media has a cloudy appearance



- **Sediment: found in the bottom of the tube**
    - Flaky – large chunks
    - Flocculent – small chunks
    - Granular – sand like
    - Viscid – thick mass that is not easily broken up by mixing
    - None
- Abundance of growth for each temperature tube should be noted as well. Make note of which tube had the most growth and which tube had the least.
- **Temperature requirements:**
    - After you have read for pattern of growth. Mix each tube up and move on to the spectrophotometer.
    - Transfer contents to a **cuvette**, make sure to follow the directions from experiment 14 on using the spectrophotometer.
    - Obtain your % Transmission readings and calculate optical density. Determine your optimum temperature.
    - This is the temperature that you will place all your inoculations at in the future.

Sodium thioglycollate medium

- This media will tell you the oxygen requirement of your organism. The oxygen indicator in this media is resazurin. Resazurin is red/pink when oxygen is present and colorless in the absence of oxygen. This media provides both an aerobic condition and an anaerobic condition.
- Do not shake this media when you obtain it as this will disperse the oxygen in the tube. Inoculate this media by stabbing it with your needle.
- After incubation compare your organism to the controls provided.

Tryptic soy agar plate

- For this media you will be reading two different tests. One is pattern of growth and the other is catalase which will be discussed in the physiological characteristics experiment. Save this plate after you have read pattern of growth.
- Do a streak for isolation on the plate. You want to be able to see the isolated colonies. Use the figure on the next page to describe your colonies.
- **Pattern of growth**
    - Color, pigment production, color of media if it changed
    - Size of colonies
    - Opacity
    - Margins of the colonies
    - Elevation of the colonies

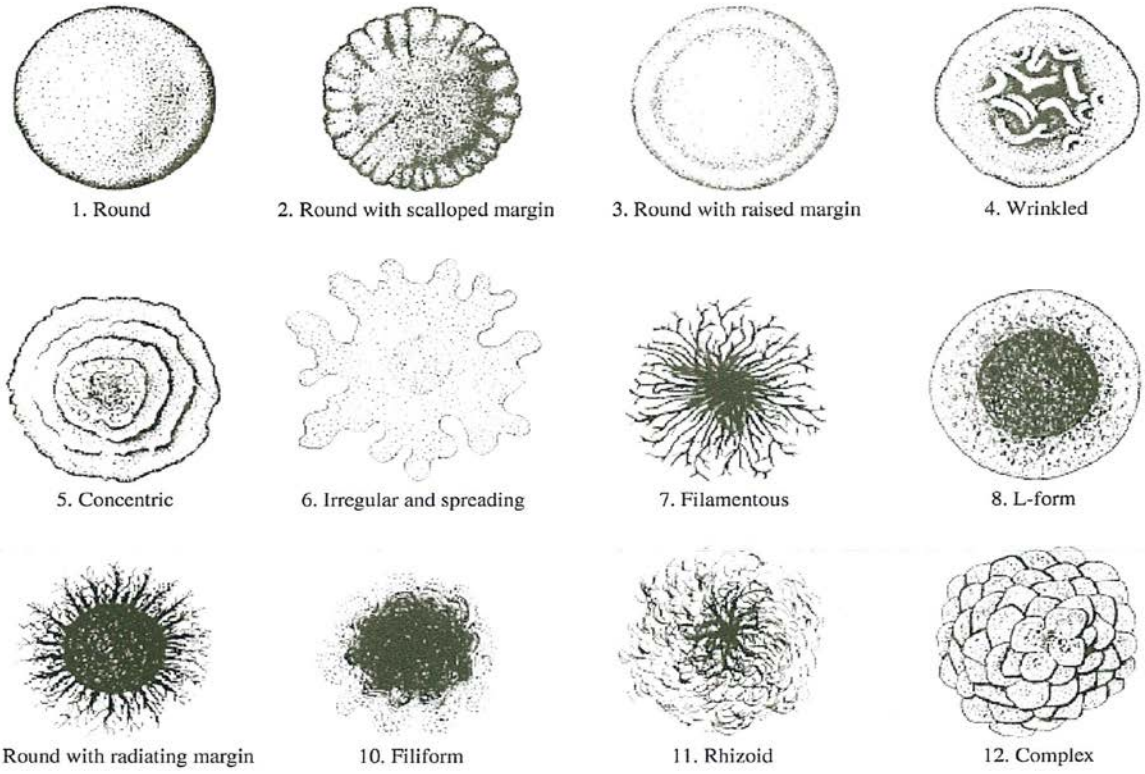
Blood agar plate

- For this media you will be reading pattern of growth and hemolysis. Hemolysis is the lysis of red blood cells. There are three different types of hemolysis.
    - Alpha-hemolysis – this is the partial break down of the red blood cells. This produces a greenish discoloration around the colonies.
    - Beta-hemolysis – this is the complete breakdown of the red blood cells. This produces a clearing around the colonies.
    - Gamma-hemolysis – this is no lysis of the red blood cells. There should be no change in the media around the colonies.

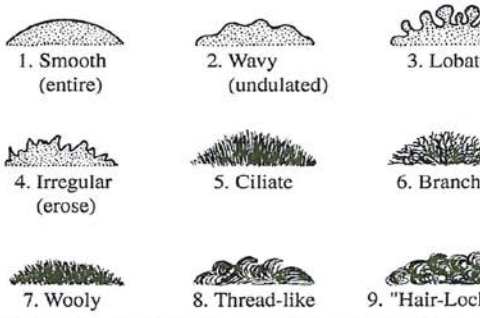
Procedure

- Do a streak for isolation on the plate. After incubation read for hemolysis and compare with the controls. Read pattern of growth like the TSA plate above.

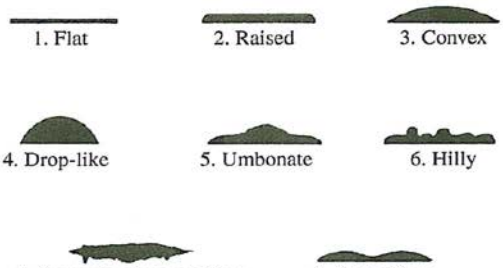
CONFIGURATIONS



MARGINS



ELEVATIONS



Experiment 23 Physiological Characteristics of An Unknown Bacteria

For this part of the experiment you will look at a variety of differential tests that will help you determine your unknown.

Catalase Test

- Catalase is a test to determine if the organism produces catalase enzyme. This enzyme degrades hydrogen peroxide to oxygen and water.
- $2H_2O_2$ + catalase $\longrightarrow$ $2H_2O + O_2$ (g)
- Bacteria that produce catalase can be detected by using hydrogen peroxide. If the organism is catalase positive gas bubbles will appear. If the organism is catalase negative no gas bubbles will appear.
- This test will be done on your TSA plate after you have read for pattern of growth.

Procedure

- Add a drop of hydrogen peroxide to the growth on your TSA plate. Record your results.
- Compare your organism to the control. Record if yours is negative or positive.

Methyl Red Test

- This test identifies those bacteria that produce formic dehydrogenylase causing acid end products from the mixed acid fermentation of glucose. The media used is called MR-VP broth. This media has peptone, glucose and a phosphate buffer that helps regulate pH changes.
- With the addition of a pH indicator methyl red after incubation we can detect the mixed acid production with a color change in the media.
- A positive result is indicated by a red/pink color change after the addition of methyl red. A negative result is indicated by either an orange or yellow color after addition of methyl red.

Procedure

- Inoculate an MR-VP broth with your organism. Incubate at your optimum temperature for five days. After incubation add 5 drops of methyl red. Mix and compare your results with the positive and negative controls. Record if yours is negative or positive.

Voges-Proskauer Test

- This test identifies those organisms that carry out fermentation of glucose to acetoin and 2,3-Butanediol. The media used is MR-VP broth, the same as the methyl red test above.
- After incubation acetoin is detected by the addition of VP reagent A (α-Naphthol) and VP reagent B (Potassium hydroxide). These reagents are collectively known as Barritt's reagents. These reagents oxidize acetoin to diacetyl which reacts with the guanidine in the media to produce a red color. This may take up to 15 minutes.

- A positive result is indicated by a brick red color after the addition of VP A and VP B. A negative result is indicated by a copper color after addition of VP A and VP B

Procedure

- Inoculate an MR-VP broth with your organism. Incubate at your optimum temperature. After incubation add 10 drops of VP A (α-Naphthol) and 8 drops of VP B (Potassium hydroxide) to the tube. Shake well to aerate the tube. Let sit for 15 minutes before reading the results.
- Compare your tube with the positive and negative controls. Record your if your organism is negative or positive.

Fermentation Test

- This test identifies those bacteria that carry out carbohydrate fermentation. The media used is a phenol red broth with the addition of a specific carbohydrate. We test a total of six different carbohydrates. They are glucose, lactose, sucrose, mannitol, trehalose, and fructose. In the phenol red broth is a peptone and the pH indicator phenol red.
- Phenol red indicator is yellow at a low pH, indicating that there was acid production from carbohydrate fermentation. Phenol red indicator is pink at high pH, indicating deamination of the peptone in the media to produce ammonia. Phenol red broth is red at neutral or close to neutral pH.
- These tubes need to be read at 24 hours and again at 48 hours. This is because some organisms are fast fermenters and will go after the peptone in the broth after they have fermented the carbohydrates.
- A positive test would be indicated by the color change to yellow, due to carbohydrate fermentation and acid production.
- A negative test would be indicated by no color change after the 48 hour reading. Another negative result would be indicated by a pink color change, due to deamination and the production of ammonia.
- Also being tested is gas production. Gas production is indicated by a bubble or displacement of the media in the Durham tube inside the larger tube holding the media. This would be a positive result for gas production.
- We utilize A or (+) for acid, Alk or (-) for alkaline or pink tubes, NC or (-) for no change in media color, and G for gas production.

Procedure

- Inoculate the six fermentation broths with your organism. Since these broths are all the same color it is recommended that you take a marker with you and label each tube as you obtain them. Incubate these tubes in the rack labeled for fermentation at your optimum temperature as you will have to read them at 24 hours and 48 hours.
- At the 24 hour reading note color change and gas production. Place back into the rack for reincubation.

- At the 48 hour mark which is usually when you next meet for class note any color changes and gas production.
- Compare your results to the positive control and the negative controls.

Oxidative-Fermentative Test

- This test identifies the ability of bacteria to oxidize or ferment a specific carbohydrate. The media used is O-F medium with the addition of a carbohydrate. In this test glucose is the carbohydrate.
- There is a pH indicator in the media that helps to detect fermentation of the carbohydrate. The pH indicator is bromthymol blue. This indicator is yellow at an acidic pH, green at a neutral pH, and blue at a basic pH.
- After inoculation one tube is overlaid with oil to create and anaerobic environment. The other tube is not overlaid with oil to allow for an aerobic environment. These tubes are also read in 24 hours and 48 hours.
- Both tubes will need to be read together. The results are as follows. If both tubes are yellow then the organism is fermentative. If the tube with no oil is yellow and the tube with oil is green then the organism is oxidative. If both tubes are green then the organism is neither a fermenter or an oxidizer.

Procedure

- Inoculate two tubes of O-F glucose media with your organism. Overlay one tube with oil **Do Not forget this step otherwise you will have to redo the test**. Incubate these tubes in a rack labeled fermentation at your optimum temperature.
- At your 24 hour reading note color change of both tubes. Place back in the rack for reincubation.
- At your 48 hour reading during your class period note color change of both tubes. Compare your tubes to the Fermentative control and the oxidative control.

Simmon's Citrate Test

- This test identifies the ability of bacteria to utilize citrate as a sole carbon source. Citrate media contains sodium citrate as the carbon source and ammonium phosphate as the nitrogen source.
- Organisms that use citrate possess citrate-permease that transports citrate into the cell to be utilized. These organisms also convert the ammonium phosphate to ammonia and ammonium hydroxide which alkalinizes the agar.
- The pH indicator in the citrate slant is bromthymol blue. Bromthymol blue is green at near neutral pH, yellow at acidic or low pH values, and blue at alkaline or higher pH values.
- A positive result is indicated by the media turning from green to blue.

Procedure

- Inoculate the citrate slant with your organism. Incubate at your optimum temperature. After incubation note your results and compare them to the positive control.

Nitrate Test

- This test identifies the ability of certain bacteria to either reduce nitrate to nitrite or nitrogen gas. The reduction of nitrate to nitrite is done by the enzyme nitrate reductase.
- To detect the presence of nitrite from the reduction of nitrate after incubation two reagents are added. These reagents are Reagent A (dimethyl-α-naphthalamine) and Reagent B (sulfanilic acid).
- If nitrite is present a dark red color will appear. This is indicated by a positive result as the nitrate in the media was reduced by the bacterial nitrate reductase to produce nitrite.
- If there is no color change after the addition of Reagent A and Reagent B then this is not definitive. Either the nitrate is still present in the tube and did not get reduced or the reduction of nitrate went all the way to nitrogen gas.
- To determine if nitrate or nitrogen gas is present in a tube with no color (after the addition of A + B) a small amount of Reagent C (Zinc) is added to the tube. Zinc will reduce the nitrate to nitrite giving a dark red color. This is indicated by a negative result as the zinc did the reduction not the bacteria. If after the addition of zinc there is still no color change this is a positive result as the bacterial nitrate reductase reduced the nitrate to nitrogen gas.

Procedure

- Inoculate the nitrate broth with your organism. Incubate at your optimum temperature. After incubation add 5 drops of Reagent A and 5 drops of Reagent B to the tube. Mix will note any color change. If your tube turned red you are done and this is a positive result.
- If there was no color change add a small amount of Reagent C. Mix well note any color change. If your tube turned red you are done and this is a negative result. However, if there was no color change you are done and this is a positive result.
- Compare your result to the controls. The positive control is after the addition of Reagent A and Reagent B. The negative control is after the addition of Reagent A, Reagent B, and Reagent C.

Starch Agar Test

- This test identifies the ability of certain bacteria to hydrolyze starch by the production of amylases. Hydrolysis of starch is detected by the addition of iodine to the media after incubation.
- Iodine complexes with the starch to cause the media to turn blue or dark brown wherever starch is present. A positive result for starch hydrolysis is a clearing or halo around the margins of growth after the addition of iodine.

Procedure

- Inoculate the starch plate with a heavy streak in the center of the plate with your organism. Incubate at your optimum temperature for 5 days.
- After incubation flood the plate with Gram's iodine and read the results right away. Be careful not to tip the plate or the liquid will pour out.
- Compare your results with the positive control.

Skim Milk Agar Test

- This test identifies the ability of bacteria to hydrolyze casein from milk with the enzyme casease. Casein is what gives milk its characteristic white color. When casein is broken down it loses its white color and becomes clear.
- A positive result would be a clearing or halo around the margins of growth.

Procedure

- Inoculate the skim milk plate with a streak in the center with your organism. Incubate at your optimum temperature for 5 days.
- After incubation check your plate for a clearing around the margins of growth. Compare your plate to the positive control. Sometimes it is easier to see if you place the plate against a dark background or a page with words on it.

Spirit Blue Agar Test

- This test identifies those bacteria that can cleave fatty acids from glycerol. They do this with the help of enzymes called lipases. Spirit blue agar contains tributyrin oil and spirit blue dye as a pH indicator.
- Those organisms that are positive will hydrolysis the oil and lower the pH resulting in a dark blue precipitate. A lightening of the plate is not a positive result.

Procedure

- Inoculate the spirit blue plate with a streak in the center with your organism. Incubate at your optimum temperature.
- After incubation compare your plate with that of the positive control. You are looking for a dark blue precipitate.

Urea Test

- This test identifies the bacteria that can hydrolyze urea into carbon dioxide and ammonia. The enzyme that is utilized to break down urea is urease.
- The pH indicator in urea broth is phenol red. When urea is hydrolyzed the pH increases due to the ammonia that is now in the tube. As a result of the ammonia the pH indicator will turn a pink color, this is a positive result.

Procedure

- Inoculate the urea broth with your organism. Incubate at your optimum temperature for 5 days.
- After incubation compare your results to the control and the uninoculated control. If your urea broth is positive it should be pink.

Phenylalanine Agar Test

- This test identifies those bacteria that deaminate phenylalanine into phenylpyruvic acid and ammonia. The enzyme that helps with this is phenylalanine deaminase.
- After incubation 10% ferric chloride is used to detect the phenylpyruvic acid. A positive result is a green color after the addition of the ferric chloride to the slant.
- Phenylalanine + phenylalanine deaminase $\longrightarrow$ phenylpyruvic acid + ammonia.

Procedure

- Inoculate the phenylalanine slant (this slant looks a lot like a TSA slant) with your organism. Incubate it at your optimum temperature.
- After incubation add four drops of 10% ferric chloride to the slant. Compare your results to the positive control. A green color should appear if your tube is positive. If it stays yellow then it is negative.

DNase Agar Test

- This test identifies the bacteria that have the ability to hydrolyze DNA into small fragments. The enzyme that hydrolyzes DNA is DNase.
- The agar we use to test this is DNase agar with methyl green. The methyl green is tied to the DNA and is released when DNA is hydrolyzed. If the bacteria produces DNase to break down the DNA in the media there is a clearing around the margins of growth. This clearing is a positive result.

Procedure

- Inoculate the DNase plate with your organism. Incubate at your optimum temperature.
- After incubation compare your plate to the positive control. You should notice a clearing around the margins of growth if your organism is positive for DNase production.

Decarboxylase Test

- This test identifies the bacteria that have the ability to produce the decarboxylase enzyme that will remove a carboxyl group from the amino acid that is present in the media. This media contains peptone, glucose, the pH indicator bromcresol purple, coenzyme pyridoxal phosphate, and a specific amino acid.

- The amino acids we use are arginine, ornithine, and lysine. After inoculation an overlay of oil is placed over each tube. This is to remove the external oxygen and promote fermentation.
- If the bacteria ferments the glucose in the media an acidic environment is produced and the pH indicator will turn a yellow color. This is a negative result as only the sugar was used.
- If the bacteria produces the correct decarboxylase enzyme to break down the specific amino acid an alkaline environment is produced and the pH indicator will turn a purple color. This is a positive result as the bacteria produced the enzyme and decarboxylation of the amino acid was done.

Procedure

- Inoculate all three decarboxylation broths (these all are the same color) with your organism. Overlay all tubes with oil. **Remember this step otherwise you cannot read your results.** Incubate at your optimum temperature.
- After incubation make sure there was oil on the top of the broth before doing your result reading. Compare your tubes to the positive control, and the two negative controls.

Gelatin Test

- This test identifies the bacteria that have the ability to hydrolyze gelatin. To help with hydrolyzing gelatin bacteria produce enzymes known as gelatinases.
- The ability to hydrolyze gelatin results in the liquefaction of the media, this is a positive result. Those bacteria that do not produce gelatinases cannot hydrolyze gelatin and it will remain solid.
- One drawback of this test is that gelatin is liquid at temperatures higher than 28°C. to overcome this issue, after incubation if the gelatin in the tube is liquid then it will need to be refrigerated for 15 minutes before it can be read. However if the gelatin is solid in the tube after incubation then this is a negative result.
- If after refrigeration the gelatin in the tube is still liquid then this is a positive result for your bacteria producing gelatinase. If after refrigeration the gelatin in the tube is solid then this is a negative result and was a result of the incubation temperature.

Procedure

- Inoculate the gelatin stab with your organism. Incubate at your optimum temperature for 5 days.
- After incubation your instructor will ask for your gelatin tubes if they are liquid. These will be placed in a rack and moved to the refrigerator for 15 minutes. If your gelatin in your tube is solid you do not need to refrigerate it and this is a negative result.
- Once the 15 minutes is up obtain your tube right away to read it otherwise if it is solid it might liquefy due to the room temperature.

- Compare your results to what is stated above for after refrigeration. Compare your results to the positive control as well.

Indole Test

- This test identifies the bacteria that produce the enzyme tryptophanase to hydrolyze tryptophan to produce ammonia, indole, and pyruvate.
- Tryptophan + tryptophanase $\longrightarrow$ pyruvate + indole + ammonia
- The indole test is done in media known as SIM or sulfur-indole-motility media.
- After incubation Kovac's reagent is added to the tube for the detection of indole. Kovac's reagent is dimethylaminobenzaldehyde, butanol, and hydrochloric acid. If indole is present a red layer on the top of the media is produced. If there is no color change then it is a negative result.

Procedure

- Inoculate the SIM agar with your organism. Incubate at your optimum temperature.
- After incubation add 10 drops of Kovac's reagent to the tube. Compare your results to the positive control.

Sulfur Reduction Test

- This test identifies the bacteria that have the ability to reduce sulfur to hydrogen sulfide. Bacteria that can do this do it in two different ways. One is with the enzyme cysteine desulfurase. The second way is with the enzyme thiosulfate reductase.
- When hydrogen sulfide is produced it reacts with the iron salt in the media to form ferric sulfide which forms a black precipitate in the media. The media used for this test is the SIM media from the indole test.
- You will not need to inoculate two tubes of SIM agar. One tube is sufficient to determine both the indole and the sulfur test.
- After incubation of your SIM agar tube check for a black precipitate anywhere in the tube then preform the indole test above. Compare your tube to the positive control.

Special Tests – indicated on the flow chart we provide

Coagulase Test

- This test differentiates *Staphylococcus aureus* from other Gram positive cocci. This organism produces coagulase enzyme.
- The coagulase test utilizes rabbit plasma to detect the enzyme. After inoculation readings are taken within 4 hours. A positive result is any coagulation of the plasma from a liquid state to a solid state.

Bile Esculin Test

- This test is used to differentiate between the members of *Enterococcus* and *Streptococci.* The bile in the media is used as the selective agent. Ferric citrate is used as a source of oxidized iron to indicate a positive result in this media.
- After incubation a darkening of the media is indicated by a positive result. No change in the media is indicated by a negative result.

Experiment 24 Examination of Water : Most Probable Number

Water safety is a concern for many people. The type of bacteria that are present in the water is of great importance. Water from lakes, rivers, and streams contain within them a variety of different bacteria. These bacteria may be harmless, but the ones that are of importance are the ones that cause disease in humans.

Water testing is done on all water treated to make sure it is safe and potable. An indicator bacteria used to test water is *E. coli*. *E. coli* is a good indicator of fecal contamination as it is not found in soil or water, but is found in the intestines of humans and other warm-blooded animals. This organism also survives longer than other intestinal pathogens.

Water testing is looking for coliform presence. Coliforms are Gram negative, rods, non-spore forming, and facultative anaerobes. They ferment lactose to produce gas and acid. There are three different tests done to determine the coliform count in water.

Presumptive test – lactose broth is inoculated with the water sample and incubated to see if gas is produced. If gas is produced it is presumed that coliforms may be present. The most probable number is determined at this time for the number of coliforms present per 100 ml of water. However a positive test does not indicate that coliforms are absolutely present. The organisms that could be present might be lactose fermenters, but not coliforms.

Confirmed test – this test uses differential and selective media to confirm that the organisms from the positive lactose broths are Gram negative and are lactose fermenters.

Completed test – This test confirms that a colony from the confirmed test is indeed a Gram negative gas producer.

After completion of these three tests it is usually a good idea to do the IMViC tests to determine that the coliform is indeed *E. coli* and not *Enterobacter*. You will get more experience with the IMViC tests during the unknown study.

Organism

- *Escherichia coli* – Gram negative rod
- *Enterobacter aerogenes* – Gram negative rod

Presumptive Test

- Note: double strength lactose broths and single strength lactose broths are just about the same color. Label the tubes as you pick them up and put them in your rack. These tubes have Durham tubes on the inside that will trap the gas production.
- Obtain five double strength lactose broths (DSLB). Label them with 10 ml as well as DSLB.
- On the bench top is a flask of water. This water already contains the indicator organism. Mix before using.

- With a 10 ml pipet and pipet aid take 10 ml of the water from the flask and transfer it into each of the DSLB tubes.
- Next obtain ten single strength lactose broths (SSLB). Label the first five with 1 ml. Label the next five with 0.1 ml.
- With a 1 ml micropipette and tip transfer 1 ml of water from the flask into each of the 1 ml SSLB tubes.
- With a 0.1 ml micropipette and tip transfer 0.1 ml of water from the flask into each of the 0.1 ml SSLB tubes.
- Place all tubes in the rack for incubation. These tubes will incubate for about 24 hours at 38°C.
- After incubation determine the most probable number (MPN) from the number of tubes that have 10% gas or more.
- MPN can be calculated from the table that follows.
- Determine the number of positive tubes in each set. For example if the first set of tubes (the 5 DSLB tubes) has all 5 tubes with gas production in them than this is a 5. IF the second set of tubes (the 5 1 ml SSLB tubes) has 2 tubes with gas production in them than this is a 2. If the third set of tubes (the 5 0.1 ml SSLB tubes) has 0 tubes with gas production in them than this is a 0. So this would be a 5-2-0. Going to the table we would see that this corresponds to 49 organisms in 100 ml of water with a 95% probability of there being between 15 and 150 organisms.
- If water is extremely turbid it is beneficial to add a second set of 5 SSLB tubes to the experiment just in case all the previous 15 tubes are 5-5-5. This would be a 0.01 ml tube and any MPN that is calculated with these tubes would need to be multiplied by 10. The first set of tubes (DSLB) would be thrown out of the data collection.

Table 45.1 MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes Are Used for Dilution (10 ml, 1.0 ml, 0.1 ml)*

Combination of Positives	MPN Index/ 100 ml	Confidence Limits		Combination of Positives	MPN Index/ 100 ml	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	10	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	S-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250

(continued)

Table 45.1 MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes Are Used for Dilution (continued) (10 ml, 1.0 ml, 0.1 ml)*

Combination of Positives	MPN Index/ 100 ml	Confidence Limits		Combination of Positives	MPN Index/ 100 ml	Confidence Limits	
		Low	High			Low	High
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

*Results to two significant figures.

From A. D. Eaton, L. S. Clesceri, E. W. Rice, A. E. Greenberg, eds. *Standard Methods for the Examination of Water and Wastewater,* 21st Edition. Washington, DC: APHA, AWWA, WEF; 2005:9-54 (Table 9221:IV).

Confirmed test

- This test is done after you have calculated your MPN. You will inoculate three different plated mediums with a single positive tube. A positive tube has 10% of the Durham tube filled with gas. It does not matter which tube you use as long as you use the same tube to inoculate all three plates.
- For each plate you will do a streak for isolation. We want to be able to isolate the colonies to determine what organisms are present in the water sample.
- The first plate media you will inoculate with a streak for isolation is Eosin methylene blue (EMB) agar. This agar isolates coliforms. The dyes eosin and methylene blue inhibit Gram positive bacteria. The lactose in the media encourages coliform growth through lactose fermentation that produces an acidic environment. A positive result on this media is indicated by a dark purple-black to blue-black colony. These colonies usually look like they have dots in the middle. Sometimes when there is a large amount of lactose fermentation there might be a green sheen that accompanies the colonies as well.

- The second plate that you will inoculate with a streak for isolation is MacConkey agar. This media contains bile salts and crystal violet which inhibit Gram positive bacteria. Also in the media is lactose and a pH indicator called neutral red. Neutral red is what will give the lactose fermenters their pink to red color on this media. A positive result is indicated by pink mucoid colonies. Precipitation of the bile salts can create a hazy look to the media under the pink colonies.
- The third plate that you will inoculate with a streak for isolation is HardyCHROM ECC agar. This media is a chromogenic media that differentiates *E. coli* from other coliforms. Selective agents have been added to this media to inhibit the growth of Gram positive organisms and non-coliforms. The chromogenic substrates in the media allow certain organisms to be distinguished by the color of their colonies. *E. coli* is identified by a pink to violet colored colony. Other coliform bacteria are a turquoise color.
- After inoculation the plates will be incubated at 38°C.

Completed test

- After incubation of the plates collect them and confirm positive colonies on the EMB agar plate and the MacConkey agar plate.
- Take your CHROM ECC plate and note the colony colors. You should have some turquoise colonies and some pink/violet colonies. Take a single pink/violet colony from this plate and inoculate half of the colony in a single strength lactose broth and the other half of the colony on a TSA slant.
- Place both tubes in the rack for incubation.
- After incubation confirm that there is gas present in the lactose broth. Gram stain the organism on the TSA slant to confirm that you have a Gram negative rod.
- Here we stop our testing. However to distinguish between *E. coli* and *Enterobacter* you would do the IMViC tests as noted above in the introduction.

Results and Questions

1. Record the data for your presumptive test here.

Number of positive tubes

5 tubes DSLB 10 ml	5 tubes SSLB 1 ml	5 tubes SSLB 0.1ml	MPN

2. What do the lactose fermenting colonies look like on EMB agar?

3. What do the lactose fermenting colonies look like on MacConkey agar?

4. If the presumptive test is positive does that mean that the water is unsafe to drink?

5. What color were the colonies on the CHROMagar ECC media?

6. After the completed test why might it be a good idea to perform the IMViC tests?

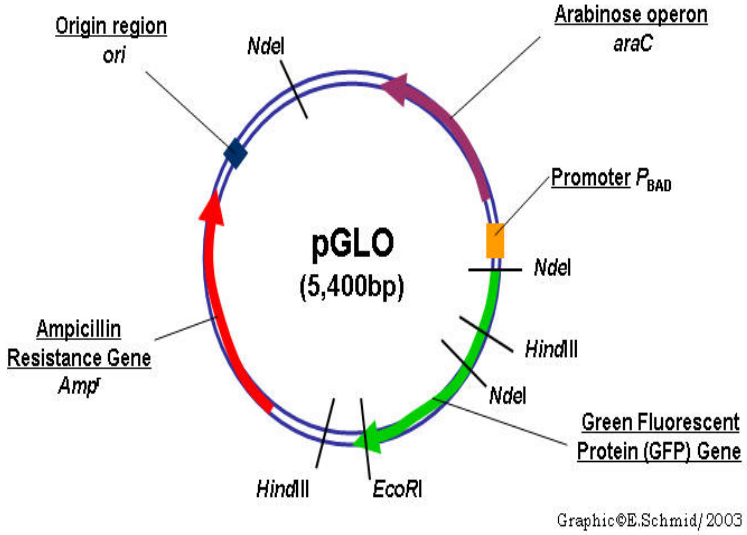
Experiment 25 **Bacterial Transformation : pGLO**

In this lab you will perform a procedure known as genetic transformation. Remember that a gene is a piece of DNA which provides the instructions for making (codes for) a protein. This protein gives an organism a particular trait. Genetic transformation literally means change caused by genes, and involves the insertion of a gene into an organism in order to change the organism's trait. Genetic transformation is used in many areas of biotechnology. In agriculture, genes coding for traits such as frost, pest, or spoilage resistance can be genetically transformed into plants. In bioremediation, bacteria can be genetically transformed with genes enabling them to digest oil spills. In medicine, diseases caused by defective genes are beginning to be treated by gene therapy; that is, by genetically transforming a sick person's cells with healthy copies of the defective gene that causes the disease.

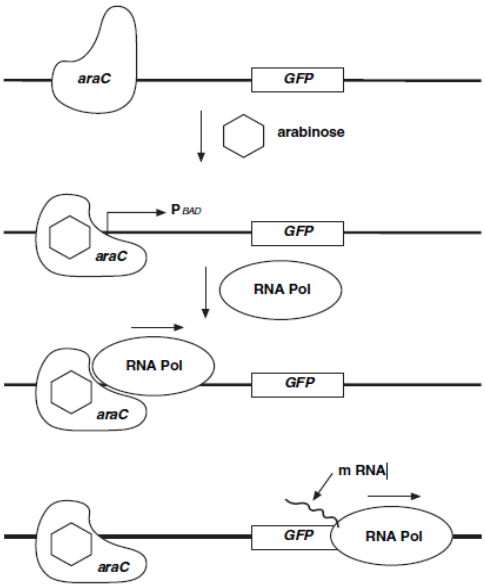
You will use a procedure to transform bacteria with a gene that codes for Green Fluorescent Protein (GFP). The real-life source of this gene is the bioluminescent jellyfish *Aequorea victoria*. Green Fluorescent Protein causes the jellyfish to fluoresce and glow in the dark. Following the transformation procedure, the bacteria express their newly acquired jellyfish gene and produce the fluorescent protein, which causes them to glow a brilliant green color under ultraviolet light.

In this activity, you will learn about the process of moving genes from one organism to another with the aid of a plasmid. In addition to one large chromosome, bacteria naturally contain one or more small circular pieces of DNA called plasmids. Plasmid DNA usually contains genes for one or more traits that may be beneficial to bacterial survival. In nature, bacteria can transfer plasmids back and forth allowing them to share these beneficial genes. This natural mechanism allows bacteria to adapt to new environments. The recent occurrence of bacterial resistance to antibiotics is due to the transmission of plasmids.

Genetic transformation involves the insertion of some new DNA into the *E. coli* cells. In addition to one large chromosome, bacteria often contain one or more small circular pieces of DNA called plasmids. Plasmid DNA usually contains genes for more than one trait. Scientists can use a process called genetic engineering to insert genes coding for new traits into a plasmid. In this case, the pGLO plasmid carries the GFP gene that codes for the green fluorescent protein and a gene (*bla*) that codes for a protein that gives the bacteria resistance to an antibiotic. The genetically engineered plasmid can then be used to genetically transform bacteria to give them this new trait.



Expression of Green Fluorescent Protein

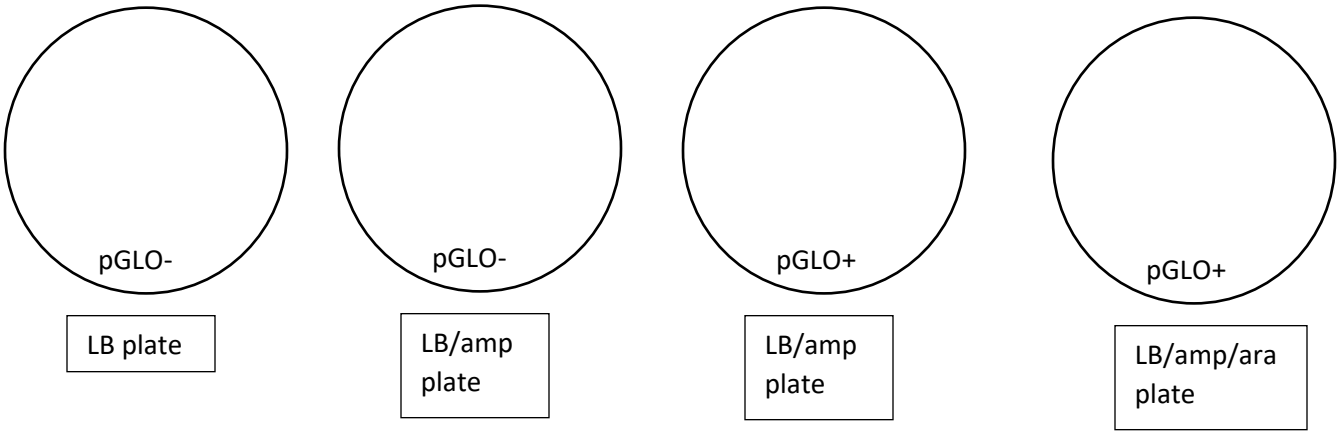


Materials per pair

- Pink microtube labeled "+" containing $CaCl_2$
- Blue microtube labeled "—" containing $CaCl_2$
- White microtube labeled "LB" containing 600µL of LB broth
- E. coli HB101 plate
- One plate labeled "LB'
- Two plates labeled "LB+amp"
- One plate labeled "LB+amp+arab"
- pGLO plasmid (to be dispensed by lab technician/instructor at window)

Protocol

1. Obtain a plate with E. coli HB101
2. Add one colony of E. coli HB101 into the microcentrifuge tube labeled "+". Spin loop in tube to assure organism has been dispersed and there are no floating chunks
3. Repeat step 2 for the tube labeled "—"
4. Take **ONLY** your pink, "+" tube to the window to have plasmid dispensed into it
5. Incubate your "+" **AND** "—" tubes on ice for **10 MINUTES**
6. While incubating, label your four plates with your name, lab section and "+ pGLO" and "— pGLO" based on the diagram below.



7. Following the 10 minute incubation on ice, place both tubes into a water bath set at 42^{O}C for **EXACTLY 50 SECONDS** (Your instructor will do this part)

8. Return the tubes to the ice **IMMEDIATELY** after removing from the water bath. Incubate on ice for **2 MINUTES**

9. After 2 minutes, obtain both of your tubes and add 250µL of LB broth from your white, LB tube to each of your microtubes (pink and blue). **USE A NEW TIP FOR EACH TUBE**

10. Incubate at 37°C for **10 MINUTES**

11. Mix the contents of each tube by flicking with your finger. Using a new, sterile tip for each tube, pipette 100µL of the contents of each tube onto the appropriately labeled plate

12. Aseptically spread the suspension evenly over the surface of the plate with your loop.

13. Incubate all plates upside down at 37^{O}C

Predictions

pGLO (-) LB	
pGLO (-) LB/AMP	
pGLO (+) LB/AMP	
pGLO (+) LB/AMP/ARA	

Following lab period

1. Obtain your four plates and record observations

2. Examine plates with UV light and record observations

Results:

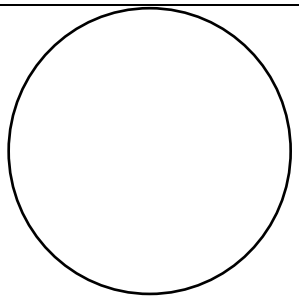
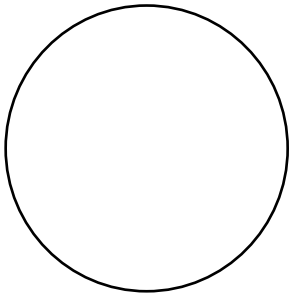
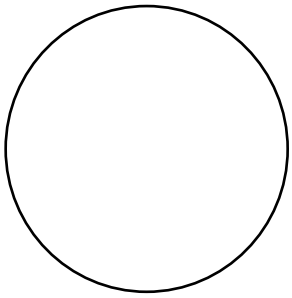
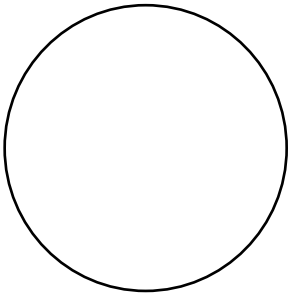
Observe each plate under normal room lighting. Then turn out the lights and hold the *UV* light over the plates. Draw that you see on each of the four plates in the designated area below. Record your observations for each plate. Address the following question:

a. How much bacterial growth do you see on each plate? (ex: a lot, a little}

b. What color are the bacteria?

c. Count how many bacterial colonies there are on each plate.

Fill in the results of your plates.

+pGLO LB/amp		Observations:
+pGLO LB/amp/ara		Observations:
-pGLO LB/amp		Observations:
-pGLO LB		Observations:

Experiment 26 Bacterial Conjugation

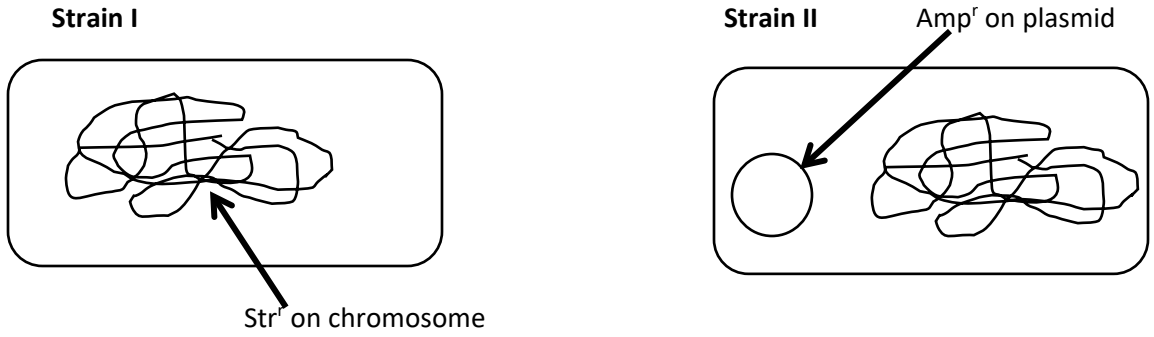
In this experiment you are introduced to a naturally occurring mechanism called conjugation, by which DNA from one cell is transferred to another cell to produce a new recombinant cell. Sometimes the DNA that is transferred codes for antibiotic resistance. The intercellular transfer of this bacterial DNA coding for resistance to antibiotics enables the new recombinant bacterial cell to express resistance to an antibiotic to which it was formerly sensitive. This transfer is also considered a type of genetic recombination.

While bacterial chromosomes normally carry all the genes necessary for growth and reproduction, bacteria also contain genes carried on extra chromosomal DNA, called plasmids. Plasmids are double-stranded circular pieces of DNA that may carry anywhere from 3-25 genes. Numerous plasmids have been described in a variety of bacteria. Plasmids contain specialized genes, can replicate independently of the bacterial chromosome, can move from one bacterial cell to another, and may even be exchanged between cells of different bacterial species.

One of the first plasmids to be described was originally called the "F (fertility) Factor." This plasmid, found in the common colon bacterium *Escherichia coli*, contains about 25 genes, most of which regulate the formation of pili, elongated appendages that extend from the surface of the cell. These pili can function as a bridge between two bacteria cells, thus allowing the transfer of DNA from the donor to the recipient. This process is called conjugation.

In 1959, it was shown that resistance to antibiotics can be transferred between bacteria during conjugation, and that this transfer involves plasmids. Plasmid-mediated drug resistance has created numerous problems for physicians and patients, because bacteria are able to transfer these "resistance genes" very rapidly. Under optimal conditions, the rate at which a conjugative plasmid can spread through a population can be exponential, showing resemblance to a bacterial growth curve.

In preparation for Lab Day 1, pure cultures of E. coli Strains I (resistant to streptomycin; Str^r) and II (resistant to ampicillin; Amp^r) are grown one or more days in TSB broth, an enriched culture medium lacking antibiotics. Samples of each strain are then transferred to a new TSB broth and allowed to conjugate. In Lab, the different tubes are plated on several LB plates containing antibiotics. This will confirm the resistance of each Strain to its respective antibiotic. If DNA transfer has occurred, it will be confirmed by growth of the conjugated strain on the LB plate containing both ampicillin and streptomycin.



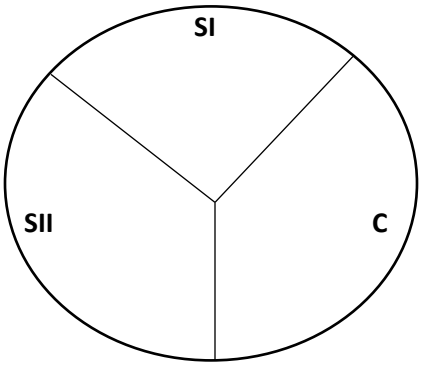
Lab Day 1

1. Obtain 1 of each of the following plates:

- LB
- LB+amp
- LB+strep
- LB+amp+strep

2. Label each type of plate as shown in the following diagram

- "**SI**" (for Strain I)
- "**SII**" (for Strain II)
- "**C**" (for Conjugated)



3. Using your loop, **aseptically** transfer 1 loopfull of organism from the Strain I test tube onto each of its corresponding sections. For example:

- 1 loopfull of the Strain I tube will go to the LB plate
- 1 loopfull of the Strain I tube will go to the LB+amp plate
- 1 loopfull of the Strain I tube will go to the LB+strep plate
- 1 loopfull of the Strain I tube will go to the LB+strep+amp plate

4. Repeat Step 3 for the Strain II tube (place onto its corresponding sections)

5. Repeat Step 3 for the Conjugation tube (place onto its corresponding sections)

7. Store all of your plates upside down in the racks

8. Record your **expected outcomes** in the table below

	***E. coli* Strain I**	***E. coli* Strain II**	***E. coli* Conjugated**
LB Agar			
LB+amp Agar			
LB+strep Agar			
LB+strep+amp Agar			

Lab Day 2

1. Obtain your plates from the previous lab period and record your **observations**

	***E. coli* Strain I**	***E. coli* Strain II**	***E. coli* Conjugation**
LB Agar			
LB+amp Agar			
LB+strep Agar			
LB+strep+amp Agar			

Results and Discussion

1. Why did Strain I not grow on the LB+amp plate?

2. Why did Strain II not grow on the LB+strep plate?

3. Why did neither Strain (alone) grow on the LB plate containing streptomycin and ampicillin?

4. Did the contents of the conjugation tube grow on the LB+strep+amp plate? If so, does this support the prediction of the recombination of antibiotic-resistant genes into a new strain? Explain.

5. Based on the results of this study, can one determine whether the Str^r gene from Strain I was transferred to Strain II or whether the Amp^r gene on the plasmid was transferred from Strain II into Strain I? Explain.

Experiment 27 Isolation and Identification of *Staphylococci*

Staphylococci are Gram positive spherical bacteria. They are non-motile and non-spore forming cocci. They are a part of the normal flora of nasal membranes, hair follicles, skin, and the perineum in healthy individuals. Infections by *Staphylococci* are introduced when the host's ability to resist infections is lowered or when a toxin from *Staphylococci* is ingested.

Staphylococcus aureus is an organism that many of the hospital infections are caused by. Further complicating matters is the fact that *S. aureus* has developed a resistance to many antibiotics including methicillin.

In this experiment you will identify unknown *Staphylococci* from your nasal isolation and from an unknown broth using differential media and biochemical tests specific to these bacteria.

Day One

Organism

- Nasal isolate
- Unknown *Staphylococci*

Procedure

- Isolate your nasal isolate using a sterile swab to swab the inside of your nose. Place the swab into a sterile M-Staph broth. Break off the very end of the swab, label, and put the tube into the rack for incubation.

Day Two

Media

- Staphylococcus Medium 110 (SM110)
    - Contains mannitol and sodium chloride.
    - Colony pigmentation is used to differentiate the different strains of *Staphylococci. S. aureus* colonies will appear yellow or orange while all other colonies will appear colorless.

Procedure

- Obtain and sign up for an unknown. Obtain your nasal isolate.
- Obtain two plates of SM110 agar.
- On one SM110 plate do a streak for isolation of your unknown organism. On the other SM110 plate do a streak for isolation of your nasal isolate.
- Incubate both plates.

Day Three

Media

- Mannitol salt agar plate (MSA)
    - Contains mannitol, 7.5% sodium chloride, and phenol red as the pH indicator.
    - The salt inhibits the growth of non-staphylococci.
    - If the mannitol is fermented the media and colonies will turn a yellow color due to increased acidity. This is evidence that the organism may be *S. aureus* or *S. saprophyticus.* This is a positive result for mannitol fermentation
    - *S. epidermidis* will produce pink colonies and no color change in the media, this is a negative result for mannitol fermentation. *S. saprophyticus* may also produce a negative result.
- Blood agar
    - We are checking for hemolysis of red blood cells.
    - *S. aureus* is β-hemolytic; meaning that there is complete lysis of the red blood cells, so there will be a clearing around the growth on the media.
    - *S. saprophytics* and *S. epidermidis* are ɣ-hemolytic; meaning there is no lysis of the red blood cells and no change in the media
- Tryptic soy agar
    - We are checking for catalase production with this plate.
- Mueller-Hinton agar (MH)
    - Looks like a TSA plate, make sure to label these plates when obtaining them.
    - We are checking for Novobiocin resistance or sensitivity.
    - To be considered susceptible the zone of inhibition must be greater than 16mm.

Procedure

- Examine your SM110 plates. Note the color of the colonies.
- Obtain two tubes of 2ml TSB. In one tube place a single colony of your nasal isolate from the SM110 plate, mix well. In the other tube place a single colony of your unknown organism from the SM110 plate, mix well. These tubes are what you will use for your inoculations.
- Obtain two MSA plates. Do a streak for isolation on one MSA plate from the nasal isolate broth. Do a streak for isolation on the other MSA plate from the unknown broth.
- Obtain two blood agar plates.
- On one blood agar plate do a streak for isolation of your unknown organism. On the other blood agar plate do a streak for isolation of your nasal isolate.
- Obtain two TSA plates. Do a streak for confluence on one TSA plate with your nasal isolate, you can use either your loop or the sterile swabs that will be provided. Do a streak for confluence on the other TSA plate with your unknown, you can use either your loop or the sterile swabs that will be provided.

- Obtain two Mueller-Hinton plates. Do a streak for confluence on one MH plate with your nasal isolate, you can use either your loop or the sterile swabs that will be provided. Do a streak for confluence on the other MH plate with your unknown, you can use either your loop or the sterile swabs that will be provided
- Take both of your Mueller-Hinton plates to either the technician or your instructor to have the novobiocin antibiotic disk place don the plate.
- Incubate all plates.

Day Four

Procedure

- Examine your MSA plates. Note colony color and if mannitol was fermented.
- Examine your blood agar plates. Note the hemolysis type. Alpha, beta, or gamma. Refer to day three for what beta and gamma hemolysis looks like. Alpha hemolysis is partial lysis of the red blood cells which makes the media take on a greenish hue.
- For the catalase test take both of your TSA plates. Add a single drop of hydrogen peroxide on the growth. A positive reaction is bubble formation. A negative result is no bubble formation.
- Look at your Mueller-Hinton plates, measure the zone of inhibition around the novobiocin disk.
- If your organism in either the nasal isolate or the unknown is mannitol positive and beta hemolytic then your will need to do a coagulase test.
- Obtain a tube of coagulase for which ever organism that is mannitol positive and beta hemolytic. Inoculate the coagulase tube with a single colony from either the MSA plate or the blood agar plate. You can use the TSA plate as long as you have not done the catalase test.
- Incubate the coagulase tubes.
- If you do not need to do the coagulase test, you can utilize the unknown flow chart to find out what your unknown organism is. For your nasal isolate there is a separate flow chart that gives an approximation of what your isolate might be. In order to completely find out what your nasal isolate is you would need to do further tests that we will not do in this experiment.
- Your instructor can confirm is your unknown organism that you got is correct.

Day Five

Procedure

- Look at your coagulase tubes. If the coagulase is still liquid, this is a negative result. If the coagulase is solid or semi-solid, this is a positive result. Use the flow charts to determine the organism you have for your unknown and what you may have for your nasal isolate.

Results and Questions

1. Record your results for your MSA plate, your blood plate, and your SM110 plate in this table.

	SM110 Plate		MSA Plate		Blood Plate
	Colony color	**growth (+/-)**	**Colony color**	**growth (+/-)**	**hemolysis**
Nasal Isolate					
Unknown					

2. Record your coagulase and catalase results.

	Catalase (+/-)	**Caogulase (+/-)**
Nasal Isolate		
Unknown		

3. Record your Novobiocin results.

	Novobiocin	
	zone of inhibition	**Rating (S, R)**
Nasal Isolate		
Unknown		

4. Identification of your organisms.

   Nasal isolate:

   Unknown:

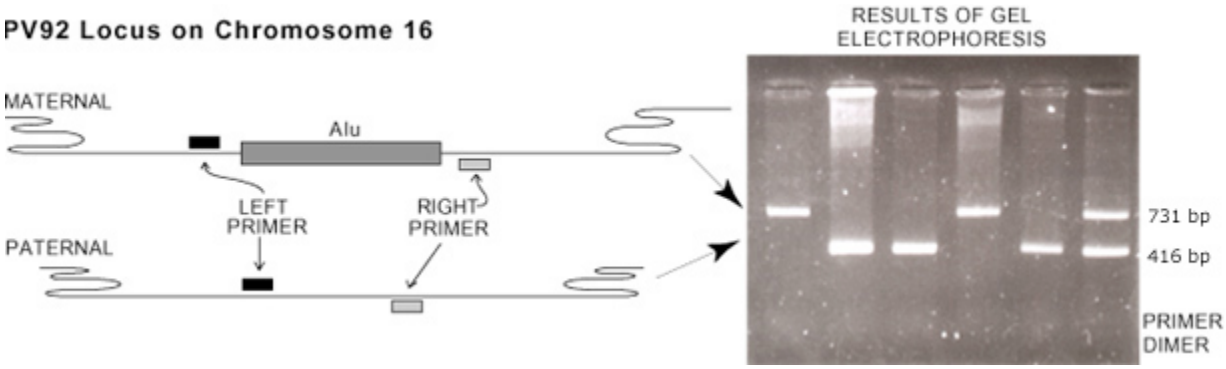
5. Describe the selective and differential properties of MSA and SM110.

6. Were you correct in identifying your unknown organism? If not what might have went wrong?

Experiment 28 Isolation of the PV92 Alu Gene

An Overview of the PV92 Alu insertion

This experiment examines PV92, a human-specific *Alu* insertion on chromosome 16. The PV92 genetic system has only two alleles indicating the presence (+) or absence (-) of the *Alu* transposable element on each of the paired chromosomes. This results in three PV92 genotypes (++, +-, or --). The + and - alleles can be separated by size using gel electrophoresis.



Alu elements are classified as SINEs, or Short INterspersed Elements. All *Alu*s are approximately 300 bp in length and derive their name from a single recognition site for the restriction enzyme *Alu*I located near the middle of the *Alu* element. Human chromosomes contain about 1,000,000 *Alu* copies, which equal 10% of the total genome. *Alu* elements probably arose from a gene that encodes the RNA component of the signal recognition particle, which labels proteins for export from the cell.

Alu is an example of a so-called "jumping gene" – a transposable DNA sequence that "reproduces" by copying itself and inserting into new chromosome locations. *Alu* is classified as a retroposon, because it is thought to require the retrovirus enzyme reverse transcriptase (rt) enzyme to make a mobile copy of itself. Here is a simple scheme to explain how an *Alu* element transposes:

- First, the inserted *Alu* is transcribed into messenger RNA by the cellular RNA polymerase.
- Then, the mRNA is converted to a double-stranded DNA molecule by reverse transcriptase.
- Finally, the DNA copy of *Alu* is integrated into a new chromosomal locus at the site of a single- or double-stranded break.

Each *Alu* element has an internal promoter for RNA polymerase III needed to independently initiate transcription of itself. However, *Alu* is a "defective" transposon, in that it lacks the enzyme functions to produce a DNA copy of itself and to integrate into a new chromosome position. However, *Alu* can obtain these functions from another transposon, called L1, a Long INterspersed Element (LINE). LINEs are essentially defective retroviruses that retain a functional rt gene. Interestingly, in addition to reverse transcribing RNA to DNA, the L1 rt also produces single-stranded nicks in DNA. In the current model, the rt enzyme produces a nick at a chromosomal locus containing the sequence AATTTT. The polyadenylated "tail" of the *Alu* transcript (-AAAA) then hydrogen bonds to the TTTT sequence at the nick site, creating a primer for reverse transcription. The L1 rt makes a staggered nick in the opposite DNA strand of the host chromosome, allowing the DNA copy to integrate. This method of insertion also accounts for the identical sequences (direct repeats) found at the ends of all *Alu* elements. So it appears that LI can provide the necessary functions for *Alu* transposition. In this sense, *Alu* is a parasite of L1, which, in turn, is a relic of a retrovirus ancestor.

Some scientists regard *Alu* as an example of "selfish DNA" – it encodes no protein and appears to exist only for its own replication. If one reduces the definition of life to "the perpetuation and amplification of a DNA sequence through time," then *Alu* is an extremely successful life form. However, other scientists

believe that transposable elements have played an important role in evolution by creating new mutations and gene combinations. Nobel laureate Barbara McClintock hypothesized that transposable elements provide a mechanism to rapidly reorganize the genome in response to environmental stress. Like *Alu*, the Ds transposable element discovered in corn by McClintock is a defective transposon and requires the help of a second element called Ac (activator).

Alu elements are found only in primates – the "monkey" branch of the evolutionary tree, which includes humans. So, all of the hundreds of thousands of *Alu* copies have accumulated in primates since their separation from other vertebrate groups about 65 million years ago. Once an *Alu* integrates into a new site, it accumulates new mutations at the same rate as surrounding DNA loci. *Alu* elements can be sorted into distinct lineages, or families, according to inherited patterns of new mutations. These studies suggest that the rate of *Alu* transposition has changed over time – from about one new jump in every live birth, early in primate evolution, to about one in every 200 newborns today. Taken together, this pattern suggests that, at any point in time, only one or several *Alu* "masters" are capable of transposing.

Once an *Alu* inserts at a chromosome locus, it can copy itself for transposition, but there is no evidence that it is ever excised or lost from a chromosome locus. So, each *Alu* insertion is stable through evolutionary time. Each is the "fossil" of a unique transposition event that occurred only once in primate evolution. Like genes, *Alu* insertions are inherited in a Mendelian fashion from parents to children. Thus, all primates showing an *Alu* insertion at a particular locus have inherited it from a common ancestor. This is called identity by descent.

An estimated 500-2,000 *Alu* elements are restricted to the human genome. The vast majority of *Alu* insertions occur in non-coding regions and are thought to be evolutionarily neutral. However, an *Alu* insertion in the NF-1 gene is responsible for neurofibromatosis I, *Alu* insertions in introns of genes for tissue plasminogen activator (TPA) and angiotensin converter enzyme (ACE) are associated with heart disease. *Alu* insertions are analogous to the insertion of a provirus in viral diseases and certain cancers.

Most *Alu* mutations are "fixed," meaning that both of the paired chromosomes have an insertion at the same locus (position). However, a number of human-specific *Alu*s are dimorphic – an insertion may be present or absent on each of the paired chromosomes of different people. These dimorphic *Alu*s inserted within the last million years, during the evolution and dispersion of modern humans. These dimorphisms show differences in allele and genotype frequencies between modern populations and are tools for reconstructing human prehistory.

DNA extraction

Materials per person:

- 5ml of sterile 0.9% NaCl (saline) in a 15ml conical vial
- Yellow microtube with 150µl of lysis buffer
- Plastic cup
- 1000µl micropipettor and tips
- 140µl and 80µl micropipettor and tips
- 2 Sterile microtubes
- Microtube lid lock

Protocol

1. Obtain your large tube of 0.9% NaCl (saline) and pour it into your plastic cup. Use the 5ml of saline to rinse your mouth vigorously for 60 seconds

2. Expel the saline back into the cup and swirl to mix the cells **Do Not get rid of liquid.**

3. Transfer 1000µl of your cheek cell solution into a new, labeled micro tube

4. Using the micro centrifuge, centrifuge your tube for 1min. **be sure to load the centrifuge so that it is balanced, and that your tube is loaded with the hinge facing out. The pellet will form below the hinge when loaded in this orientation.

5. Carefully decant the supernatant (liquid) into the bleach waste container **make sure not to disturb the pellet**

6. Once the liquid has been poured off, mix the cheek cell mixture from step 2 and transfer 1000µl to the same micro centrifuge tube. Re-suspend the cells by running the tube along the micro tube rack. Centrifuge your tube for 1min. Pour off the liquid into the bleach waste container.

7. Add 140µl of lysis buffer from the yellow tube to re-suspend your cells. Do this by running your tube along the micro tube rack. Carefully clamp your tube shut with a lid lock

8. Place your tube into the 55°C water bath for 15min. This will help the proteinase K in the lysis buffer to denature\degrade the proteins and nucleases that are present in the cell while protecting the DNA.

9. Mix the sample by running the tube over the micro tube rack. Do this for 20 seconds.

10. Once mixed place the tube into the 99°C heat block for 15min. This step deactivates the proteinase K. **Do Not use the timer function on the heat block it turns the unit off.**

11. Remove the lid lock and place it back into the beaker. Centrifuge your tube for 2min **be sure to load the centrifuge so that it is balanced, and that your tube is loaded with the hinge facing out.

12. Remove 80µl of the supernatant (which contains your DNA) and place in a fresh, labeled tube. ****be sure not to disturb the pellet or remove any of the cellular debris with your DNA sample****

13. Place your labeled DNA sample in the rack at the front of the room to be refrigerated until the next class meeting.

An overview of the PCR process

To preform PCR, the template DNA and the molar excess of primers are mixed with the four "free" deoxynucleotides (dATP, dCTP, dGTP, and dTTP), and a thermostable DNA polymerase. The most commonly used DNA polymerase is *Taq* DNA polymerase. This enzyme, originally purified from a bacterium that inhabits hot springs, is stable at very high temperatures. These components (template DNA, primers, the four deoxynucleotides, and *Taq* DNA polymerase) are mixed with a buffer that contains Mg^{+2}, an essential ofactor for *Taq* polymerase. The PCR reaction mixture is subjected to sequential heating/cooling cycles at three different temperatures in a thermal cycler.

- In the first step, known as "denaturation", the mixture is heated to near boiling (94°C - 96°C) to "unzip" the target DNA. The high temperature disrupts the hydrogen bonds between the two complementary DNA strands and causes their separation.
- In the second step, known as "priming", the reaction mixture is cooled to 45°C - 65°C, which allows the primers to base pair with the target DNA sequence.
- In the third step, known as "extension", the temperature is raised to 72°C. This is the optimal temperature at which *Taq* polymerase can add nucleotides to the hybridized primers to synthesize the new complementary strands.

These three steps – denaturation, priming, and extension – constitute one PCR "cycle". Each PCR cycle doubles the amount of the target DNA in less than five minutes. In order to produce enough DNA for analysis, twenty to forty cycles may be required. To simplify this process, a specialized machine, called a "thermal cycler" or a "PCR machine", was created to rapidly heat and cool the samples.

PCR amplification

Materials per person:

- DNA samples from the previous class meeting
- PCR tube with 20µl of complete master mix (located on ice at the front of the class)
- 10µl micropipettor and tips

Protocol

1. Obtain your DNA sample from the last class meeting
2. Carefully pipette 10µl of your DNA into the PCR tube **give this tube a unique label**
3. Mix your cells with the master mix by pipetting up and down

4. Use a quick flick to make sure that all the liquid is in the bottom of the tube when you are finished
5. Quickly place your tube in the thermocycler and record the location of your tube on the PCR signup sheet
6. The thermocycler will be programmed with the following cycles:

Temp (°C)	time	purpose	# of cycles
94°C	4min	Hot start- polymerase activation & initial denaturation	1
94°C	1min	Denaturation	40
65°C	1min	Priming	
72°C	2min	Extension	
72°C	10min	Final extension	1
4°C	overnight	Cold storage	1

7. Your PCR tubes will be stored at 4°C until the next lab meeting

An overview of the gel electrophoresis process

Gel electrophoresis is a laboratory method used to separate mixtures of DNA, RNA, or proteins according to molecular size. In gel electrophoresis, the molecules to be separated are pushed by an electrical field through a gel that contains small pores. The molecules travel through the pores in the gel at a speed that is inversely related to their lengths. This means that a small DNA molecule will travel a greater distance through the gel than will a larger DNA molecule.

As previously mentioned, gel electrophoresis involves an electrical field; in particular, this field is applied such that one end of the gel has a positive charge and the other end has a negative charge. Because DNA and RNA are negatively charged molecules, they will be pulled toward the positively charged end of the gel.

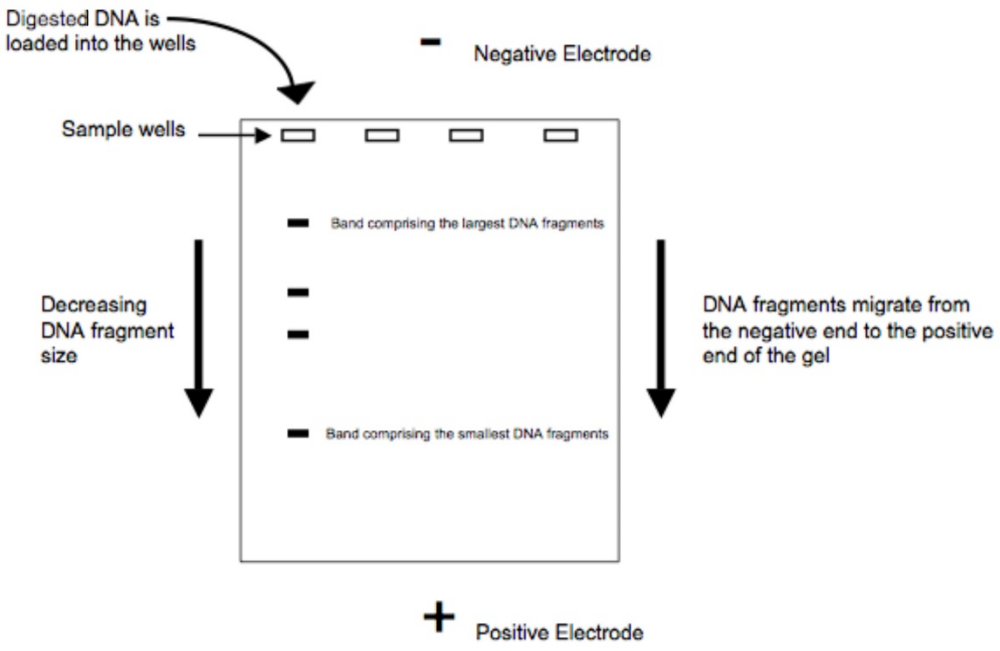


Figure 7 - The basic process of running digested DNA in an electrophoretic gel

Gel electrophoresis

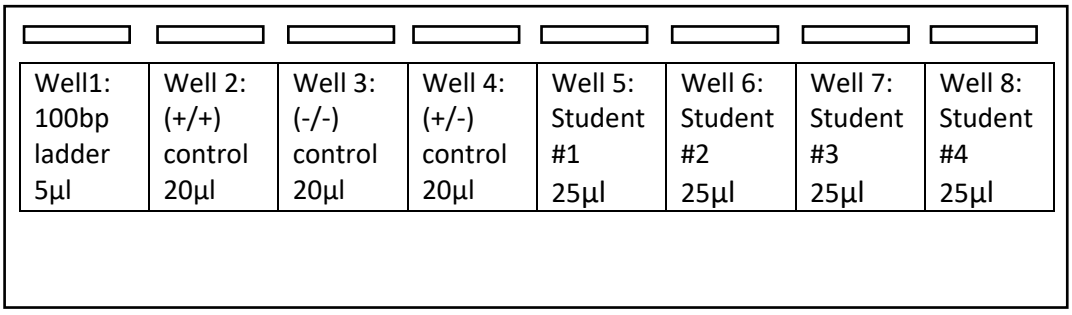
Materials per group of 4:

- Amplified samples from the previous class meeting
- Amplified controls (one set per gel)
- 100bp ladder (one set per gel)

- Loading dye (5µl/sample)
- 5µl micropipettor and tips
- 25µl micropipettor and tips
- Light box for viewing gels
- Gloves

Protocol

1. Obtain your PCR amplified samples from the last class meeting.
2. Add 5µl of loading dye to your PCR tube containing your amplified cheek cell DNA and mix well
3. On the bench tops where each gel box is there is an ice bucket that has PCR tubes with a (-/-) control, a (+/-) control, a (+/+) control, and a larger tube with ladder in it. Each of these tubes already has loading dye inside, they can be loaded on the gel according to step 4.
4. Load the gel according to the diagram below. When loading your samples, **do not touch the gel,** hover above the well you are trying to load. Slowly push down on the plunger to dispense the sample into the well, being sure not to dispense any air bubbles or puncture the well.

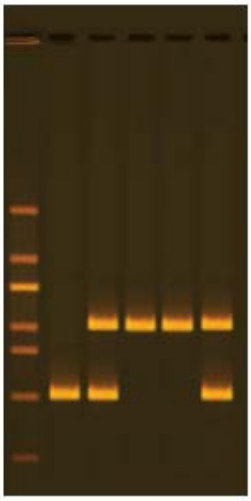


Well1: 100bp ladder 5µl	Well 2: (+/+) control 20µl	Well 3: (-/-) control 20µl	Well 4: (+/-) control 20µl	Well 5: Student #1 25µl	Well 6: Student #2 25µl	Well 7: Student #3 25µl	Well 8: Student #4 25µl

5. After your gel is loaded with all the samples put the top on the gel box, making sure to match the corresponding colors. Red with Red and Black with Black. The voltage on the power source will already be set to 100V (volts). Once the lid is on place press the Run button and time for 30 minutes. (the purple band on the gel should be at the halfway mark on the gel tray.)

6. **WEARING GLOVES.** After gel run is complete press the Stop button, take off the lid, and carefully remove the tray and gel from the box. Take it over to the light box. Put on the safety goggles to shield eyes before turning on the light box. View the gel. Below is a figure of what the gel might look like.

Experiment Results and Analysis



The results photo shows an example of the possible PCR products from different genotypes.

Lane	Recommended	Molecular Weight	Result
1	EdvoQuick™ DNA Ladder	---	---
2	Control DNA*	400 bp	Null for Alu insertion (-/-)
3	Student #1	700, 400 bp	Heterozygous for Alu insertion (+/-)
4	Student #2	700 bp	Homozygous for Alu insertion (+/+)
5	Student #3	700 bp	Homozygous for Alu insertion (+/+)
6	Student #4	700, 400 bp	Heterozygous for Alu insertion (+/-)

DNA Fingerprint for PV92 Alu

Part One: DNA Template Preparation

1. What part of the cell is needed for this study?

2. What part of the genome is needed for this study?

3. What is the function of Proteinase K?

4. How are these type of studies used in the “real” world?

Part two: PCR Amplification

1. Why is it necessary to have a primer on each side of the DNA segment to be amplified?

2. How did *Taq* DNA polymerase acquire its name?

3. Why are there nucleotides (A,T,G,C) in the master mix? What are the other components of the master mix and what are their functions?

4. Describe the three main steps for each cycle of PCR amplification and what reactions occur at each step.

5. Explain why the precise length target DNA sequence does not get amplified until the third cycle. Illustrate your answer.

Part three: Gel electrophoresis of Amplified PCR Samples

1. Explain the difference between an intron and an exon.

2. Why do the two possible PCR products differ in size by 300 base pairs?

3. Explain how agarose electrophoresis separates DNA fragments. Why does a smaller DNA fragment move faster than a larger one?

4. What kind of controls are run in this experiment? Why are they important? Could others be used?

Part four: Analysis of Data

1. What is your genotype for the Alu insert in your PV92 region?

2. Class Data:

Category	**Number**	**Frequency (# of genotypes/total)**
Homozygous (+/+)		
Homozygous (-/-)		
Heterozygous (+/-)		
Total		

3. If you have no genotype for the Alu insert what could have went wrong?

Appendix A Tables
Table II Four-place Logarithms

N	0	1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670
37	56822	5694	5705	5717	5729	5740	5752	5763	5775	5786
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316
54	7324	7332	7340	7348	7356	7364	7372	7380	7338	7396

Appendix A Tables
Table II Four-place Logarithms

N	0	1	2	3	4	5	6	7	8	9
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551
57	7559	7566	7574	7582	7589	7567	7604	7612	7619	7627
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846
51	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686
74	8692	9698	8704	8710	8716	8722	8727	8733	8739	8745
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238
84	9243	9248	9253	9258	9263	9269	9274	9279	9384	9289
85	9594	9299	9304	9309	9315	9320	9325	9330	9335	9340
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489
89	9494	9499	9504	9509	9513	9518	9523	9528	9532	9538
90	9542	9547	9552	9557	9562	9566	9571	9579	9581	9586
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996
100	0000	0004	0009	0013	0017	0022	0026	0030	0035	0039

Descriptive Chart

STUDENT: _____

LAB SECTION: _____

Habitat: _____ Unknown No.: _____

Organism: _____

MORPHOLOGICAL CHARACTERISTICS

Cell Shape:

Arrangement:

Size:

Spores:

Gram's Stain:

Motility:

Capsules:

Acid Fast Stain:

CULTURAL CHARACTERISTICS

Colonies:

Nutrient Agar: Colony morphology:

Blood Agar:

Hemolysis type:

Colony morphology:

Agar Slant:

Nutrient Broth:

Oxygen Requirements:

Optimum Temperature:

PHYSIOLOGIAL CHARACTERISTICS

	TESTS	RESULTS
Fermentation	Glucose	
	Lactose	
	Sucrose	
	Mannitol	
	Trehalose	
	Fructose	
	OF Glucose (circle one)	Fermentative
		Oxidative
		Neither
Decarboxylase	Arginine	
	Lysine	
	Ornithine	
Hydrolysis	Gelatin Liquefaction	
	Starch	
	Casein (skim milk)	
	Lipase (spirit blue)	
IMViC	Indole	
	Methyl Red	
	V-P (acetylmethylcarbinol)	
	Citrate Utilization	
Other	Nitrate Reduction	
	H_2S Production	
	Urease	
	Catalase	
	DNase	
	Phenylalanase	
	Special Tests: Oxidase, Antibiotic Sensitivity, Bile Esculin, Salt Tolerance, etc (Circle test and list results)	

SPECIMEN SIZE DETERMINATION

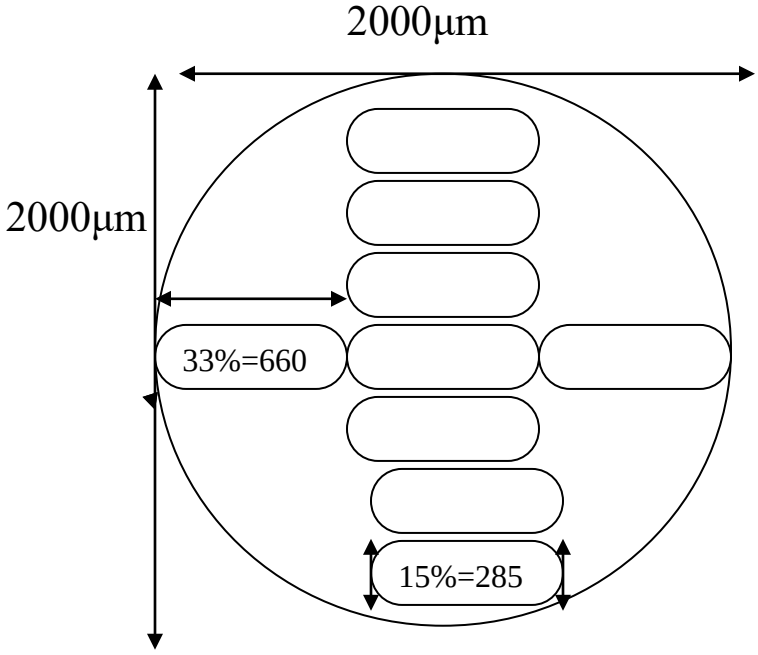
Exact measurements can be accomplished with the use of micrometers. (see lab exercise #4). In the absence of this equipment, size determinations can be approximated with a good deal of accuracy by using the following table and formula.

Objective	Objective Magnification	Ocular Magnification	Total Magnification	Diameter of field view in microns
scanning	4X	10X	40X	5000
low	10X	10X	100X	2000
high dry	40X	10X	400X	500
oil immersion	100X	10X	1000X	200

Micrometers (microns) = μm

SIZE = diameter of field of view (μ) X % of diameter that the specimen covers

To determine the approximate size of an object in the field of view you must approximate the amount of space the object takes and multiply by the diameter field of view. eg. specimen length takes up approximately 30% and the width approximately 15% of the field of view at low power.
length = .33 X 2000 = approximately 660μm or 2000/3= 666μm
width = .15 X 2000= approximately 300μm or 2000/7 = 286μm
Therefore the approximate size of specimen is 666μm X 286μm



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