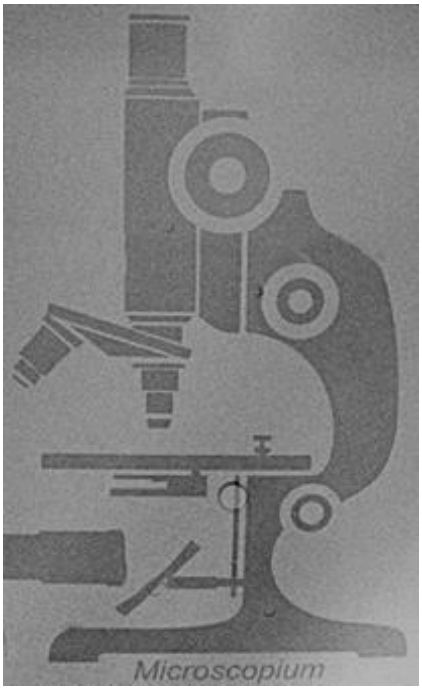
Introduction to Microbiology

Biosci 221
Laboratory Manual



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College of the Canyons
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COLLEGE OF THE CANYONS

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Safety

At College of the Canyons we are very concerned about safety in our Intro to Microbiology labs. Safety is more important than anything else and many of our rules, techniques, and behaviors we teach are to ensure the safety of our students, staff, instructors, etc. In 2013 the American Society for Microbiology (ASM) established guidelines for teaching labs. Most of the organisms we use in our labs are BSL-1; however, we follow the BSL-2 guidelines because we feel that they instruct students on preferred safety procedures and we occasionally use *Staphylococcus aureus*, a BSL-2 bacterium. ASM worked on guidelines due to infections acquired from clinical and teaching labs, noted below. The organism in question is a BSL 2 bacterium and is NOT used in our laboratories.

"The low reported incidence of infections associated with microbiology teaching laboratories suggests that these laboratories are relatively safe (3). However, the importance of biosafety in the laboratory is highlighted by recent multi-state infections by Salmonella Typhimurium associated with clinical and teaching laboratories. Between August 2010 and June 2011 the Centers for Disease Control and Prevention (CDC) linked Salmonella Typhimurium infections in 109 individuals ranging in age from one to 91 years old to a strain used in clinical and teaching laboratories. The infections included twelve hospitalizations and one death (1)." [1]

Learning Objectives:

After this lab you should be able to:
1. List important safety rules.
2. Identify infractions in safety.
3. Comply with and perform the correct safety procedures during lab.
4. Define Biosafety Levels (BSL-1-4).
5. Compare and contrast BSL-1 and BLS-2 organisms and their lab safety requirements.

Introduction:

You will receive instruction on many safety concerns. Keep the handouts to learn from and refer to. Some safety protocols will be introduced later but are referenced here and on the included handouts. The most important things are:
1. Be prepared—read ahead and know what you will be doing in lab on any given day.
2. Follow ALL safety instructions.
3. ASK if you don't know what to do.

Materials:

Included in this lab manual

- Autoclave Instructions (Separate)
- Biosafety Level (BSL) Chart
- Disposal of Biohazardous Materials
- Safety Rules-General and Microbiology specific
- Student Agreement (separate)

Procedures:

Review the following and the handouts. Sign the acknowledgement form.

College of the Canyons
Biological Science Department
Laboratory Safety Guidelines

Each laboratory is a restricted area. Enrolled students may work in a lab only when there are authorized personnel present. Friends of students in lab classes will not be allowed to "visit" inside the laboratory. Students are not permitted into the storage rooms or prep areas unless given specific permission by their instructor or lab personnel.

Ensuring safety in the laboratory is the responsibility of everyone working in the lab. Please follow these guidelines carefully.

GENERAL GUIDELINES:

USE COMMON SENSE WHEN WORKING IN THE LAB.

Be prepared for your work in the lab. Read all procedures thoroughly before entering the lab. Follow all written and verbal instructions carefully. If you do not understand a direction or part of a procedure, ask the instructor before proceeding.
Do not eat, drink or smoke in the lab. Do not use laboratory glassware as containers for food or beverages.
Always wear close-toed shoes in the lab.
Wear safety goggles whenever working with chemicals or when there is an impact risk.
Long hair should be tied back when working with flames, chemicals or dissections.
Observe good housekeeping practices. Work areas should be kept clean and tidy at all times. Keep aisles clear. Push your chair under the desk when not in use.
No open flames are permitted in the laboratory unless specifically indicated by the instructor. When burners or hot plates are being used, caution should be exercised to avoid thermal burns. If you sustain a thermal burn immediately flush the area with cold water and notify the instructor.
If there is a blood spill, immediately notify the instructor.

ANY ACCIDENTS OR INJURIES THAT OCCUR IN THE LAB MUST BE REPORTED TO THE INSTRUCTOR AT ONCE.

Familiarize yourself with the location of the Fire Extinguisher. There is a telephone in each lab room for EMERGENCY use only. In case of emergency dial 7 to reach the school operator who will contact and direct the emergency personnel.
Broken glass is to be disposed of in the broken glass container and reported to the instructor.
Keep hands away from face, eyes, mouth and body while using chemicals or preserved specimens.
Wash your hands with soap and water after performing all experiments. Clean, rinse and wipe dry all work

surfaces and apparatus at the end of the experiment. Return all equipment cleaned and to the proper area.
Handle all living organisms used in a lab activity in a humane manner.
Never use mouth suction to fill a pipet. Use a rubber bulb or pipet pump.
When removing an electrical plug from its socket, grasp the plug, not the electrical cord. Hands must be completely dry before touching an electrical switch, plug or outlet.

HANDLING CHEMICALS

Wear safety goggles whenever working with chemicals.
Chemicals and biological stains should be used with caution. Follow specific directions regarding all chemicals used during lab. Check the label on chemical bottles twice before removing any of the contents. Take only as much chemical as you need.
If any chemical comes into contact with your skin, immediately flush the area with water for several minutes and notify the instructor. A strong base feels soapy on your skin but will still cause a severe burn.
Familiarize yourself with the location of the chemical eyewash. If any chemical is splashed into your eyes, hold eyelids open and flush with water for 15 minutes. Notify the instructor.
Dispose of all chemical waste properly. Do not pour chemicals down the sink unless told to do so by your instructor. Check the label of all waste containers twice before adding your chemical waste to the container.

DISSECTIONS—Special Precautions

Students should consult with the instructor regarding the pros and cons of wearing contact lenses during dissections.
Safety glasses or other protective eyewear is recommended for all students performing dissections.
Protective gloves should be worn during dissections. If your skin comes in contact with a chemical preservative, immediately run water over the area and notify the instructor.
Do not remove preserved specimens from the laboratory.
Preserved biological materials are to be treated with respect.
When using scalpels and other sharp instruments, always carry with tips and points pointing down and away. Notify instructor of any cuts or other injuries.

MICROORGANISMS—Special Precautions

When working with microorganisms, lab coats must be worn at all times.
The laboratory benches must be cleaned with Sanisol (a disinfectant) before and after all lab work.
If cultures are spilled in the lab, they must be disinfected. Pour Sanisol over the spill, let stand for 5 minutes, then wipe up and dispose of towels in a biohazard receptacle.
Never remove cultures from the laboratory.
When finished working with a specific culture place it in the biohazard receptacle provided.

Agreement: (You will be given a separate sheet to sign and turn in, do not turn this in)

I have read and agree to follow the above safety rules for the Biological Science Laboratories.
I realize that I must obey these rules to ensure my own safety and that of my fellow students and instructors.

_____ _____
Student Signature Student Name (print)

_____
Date

Microbiology Specific Guidelines:
These procedures are specific to Microbiology (based on the 2012 ASM Guidelines for BSL 2 teaching labs) and are **in addition** to the General Safety Guidelines.

1. Safety goggles or safety glasses with side shields are required for laboratory procedures involving bacterial cultures.
2. Wear closed-toe shoes that cover the top of the foot, and heel. You will not be allowed to participate if you don't have on the proper shoes.
3. Wear gloves when handling BSL 2 microorganisms or hazardous chemicals, including stains.* 4. Wear laboratory coats. Coats should cover to the knees. Long pants are recommended. You will not be allowed to participate if you don't have on the proper attire.
5. Wash hands after entering and before exiting the laboratory.
6. Tie back long hair.
7. Do not wear dangling jewelry, scarfs, etc.
8. No food, gum, or water in the lab.
9. Do not touch face, including eyes, or use cosmetics in lab.
10. Keep hands, pens, pencils, etc., away from mouth (no nail biting!).
11. Do not use personal pens, pencils, calculators, etc. in lab. These will be provided for you.
12. Cell phones, laptops, etc. are not allowed in lab. If they become contaminated there is no way to disinfect them, and they are a distraction. No exceptions whatsoever. Computers are available in lab when needed.
13. Sanitize lab bench before and after lab activities.
14. Use test tube racks when moving cultures and store plates and test tubes as instructed.
15. Note the location of biohazardous waste and sharps receptacles. Review the section on biohazardous waste disposal.
16. Notify instructor of all spills or injuries. Clean up spills as instructed.
17. If you are pregnant, immune-compromised, or live or work with someone who is, inform your physician that you are taking this class.

***Special instructions for wearing gloves:**
Wearing gloves often makes the wearer feel safe, and this is one reason for wearing them. However, one must also be aware that contamination that gets on gloves can then be transferred to other surfaces, people, pens, pencils, microscopes, etc. So it is very important to follow proper glove protocols.
1. Wear gloves when working with BSL 2 organisms and when staining. Your instructor will let you know when this is necessary.
2. Nitrile gloves are recommended as some people have latex sensitivity. Gloves should fit snuggly. Loose gloves are hazardous in themselves.
3. Gloves are flammable/melt. Use caution when using near a heat source like the Bact-incinerators and hot plates.
4. If gloves become contaminated with a bacterial culture or chemical, or there is a hole in the glove, remove them aseptically as instructed, and put on a new pair.
5. ALL gloves are discarded in the biohazard waste bin.
6. Do not touch other surfaces while wearing gloves, including (but not limited to) things like your face, drawer handles, incubator door, microscope, door handles, sink handles, personal items, pens, pencils, calculators, computer, chairs, etc.

Video on the proper way to glove and un-glove:
https://youtu.be/S4gyNAsPCbU

Biosafety Levels (BSL) for Infectious Agents: Summary only

BSL	Agents	Practices	Safety Equipment (Primary Barriers)	Facilities (Secondary Barriers)
1	Not known to consistently cause disease in healthy adults. Supervision by a scientist with training in microbiology or related field.	Controlled access. Wash hands after working and before leaving lab. No eating, drinking, handling contact lenses, applying cosmetics, storing food. No mouth pipetting. Sharps/broken glassware precautions and disposal. Minimize splashes/aerosols. Decontaminate work surfaces, materials, and contaminated waste. Biohazard posting. Pest control.	Lab coats recommended, Protective eyewear, Gloves, as needed.	Limited access. Sink.
2	Associated with human disease which pose a moderate hazard.	BSL-1 plus: Limited access. Biohazard signage with BSL-2 Demonstrated proficiency with BSL-1. Biosafety lab manual provided.	Lab coats recommended, Protective eyewear, face shields (as needed), Gloves, required. Biosafety Cabinets used when splashes/aerosols may occur.	BSL-1 Plus: Autoclave Eyewash station.
3	Indigenous or exotic agents that may cause serious or lethal disease via inhalation.	BSL-2 plus: Specific training. BSC.	BSL-2 plus: BSC. Protective clothing Respiratory protection as needed.	BSL-2 plus: Restricted access. Physical separation. Self-closing double doors. Negative airflow. Ducted ventilation systems with directional airflow, air not recirculated to other areas. Specially designed lab facilities, clothing areas, etc.
4	Dangerous and exotic agents, high individual risk of aerosol transmission, life threatening, frequently fatal disease, for which there are no vaccines or treatments, or agent with unknown risk.	BSL-3 plus: Training in extremely hazardous infectious agents. Clothing change, shower on exit.	BSL-3 plus: Class III cabinets or Positive pressure supplied air protective suit. Medical surveillance.	BSL-3 plus: Specially designed facilities to prevent dissemination. Secured facility. Decontamination protocols. Redundant systems. HEPA filtered ventilation systems.

Summarized from; for more detail please visit**:**
http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5_sect_IV.pdf

Disposal of Hazardous Materials:
NEVER MIX CONTAMINATED REUSABLE AND DISPOSABLE ITEMS TOGETHER
NEVER PLACE USED ITEMS WITH STERILE ITEMS
NEVER DISPOSE OF ANY CONTAMINATED MATERIALS IN THE REGUALR TRASH
NEVER REMOVE ANY CONTAMINATED ITEM OR CULTURE FROM THE LABORATORY

Discard Cart:

A discard cart is provided in the lab for the disposal of biohazardous materials.

- Small biohazard sharps container--for any sharp item (broken loop, glassware, slide, etc.) that has media in it, or that is contaminated with bacteria.
- Test tube racks—contaminated test tubes. Sort the tubes according to size and place in the correct sized rack to prevent spills. Fill racks from back to front, not randomly. Remove ALL tape from caps and tubes before discarding. Test tubes are NEVER discarded in the biohazard trashcan on the floor.
- The cart may sometimes have containers for pipette disposal, flasks, etc.

If a spill occurs on the cart, thoroughly spray the rack, area, etc., with Sanisol, wait 5 minutes and then with gloves on, wipe up area with paper towels (paper towels and gloves are discarded in the biohazard trashcan on the floor).

Biohazard Trashcan:

The biohazard trashcan located on the floor next to the cart should always have a biohazard plastic liner. Do not discard anything if there isn't a liner—let your instructor know. DO NOT overfill the biohazard trash can; inform your instructor when it is ¾ full. This trashcan is for contaminated disposable items—NO GLASS ITEMS, ever. For example,

- Plastic petri dishes
- Gloves
- Contaminated paper towels
- Contaminated swabs, etc.

Specifics on different materials:

Glass Test Tubes: These tubes are autoclave, washed, and re-used. Remove all tape and place upright in the appropriate sized rack on the discard cart. For broken tubes—remove cap if possible and place cap on the cart; place the broken tube in the small biohazard sharps container on the cart (clean, dry broken tubes may be discarded in the cardboard sharps box).

Plastic Petri Dishes: DO NOT remove markings; it is not necessary since the plates will be autoclaved and discarded. These are placed in the biohazard trashcan.

Contaminated paper towels and gloves: Place in the biohazard trashcan. Remove gloves carefully as instructed.

Uncontaminated paper towels: should be thrown away in a regular trashcan, not the biohazard trashcan. All gloves are discarded in the biohazard trash can.

Pipettes: All serological pipettes should be discarded in pipette trays, on each lab bench, containing Sanisol. A small biohazard container is usually provided at each station for swabs, microfuge tubes, and transfer pipettes.

Scalpels, forceps, spatulas, etc.: These items are usually placed in the pipette trays. Follow directions given by your instructor.

Glass Slides: All microscope slides once stained, will be discarded in dishes of sanisol on each lab bench. Broken contaminated slides are disposed of in the small biohazard sharps container on the discard cart (broken clean slides may be disposed of in the cardboard sharps box).

Flasks, weigh boats, blenders, etc.: When these types of additional materials are used, you will be instructed on how and where to discard them. Usually, there will be a separate cart with tubs for flasks; weigh boats are usually washed and placed on the drain board, etc. Pay careful attention to instructions.

Questions: Answer after reviewing all handouts

1. What Personal Protective Equipment (PPE) should you wear when:

    a. Staining bacteria

    b. Writing pre-lab lecture notes

    c. Using a BSL 2 culture like *Staphylococcus aureus*

2. What should you do if you break clean glassware or slides?

3. Give examples of some things you should not wear in lab.

4. What is a major concern while wearing gloves?

5. Where does one dispose of bacterial cultures in petri dishes when finished with them?

6. Where does one dispose of bacterial cultures in test tubes when finished with them?

7. Draw a diagram of the lab and indicate where the eyewash station, shower, fire blanket, and fire extinguisher are.

8. Describe the procedure for leaving lab once you are finished with your work.

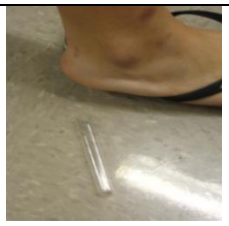
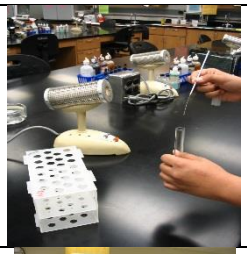
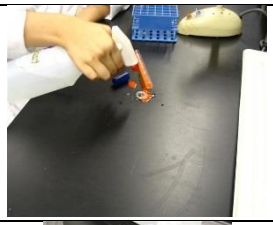
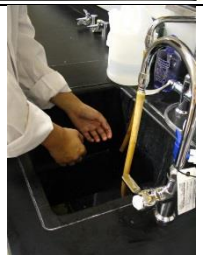
Conclusion:

Describe why lab safety is especially important in a microbiology lab and give important procedures for working with live bacteria.

Resources:

1. Emmert, Elizabeth A.B., ASM Task Committee on Laboratory Biosafety. "Biosafety Guidelines for Handling Microorganisms in the Teaching Laboratory: Development and Rationale." *J. Microbiol. Biol. Educ.* May 2013 vol. 14 no. 1 78-83. doi:10.1128/jmbe.v14i1.531. Web. 22 Oct. 2016. http://www.asmscience.org/content/journal/jmbe/10.1128/jmbe.v14i1.531#b3-jmbe-14-78

Safety Rule Reminder: Biosci 221

SAFETY RULE:			*WHY?*
Closed-toe and closed-heel shoes			*Safe shoes prevent cuts, contamination from spills, slipping, and protect from dropped items.*
No Food or Drink!			*It's too risky to take a chance with contamination! Keep all personal items off the lab bench too.*
Keep Hair Tied Back			***Fire! & Burns!***
Clean Spills Properly			*Contamination <u>will</u> s p r e a d and that puts yourself and others at risk!*
Keep Hands and other items away from face and mouth!			*Eyes, nose and mouth are "portals of entry" for bacteria.* *Always wash your hands before you leave the lab and after any contact with cultures.*
Use Common Sense!		Think, ask, understand, **THEN**, do! 	*Protect Yourself; Protect your cultures; Protect Others!*

The Metric System, Measurement, and Lab Equipment Review

As you know, the metric system is the unit of measurement (International System of Units, or SI) in most countries and the only system adopted by the international science community. Although we don't often use the metric system in the United States to quantify things in our daily lives, it is what we use exclusively in science. Interestingly, the U.S. has been moving to convert to the metric system for decades, and many things are labeled with the metric system. Think of a two-liter bottle of soda, a 5K charity run, a 600mg dose of Ibuprofen, etc. The Metric System has been in use since it was invented in France in the 1790's; perhaps one day we will catch up!

Learning Objectives:

After this lab you should be able to:

1. Properly identify and use various types of glassware, pipettes, and other basic lab equipment.
2. Measure weight, length, and volume in the Metric System.
3. Make unit conversions within the Metric System.

Introduction:

In today's lab you will re-familiarize yourself with basic lab equipment used for measuring. It is important to become proficient with the use of these items and the metric system because it is what scientists use, but equally importantly, it is what you will use during all subsequent lab activities. The metric system is a decimal based system that is very easy to use and convert within, once you get the hang of it. The practice in this lab should help make using the metric system more natural and automatic.

The basic units in the metric system are:

Weight: gram (g)

Length: meter (m)

Volume: liter (L)

Temperature: Celsius (C) *(not technically metric, but the system that scientists use)*

There are several benchmarks for getting a feel of the metric system. For weight, a dollar bill weighs one gram and a penny weighs 2.5g. So if weighing something out that is 25g, think about the weight of ten pennies in your hand.

For length, the meter is 39.37 inches, so slightly longer than a yard (which is 3 feet). A 100-yard football field is slightly more than 91 meters. On a smaller scale, the length of a centimeter is about the width of a standard paperclip or the average width of the nail on your pinky finger! And a millimeter is about the width of your ATM card.

You are already familiar with one and two liter (2L) volumes due to the packaging of soda and water bottles. An additional point of reference is that 1 cup (8 ounces) is about 237 milliliters. So if you had to measure a volume of 250ml it would be slightly more than one cup.

Finally, think about temperature. Your body temperature is probably about 98.6 ^{o}F, which is 37^{o}C. You will notice in our labs that we often set the incubators to 34-35^{o}C, which is a little below body temperature at 95^{o}F. Room temperature, 70^{o}F, is 21^{o}C. Now you know that if you are travelling and the temperature is 25^{o}C, it will be a beautiful day (77^{o}F). Of course 100^{o}C is boiling (212^{o}F).

The good news is that we are not going to ask you to convert from standard U.S. measures to metric very often. Rather we just want you to use the metric system and eventually it will become familiar and easy. The other good news, though it takes a while to catch on sometimes (as you may know from previous coursework), it is very easy to convert within the metric system. You just have to get the hang of it, use it, and it will stick.

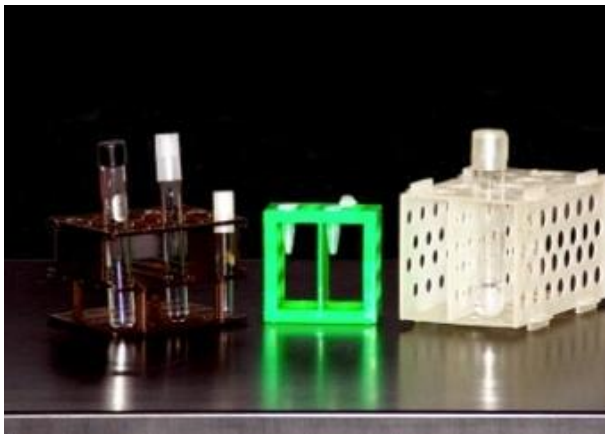
These are the prefixes that are most commonly used in basic science labs. The prefixes are the same for gram, meter, and Liter:

Prefix	Abbreviation	Decimal equivalent	Exponential Equivalent
pico	p	0.000000000001	10^{-12}
nano	n	0.000000001	10^{-9}
micro	u	0.000001	10^{-6}
milli	m	0.001	10^{-3}
centi	c	0.01	10^{-2}
none		1.0	10^{0} =1
kilo	k	1000.0	10^{3}
giga	g	1,000,000,000.0	10^{9}

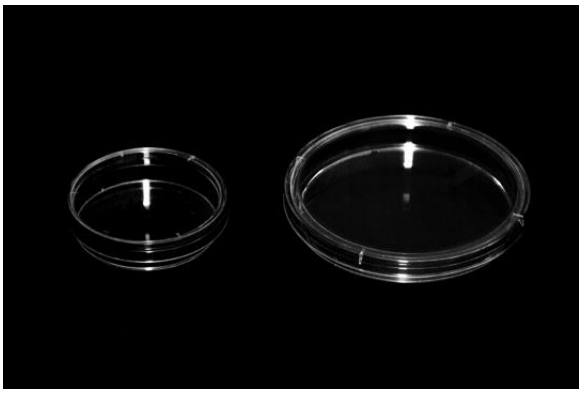
It should be noted that the common term for a micrometer is "micron" (um).

Equipment:

Test Tubes and Racks



Petri Dishes



Glassware

Pipettes

Pre-lab questions:

1. What is the equivalent term for milligram in volume and length?

2. How much larger is a centimeter than a micron?

3. If you were traveling to Germany and the posted temperature was 32°C, what clothing might you need?

4. A typical soft drink may have as much as 39g of sugar. How many teaspoons is this? (Google this!).

Materials:

250 ml beaker with marked graduations
100 ml graduated cylinder, 25ml graduated cylinder
Digital scale
Glass slides
Standard petri dish
Small petri dish
Small metric rulers
Standard test tube
Durham test tube
4 empty standard petri dishes labeled 5 ml, 10ml, 15ml, 20ml
TSA Plate
Small squares of Parafilm
Transfer pipette
1 ml serological pipette
5 ml serological pipette
Microfuge tubes and racks
p20 Micropipette and tips
p200 Micropipette and tips
p1000 Micropipette and tips
Colored water or coloring dye
Beaker of RT water with thermometer beside the beaker
Beaker of water in an ice bath with thermometer beside the ice bath
Beaker of water on a hot plate, boiling with boiling stones, with thermometer beside the hot plate

Procedures:

Weight:

1. Weigh an empty 500ml beaker and record its weight to the nearest tenth of a gram (remember to tare the scale first). Then measure 50ml of water in a graduated cylinder, add it to the beaker, and weigh the beaker again.

Weight of empty beaker: _____

Weight of beaker plus water: _____

Calculate the weight of the water: _____

How much would 1ml of water weigh? _____

2. To get some reference points for weight, pick several items to weigh, including your cell phone. Other items could be a pen, a coin, your wallet, etc. Take the weight of the item in grams on the scale and convert to the other values. Give the values below. NOTE: Enjoy having your cell phone out for this, in the future you will never have electronic items out in class.

Item	kg	g	mg
Cell Phone			

Length:

1. Measure the l x w x h (mm) of a standard glass slide and give the dimensions below:

_____mm x _____mm x _____mm
l w h

2. Measure the diameter and height of standard and small petri dishes:

Standard petri dish dimensions (d x h) in mm:

Small Petri dish dimensions (d x h) in mm:

3. Measure the height and diameter of a standard test tube and a small “Durham” test tube:

Standard test tube dimensions (d x h) in mm:

Durham tube dimensions (d x h) in mm:

4. View the bacterium *E. coli* via the microscope on display. Bacteria are very small organisms. E coli averages about 3um in length. As you look in the microscope, keep in mind that the *E. coli* is magnified 1000x. So it is very small indeed! The average lengths of several bacteria are given in um below. Convert these values to cm and mm.

Bacterium	um	mm	cm
E. coli	2		
Bacillus cereus	4		
Streptococcus	1		
Rhodospirillum	8		

Volume:

1. Fill a 2500ml beaker with 100ml of water using the 100ml marking on the beaker. Pour the water into a 100 ml graduated cylinder. What does the volume of the water read in the graduated cylinder (remember to read at the meniscus)?

Volume in the graduated cylinder: _____

If the volume is different than 100ml calculate the percent error of the 500ml beaker:

% Error = Exp-Obs/Obs x 100 (Note: there is no negative % error)

% Error =

2. Use a 25ml graduated cylinder and measure the following volumes of water- 10ml, 15ml, and 25ml. Pour the water from each test tube into the 3 labeled petri dishes. Compare the volume of water in the 3 petri dishes with the volume of media in a normal TSA plate.

a. What is the probable volume of media in the TSA plate?

TSA Plate volume = _____ml

b. How many TSA plates could be made from 1L of prepared liquid media?

c. Empty the petri dishes when done.

Pipetting practice:

As you do these exercises think about when and why you should use the different types of pipettes. Also make sure that you know how to measure with the serological pipette—this is a common source of confusion and therefore measurement mistakes.

Place a square of Parafilm on your lab bench. Use the tube of water at your bench (if colored water is available use it, or add coloring to the water).

1. Transfer pipettes:
Using a transfer pipette transfer 0.5ml from the beaker to the Parafilm (the water should bead up on the Parafilm). Using a 1.0 ml serological pipette withdraw 0.5 ml (read from the bottom up) of water from the beaker and dispense onto the Parafilm a few cm away from the first drop.

a. Do the drops look the same size?

b. Using the 1.0 serological pipette, pipette up the first drop you made with the transfer pipette. What was the actual volume of the water dispensed from the transfer pipette?

_____ml

c. Is this what you expected? Why or why not do you think?

d. When would it be appropriate to use a transfer pipette vs. a serological pipette?

e. When is it more appropriate to use a serological pipette than a transfer pipette?

2. Serological pipettes:
Dry off the Parafilm square. Then, use a 5 ml pipette to transfer 1ml of the colored water to the Parafilm. Do the same with a 1ml serological pipette.

a. The drops should look identical. Do they?

b. Dry off the Parafilm again. Use a 5ml pipette and a 1ml pipette each, and transfer the following volumes to the Parafilm in two separate rows as in the pattern below: 1ml, 0.5ml, 0.1ml. Draw a picture of your drops below.

	1.0ml	0.5ml	0.1ml
5ml pipette			
1ml pipette			

c. When would you use a 5ml pipette instead of a 1ml pipette?

d. When would you use a 1 ml pipette instead of a 5ml pipette?

e. Fill a microfuge tube with the colored water using a transfer pipette, using a 5ml pipette to withdraw the contents. How much volume will the microfuge tube hold?

3. Micropipettes:
Micropipettes are most often used for very accurately measuring very small volumes. It would be difficult to measure 0.01ml with a 1.0ml pipette but easy with the appropriate micropipette. Each pipette uses a different pipette tip that fits snugly, and is calibrated with the pipette to withdraw and dispense the correct amount. Pipette tips can be ejected from the pipette without touching them, which can be very important when transferring microbial cultures! Pipette tips are disposed of in small biohazard buckets that are provided.

Your instructor will review how to withdraw and dispense liquids and eject the pipette tips. Make sure that you understand how to do this prior to using a pipette.

The most commonly used micropipettes are the p20, p200, and p1000. The values 20, 200, and 1000 refer to the max volume (in ul) each pipette can deliver. Convert the following:

20ul = _____ ml

200 ul = _____ml

1000ul = _____ml

Turning the thumbwheel gauge on the top portion of the pipette sets volumes for each. Be very careful when adjusting these. Do not over rotate the dial attempt to adjust the pipette to higher or lower than the range for that pipette. Doing so can damage the pipette. The range for each is:

p20 measures 2ul-20ul

p200 measures 20-200ul

p1000 measures 200-1000ul

a. The gauge for each pipette is unique in the range it represents. Note what the gauge looks like, and what it actually represents:

p20	p200	p1000
1	1	1
0	0	0
0	0	0
10.0ul	100.0ul	1000.0ul
_____ml	_____ml	_____ml

b. Make sure your piece of Parafilm is dried off again. With the p20 set the gauge to 100 and withdraw 10.0ul from your beaker of colored water and dispense onto the Parafilm. Set the p200 to 100 and withdraw and dispense 100.0ul of water onto the Parafilm. Set the p1000 to 100 and dispense 1000.0ul onto the Parafilm. Compare the sizes of the drops. Draw below:

c. Micropipette challenge: Each person in your group should do the following. Use a p200 and dispense the following amounts of water (from your beaker of colored water) into a microfuge tube, resetting the thumbwheel as needed:

Transfer 20ul, then an additional 35ul, and finally an additional 65ul. What is the total volume you have dispensed?

Total volume = _____ul

Compare your microfuge tubes to your lab partners'. Do all the tubes appear to have the same volume?

Now, exchange microfuge tubes with each other so that no one has their own tube. Using a p200 set the gauge to the total volume you determined above and withdraw the liquid from the microfuge tube.

Were you able to withdraw the entire volume of the tube or is there some left in the tube?

Were there any air bubbles in the tip of the pipette?

What could be some sources of error in transferring these volumes?

Temperature:

Observe and record the temperature in Celsius in the three water samples. Handle the thermometers with care; they are easily broken. Place the thermometer in each beaker without letting the thermometer hit the bottom of the beaker and hold it for one minute, record the T and remove the thermometer and place it gently on the lab bench.

	Temperature (C)
RT beaker	
Ice bath beaker	
Hot plate beaker	

Questions:

1. What do the following prefixes mean in relationship to the standard base unit (g, m, L)?

Pico = _____ Micro =_____

Kilo = _____ Milli = _____

Centi = _____

2. Circle the value that is larger than the other?

5cm or 40mm	150ml or .200L	1g or 1kg
2mg or 3 cg	100 mm or 10km	100L or 1000ml

3. Fill in the following table:

kg	g	mg	ug
2			
		2	
	2		
			2

4. Determine the equivalents:

27g/L = 27g/_____ ml	0.1ml = _____ul	200g = _____ug
_____mm= 30um	_____cm = 6m	5 cm= _____mm

5. Interpret the following micropipette displays and give the correct volume in ul:

p20	p200	p1000
1	1	0
3	2	8
0	1	4
_____ ul	_____ul	_____ul

6. Draw the following:

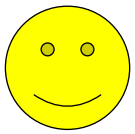
Beaker:

Erlenmeyer flask:

Graduated cylinder:

Transfer pipette vs. serological pipette (draw each to compare):

7. When is it appropriate to have electronic devices out in class? Check the correct box:

_____ Never 

Conclusion:

1. Metric System-
Reflect on what you have learned, or re-learned about the metric system. Did you remember the prefixes and their values? Did you remember how to convert from cm to mm, or kg to gram for example? How confident do you feel about understanding and using the metric system?

2. Equipment:
Do you feel confident about which measuring tools to use? For example, when might a transfer pipette be appropriate over a micropipette? Do you feel confident measuring 0.3ml with a 1.0ml serological pipette?

3. What do you need more assistance or practice with? What do you need to do to understand the metric or measuring better?

Resources:

Update of sorts on the current status of the teaching and use of the metric system in the U.S.:
http://www.theatlantic.com/education/archive/2016/06/why-the-metric-system-hasnt-failed-in-the-us/487040/
Metric Practice:
https://www.khanacademy.org/math/pre-algebra/rates-and-ratios/metric-system-tutorial/v/unit-conversion
More than you might want to know about the history of the metric system, but a short read:
http://www.us-metric.org/origin-of-the-metric-system/
Micropipettes:
https://www.mcdb.ucla.edu/Research/Goldberg/HC70AL_Su14/pdf/How%20to%20Use%20a%20Micropipettor.pdf

Microscopy

Animate Life: Seeing the Invisible
Van Leeuwenhoek's first glimpses of the microbial world
https://youtu.be/ePnbkNVdPio
"Most of life is invisible"

Imagine being the first person to see microscopic life.

Learning Objectives:

After this lab you should be able to:
1. Prepare wet mounts and hanging drop slide of live specimens for observation.
2. Identify parts of the microscope and define their functions.
3. Correctly focus samples for observation
4. Demonstrate correct use, care, cleaning, and storage of the microscopes.

Introduction:

Van Leeuwenhoek, in the late 1600's, was the first person to see the microbial world. Although microscopes had been invented earlier, Van Leeuwenhoek perfected a technique to produce lenses that were unmatched for centuries. With these lenses he made thousands of observations on all manner of specimens. Primarily, as a cloth merchant, he was hoping to better observe the quality of his cloth goods. Instead, he discovered an entirely new world. For microbiologists, this is the beginning of their entire field, and the seminal event in natural science. Prior to Van Leeuwenhook no one knew microbes even existed. He truly changed man's perception and knowledge of the world around us in a most fundamental way. An eccentric non-scientist he is now revered for his curiosity, lens crafting, and discovery of microscopic life. In this lab you will be introduced to techniques in microscopy that Van Leeuwenhook could never have dreamed of. Electricity, Nobel Prize winning advances in optics, knowledge of atomic forces and particle physics, have led to microscopes that can allow us to observe small molecular structures on the nano-scale. As you view the live Protists in lab today you will be expecting to see small organisms swimming around, and you may take that for granted. Imagine not expecting to observe anything living and the surprise you would feel at seeing wiggling "animalcules" in your field of view!

Light Microscopy:
The light microscope uses visible light and a series of lenses in order to view microscopic specimens. The condenser lens sits above the light source and below the stage and specimen. The condenser lens focuses the light as it goes through the specimen and can be adjusted for optimization. The objective lenses magnify the specimen, capture the transmitted and reflected light and create a **real image** of the specimen. The ocular lens further magnifies the image and creates a **virtual image** for viewing. The total magnification of a specimen is obtained by multiplying the magnification of the objective lens by the ocular lens.

What you observe through the microscope is your **field of view**. As one increases the magnification, your field of view decreases. At low magnification you might see an entire organism. As you move to higher magnification you zoom in onto a smaller portion of the organism. The **depth of field** is the depth of the plane of focus—how "deep" something is in focus. As one increases magnification, the depth of field decreases. **Working distance** is the distance between your specimen/slide and the objective lens. As magnification increases the working distance decreases substantially. When using oil immersion the working distance is very reduced and one must be extremely aware NOT to use the coarse focus with any objective other than low power (10x).

Refraction of light is an important concept to understand when learning how to use a microscope. As light moves through different media (air, glass, water, etc.), from one to another, the light "bends" or refracts. The angle of refraction depends on the types of media the light is passing through. Different media will have a different refractive index. This is why you need to wear swim goggles in order to see

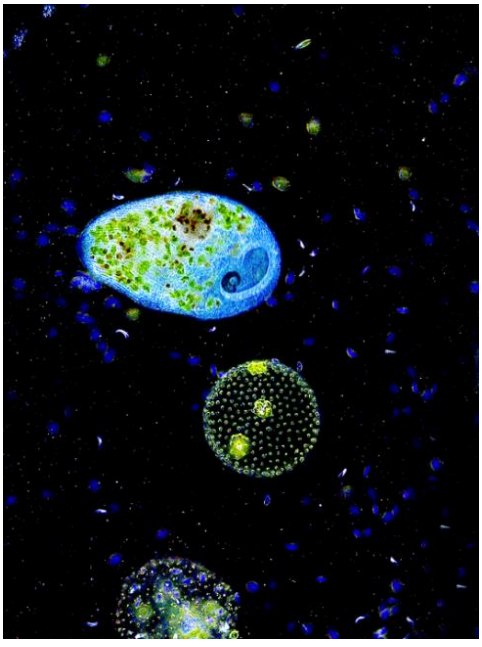
clearly underwater. As light moves from air to water it refracts, wearing goggles creates an air space in front of your eyes and the light bends again, in essence correcting itself in terms of your ability to see clearly.

Another important concept in microscopy is **resolution.** Resolution is the ability to distinguish two points as separate points. If the points are too close together they cannot be "resolved" and actually look like one single point. It's convenient to think of this as clarity. In a light microscope the resolving power is limited to objects that are about 0.2Um apart.

Your microscopes have a special 100x **oil immersion** lens. This lens is actually immersed in a drop of oil that one places directly on top of a stained specimen on a slide. The oil used has the same refractive index as glass. Normally, light passes from the glass slide/specimen through air, and then into the glass objective lens. As the light moves from glass to air to glass, it refracts and light is lost. When using oil immersion, light passes from glass to oil (with the same refractive index as glass) to glass. In this case, the light does not refract and optimal light collection in the objective lens is obtained. Oil immersion does not increase magnification but does increase resolution (clarity).

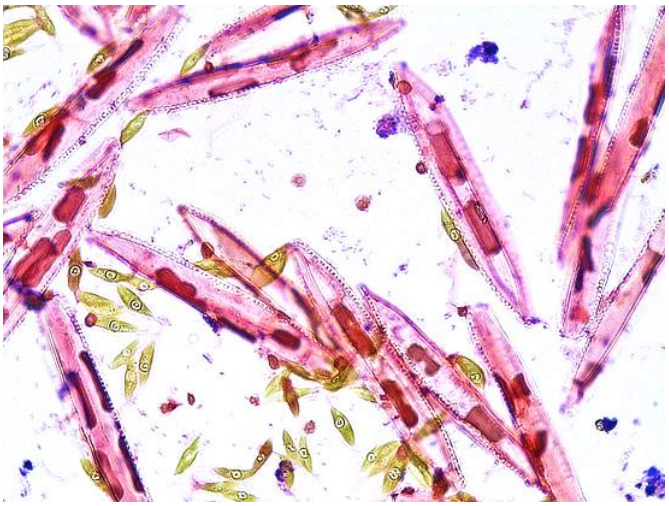
There are several types of light microscopy. The Olympus microscopes have a special turret under the stage that adjusts for different viewing options. 0 is for Bright-field, DF is Dark-Field, PH 1, 2, 3, are for Phase settings.

Bright-field (BF) microscopy is probably what you are most familiar with. The light passes through the specimen and is collected in the objectives. While there is refraction, much of the light passes through the specimen and into the objective. Unstained specimens are difficult to see so microscopic cells are typically stained to increase contrast so that they can be seen. The stained specimens appear dark against a "bright" background.



DF microscopy of *Stentor* and *Volvox.* 400x
KBurke. CC-BY-NC-SA 4.0

In



Gram stain of freshwater diatoms, euglenoids, and bacteria. 400x
KBurke. CC-BY-NC-SA 4.0

Dark-field (DF) microscopy, some of the rays of light are blocked as they pass through the condenser. This is via a special occulting disc that essentially scatters the light and the light reflected off the specimen is collected in the objective. This gives a very vivid and beautiful effect when viewing live specimens like Protists. Make sure that you view the live Protist samples with Dark-field, you won't be disappointed!

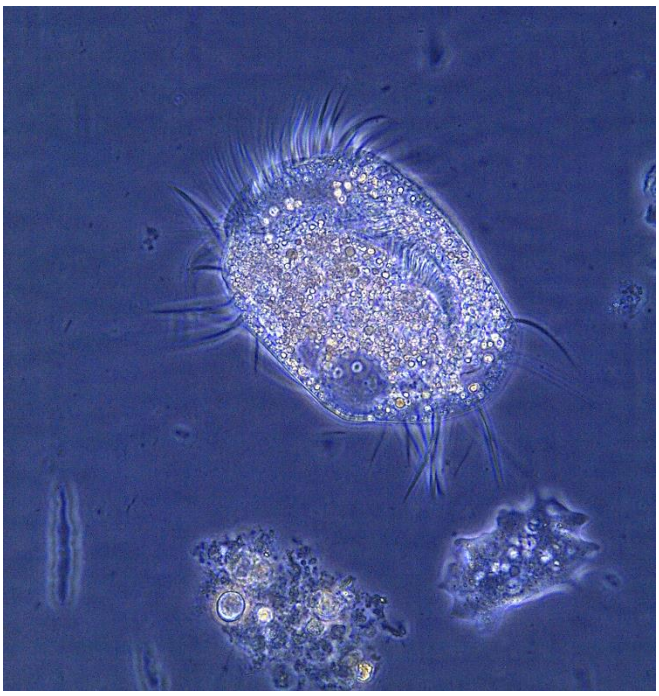
Phase Contrast microscopy is a technique for viewing live specimens, which enables one to see the details and finer structures of cells. As stated above, most live cells are difficult to see in BF microscopy because of the lack of contrast. Staining does not usually help see fine cell details, unless sophisticated differential staining techniques are used. In addition, staining often kills cells, and can alter specimens. Further, stained cells are not moving, feeding, metabolizing, dividing, etc. So phase microscopy has many advantages. To understand how phase contrast works think about a typical eukaryotic cell. This cell would have a nucleus, cytoplasm, perhaps cilia on the outside, etc. Each of these cell structures have different densities, and so refract light differently.

If you follow the light pathway in a phase scope it first passes through a condenser annulus. Only light in parallel waves passes through the openings in the annulus and illuminates the specimen. Some light passes straight/undeviated around the specimen. Other light passes through the specimen and is diffracted by the specimen. Different parts of the specimen will diffract the light differently because of the relative thickness of different parts of the sample (the diffracted light is also scattered). This causes a "phase shift" because when light is diffracted it slows. The thicker the specimen (or part of the specimen) the more the light wave is diffracted and thus, slowed.

Undeviated light surrounding the specimen is not slowed because it is not diffracted (no change in the medium the light is passing through, so no bending—or not very much!).
So the thickness of different parts of the specimen will refract (bend) the light to different degrees. Therefore the location of the light emerging from the specimen into the objective is different than the light passing through the surrounding media. This is the phase shift.

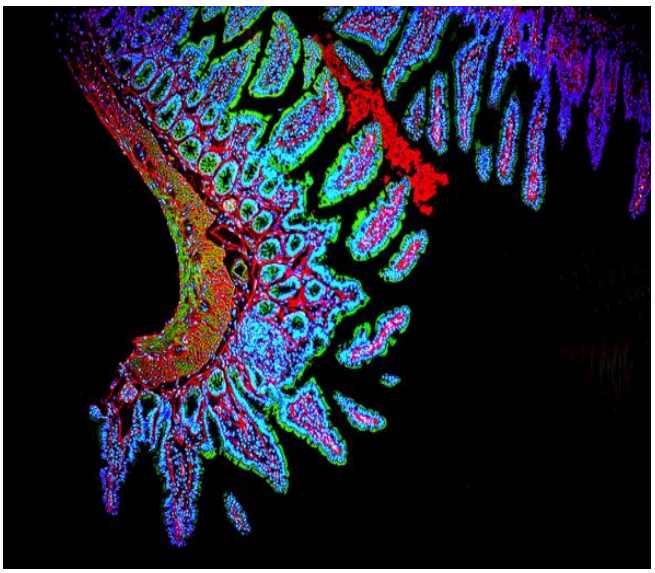
The undeviated light and diffracted light is then collected in the objective and passes through a phase plate in the objective which alters the undeviated light to further enhance contrast. Ultimately they, diffracted light and undeviated light waves, combine and are focused in the objective to form the image we see. The image depends on the intensity differences in the undeviated and diffracted light waves, which causes contrast. In regular bright-field microscopy, there are no "phase" differences and so very little contrast.

This ultimately increases contrast between the background light, and light passing through cell structures giving a 3-D effect and the ability to see many of the cell structures. An artifact of this, however, is a halo effect around cells.



Phase contrast microscopy of *Euplotes* 400x
KBurke. CC-BY-NC-SA 4.0

The invention of this technique earned Frederick Zernike a Nobel Prize in 1953!



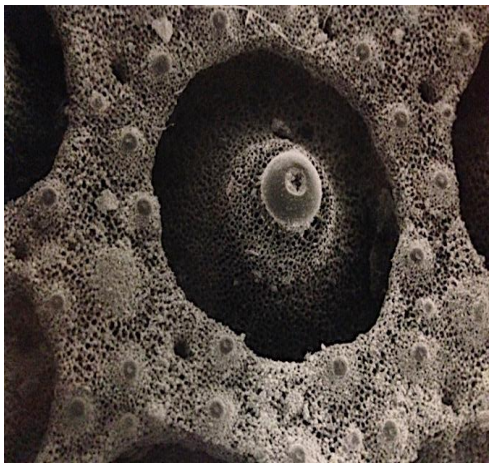
Rat gut. 400x
KBurke. CC-BY-NC-SA 4.0

Fluorescent microscopy is a technique that utilizes fluorescent dyes to stain different parts of cells. Often the dyes are attached to antibodies that very specifically attach to different bacteria or cell structures. These dyes are called fluorophores. They absorb certain wavelengths of light and emit others. Fluorophores come in a range of colors that span the visible spectrum. Three of the most common fluorophores used are **DAPI** (emits blue), **FITC** (emits green), and **Texas Red** (emits red).

A conventional light microscope uses visible light (400-700 nanometers) to illuminate and produce a magnified image of a sample, while a fluorescence microscope uses higher intensity **ultra violet light** that excites fluorescent molecules in a specimen. Following excitation, the fluorescent molecules will then **emit** a longer wavelength light, which produces the magnified image of the sample.

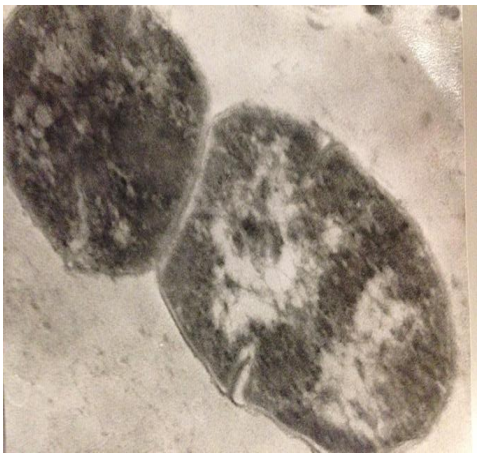
Scanning and Transmission Electron Microscopy (TEM/SEM): Your instructor may also discuss electron microscopy, which utilizes electrons instead of visible light. You should refer to your textbook to read more about this and other types of modern microscopic techniques.

TEM:



SEM, Sea Biscuit test
KBurke. CC-BY-NC-SA 4.0

SEM:



TEM, *Streptococcus agalactiae*
KBurke. CC-BY-NC-SA 4.0

Measurement: Using the ocular micrometer

The ocular micrometer is a ruler located in one of the ocular lenses in the microscope. This ruler is fixed. However, as you increase magnification, the value of each of the individual spaces changes because the image is magnified. At low power (10x) for example, one might be able to see an entire organism, whereas at high dry (400x) or oil immersion (100x), one would only be able to see part of the organism because it is magnified. There is an inverse relationship between the value of each space in the slide and the magnification. As magnification increases, the value of each space will decrease proportionally. Your instructor will go through calibration of your ocular micrometer.

Pre-lab questions: Read the Microscopy chapter in your textbook prior to lab.

1. Compare and contrast Bright-field, Dark-field, and Phase Contrast microscopy.

2. What would the total magnification be for the following?

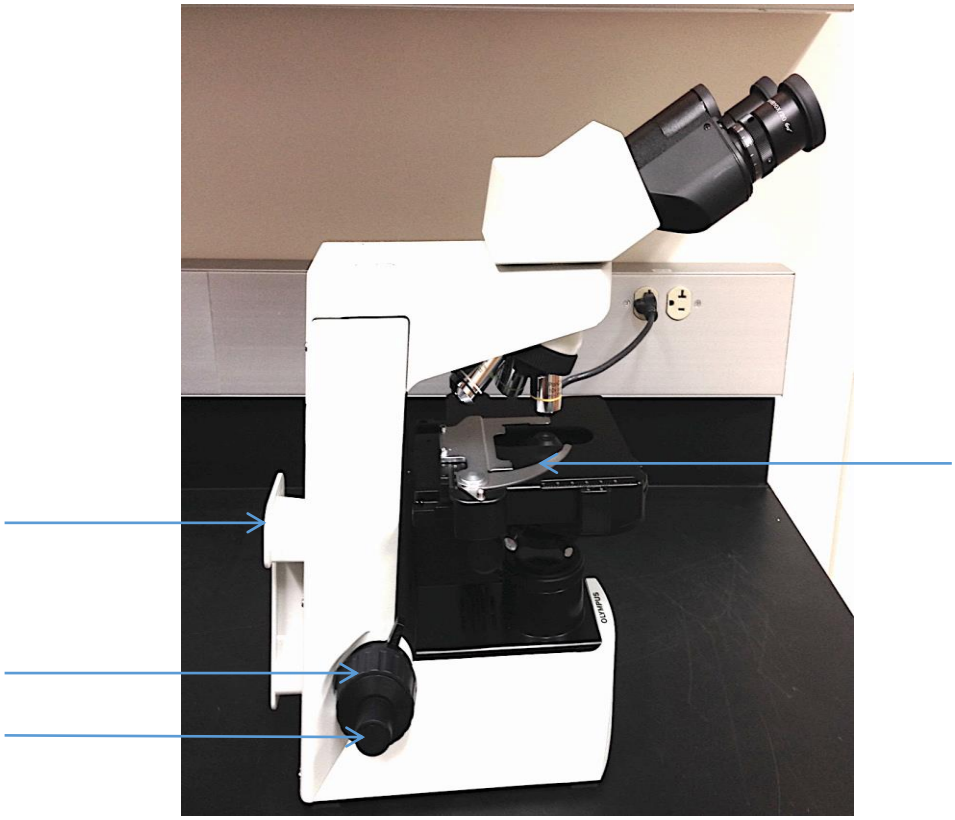
Objective magnification	Ocular magnification	Total magnification
20	15	
16	10	
100	15	
100	10	

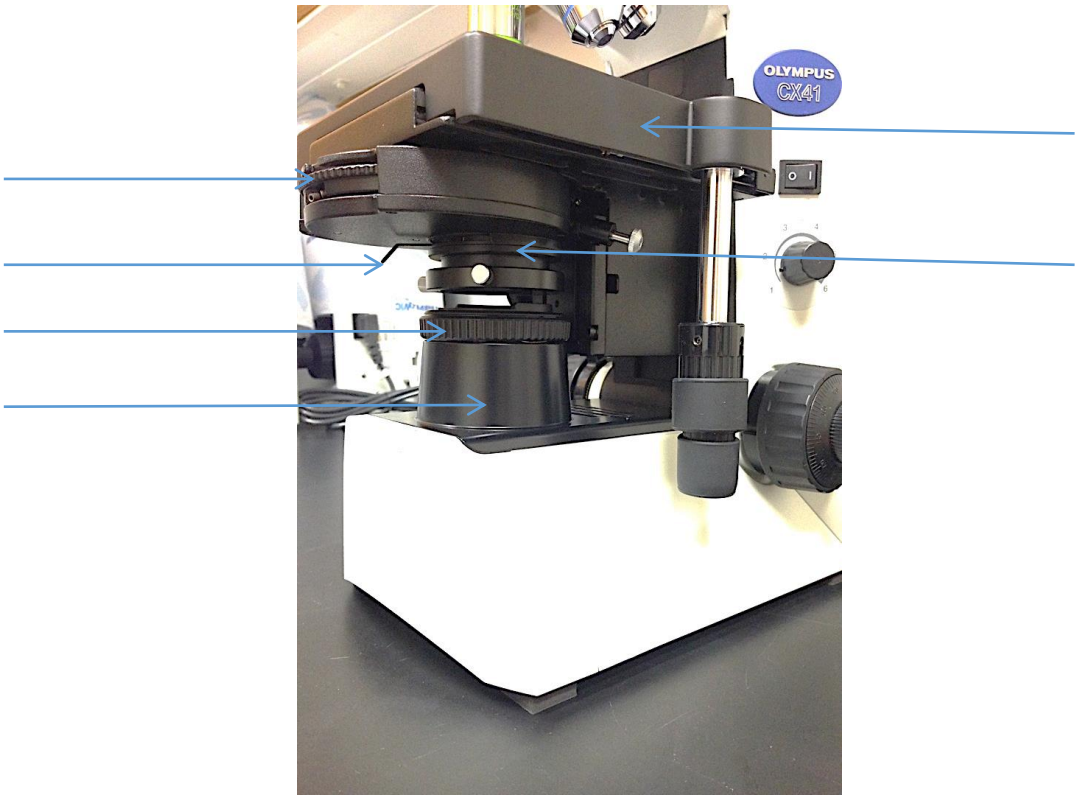
3. Why is it often necessary to stain specimens?

4. Give an advantage of dark-field or phase contrast microscopy over bright-field:

Parts of the Compound Microscope:

Label the parts of the microscope indicated by the arrows:




OLYMPUS
CX41

Materials:

Wet Mount Materials:
Glass slides
Hanging drop slides
Coverslips
Vaseline
Detain/Protoslo

Live Material:
Mixed Protists

Prepared Slides:
Amoeba proteus
Trypanosoma gambiense (West African trypanosomiasis)
Plasmodium vivax (malaria)
Paramecium caudatum
Giardia lamblia (giardiasis)
Cryptospiridium parvum
Entamoeba histolytica (Amoebic dysentery)
Trichomonas vaginalis
Balantidium coli

Procedures:

1. Use and care of the microscope:

1. ALWAYS carry the microscope upright by holding the instrument with one hand on the arm of the scope and the other under the base.

2. Clean the oculars and objectives before you start: use ONLY special LENS PAPER for cleaning the lenses and lens cleaner; NEVER use Kimwipes, paper towels, or any other paper or cloth. You may use Kimwipes for cleaning dirty slides, stage, etc.

3. Connect the light source, turn on at the switch, and adjust light to a medium intensity. Make sure that the stage diaphragm and field diaphragms are open.

4. Place the slide specimen over the opening on the stage under the low power (4X or 10X) and center it over the light.

5. Before looking into the microscope, turn the coarse adjustment so that the stage, with slide, is at its highest position. Do not do this on anything but low power, at high power the stage can hit the objective. Also, only use the coarse adjustment with the low power objective. NEVER use the coarse adjustment with any other objective.

6. The substage condenser, if moveable, should be raised to its highest position. You may need to adjust it later.

7. For the safety of your sight, keep both eyes open when looking into a microscope. This will take some practice, but you will soon master the technique. The oculars can be adjusted to match your intraocular distance, the distance between your eyes, for maximum comfort and clear viewing. The oculars can also be individually focused by looking through each, one at a time, and then rotating the ocular in or out—this is especially helpful if there is a discrepancy between the vision of each eye.

8. Look into the ocular and gradually turn the coarse adjustment knob, lowering the stage until the object on the slide comes into view. When the object is in approximate focus, use the fine adjustment knob to get the sharpest view.

9. Use the iris diaphragm to change the amount of light coming through the object on the slide when needed. The use of this light control is very important. When plant or animal tissues are not stained or poorly stained, they may be made more visible by DECREASING the amount of light. Open and close the diaphragm several times to note differences in the appearance of the object being viewed.

10. Adjust the sub-stage condenser to provide a clear white background. Practice moving the condenser up and down to see the effect the condenser has on image clarity. Proper condenser height allows for the light rays to cross through the specimen at the optimum point.

11. The microscopes are "parfocal"; if the image is in focus at low power it will be nearly in focus at a higher power. To view specimen at higher magnification, first focus on low power then center the object in the field of view and rotate the nosepiece until the high dry power objective clicks into place. You should only need to focus slightly with the FINE ADJUSTMENT KNOB ONLY. You may need to turn the light adjustment to the next highest number and adjust the diaphragm accordingly.

12. Never use the coarse adjustment knob with the high dry power objective. When using slides with thick covers or thick preparations, look at the position of the objective at low power and watch as you turn the nose piece to high power . . . NEVER FORCE the high power into place.

13. The 100x objective is for OIL IMMERSION only. You will be instructed on this separately in more detail. In short, once your specimen is in focus on high dry power, move the 100x objective towards the slide, but not quite in place. Place a drop of immersion oil directly on the area of the slide that you are viewing and then click the 100x objective into place. Use the fine focus carefully to sharpen the image. The lens will be immersed in the oil. This technique allows maximum light rays to be focused through the specimen for improved resolution at the highest magnification (1000x) that your microscope will allow. NEVER USE OIL ON ANY OTHER LENS, AND DO NOT USE THE 100X LENS WITHOUT OIL. **Be very cautious not to drag the 40x lens through the oil.** Always clean all lenses after using oil immersion. Particularly, **check the 40x for oil**, and clean the 100x completely.

13. Before putting the microscope away: remove all slides, clean stage, set the low-power objective into place, move the stage to the maximum distance away from the objectives, turn off light at the switch, and unplug the microscope by the plug – NEVER pull the cord. If oil was used, remove any oil with the lens cleaner and lens paper. Gently coil cord around the cord holder or wrap with velcro strap.

    YOUR INSTRUCTOR MUST CHECK YOUR MICROSCOPE BEFORE YOU PUT IT AWAY.

    Replace the cover and return the microscope to the appropriate cabinet and numbered space, oculars facing in.

NOTE: The objective of today is to learn to use the microscope. Concentrate on successfully finding and focusing on the specimens; utilizing components of the microscope (open and close diaphragms, adjust condenser up and down, dark-field and phase contrast, moving between objectives, etc.) so that you feel comfortable and confidant. Today you do not need to see everything and draw everything. DO prepare and observe a wet mount and a variety of prepared slides in order to get familiar with the microscope and its capabilities. You will have additional time to concentrate on the organisms in the next lab.

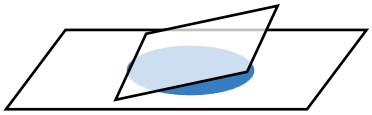
2. Live material-Wet Mount and Hanging Drop Slides:

Observe the live Mixed Protist Survey via a wet mount and a hanging drop slide. View the specimen with Bright Field, Phase Contrast, and Dark Field if your microscope is equipped with it. Take measurements. Draw what you observe.

Wet mount:

a. Place one drop of sample onto a cleaned slide. Add a drop of Detain if desired to slow down the movement of the organisms.

b. Observe under the microscope (low and high power) with bright-field, dark-field, and phase contrast. Draw some of your observations.

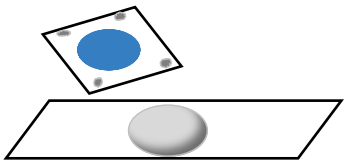


Hanging Drop:

a. Place 4 small dabs of Vaseline in the corners of a cover slip. Place one drop of sample onto the center of the coverslip. Add a drop of Detain if desired to slow down the movement of the organisms.

b. Invert the coverslip over a depression slide so that the drop of sample hangs down from the coverslip over the well in the slide.

b. Observe under the microscope (low and high power) with bright-field, dark-field, and phase contrast. Draw some of your observations.



3. Prepared slides:

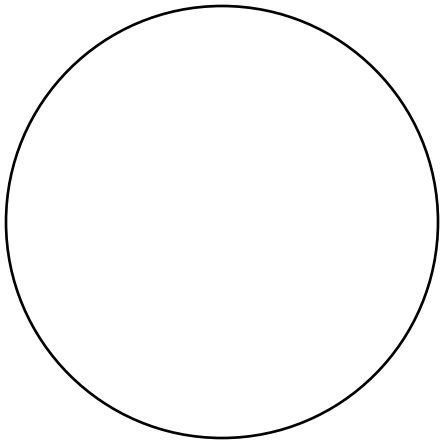
Examine a few of the prepared slides (use Bright Field only). Observe and draw.

Results:

1. Ocular micrometer

Objective	Value of each space in the ocular micrometer (um)
10x	
20x	
40x	
100x	

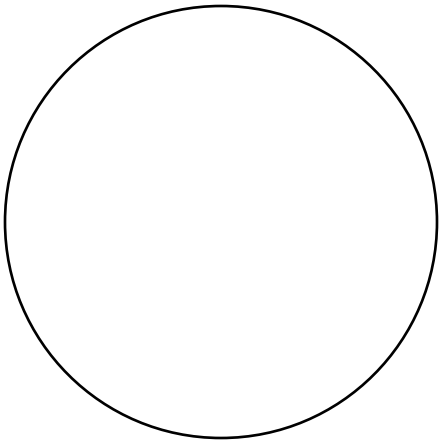
2. Draw your observations below. Label each with total magnification; take a measurement of each if possible.



Organism _____

Size _____

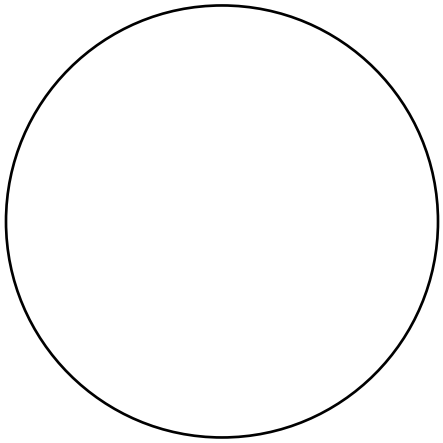
Magnification _____



Organism _____

Size _____

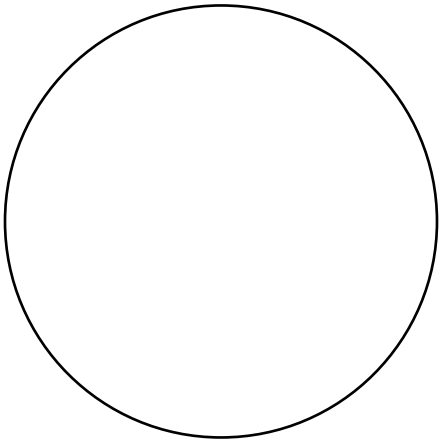
Magnification _____



Organism _____

Size _____

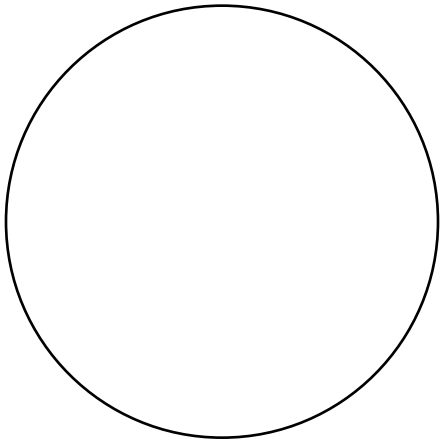
Magnification _____



Organism _____

Size _____

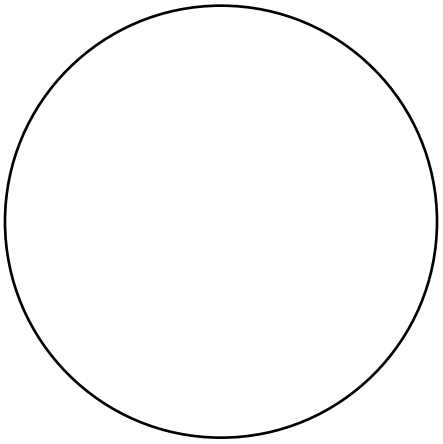
Magnification _____



Organism _____

Size _____

Magnification _____



Organism _____

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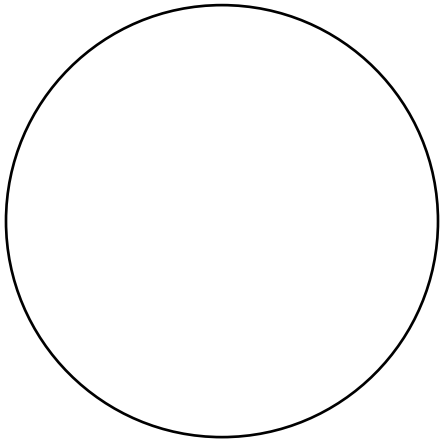
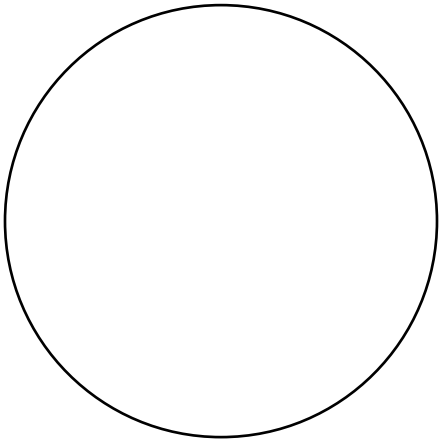


Organism _____

Size _____

Magnification _____



Organism _____

Size _____

Magnification _____

Questions:

1. What is the function of the condenser?

2. What is the function of the iris diaphragm?

3. At what magnification(s) is it ok to use the coarse focus?

4. Define parfocal.

5. What is the relationship between value of the spaces in the ocular micrometer and magnification?

6. What was the best specimen you observed today, and why?

Conclusion:

Assess your ability to find and focus the specimen:

What do you need to do in order to improve your ability to find and focus on specimens? What will you try in order to improve your skills?

What do you need help with next time?

Resources:

Read the chapter on microscopy in your textbook.

Environmental Sampling

Microbes are ubiquitous. No doubt you've probably heard that by now in class or lab. In order for you to develop skills with aseptic technique, it helps for you to see this firsthand. What microbes are in the air? What microbes are found on the surfaces of things around you? We will continually stress the importance of protecting your cultures (and yourself and others) from contamination. This exercise will show you where sources of bacterial and fungal contamination can be found, and how common microbes are in our environment.

Learning Objectives:

After this lab you should be able to:
1. Observe sources of bacteria and fungi from the environment in, and around, the laboratory.
2. Define the term ubiquitous.
3. Describe some consequences of exposing your cultures to the air or non-sterile objects.
4. Apply your observations to the practice of Aseptic Technique.

Introduction:

Bacteria and fungi can be airborne. Spores and cells can float on the wind, dust particles, soil that gets disrupted, etc. At some point they may land on a surface. You will sample the air by placing an open agar filled petri dish inside or outside for 15 minutes.

Bacteria and fungi can land on a surface via the air, or they can be transferred in a variety of ways. Hands, the bottom of shoes, objects like purses, notebooks, etc., may pick up bacteria from one surface and transfer it to another. You will sample surfaces by swabbing and then streaking onto an agar plate.

Pre-lab questions:

1. What does the word “ubiquitous” mean?

2. Why should one be concerned that there are bacteria and fungi in the air and on hands, surfaces, etc., in the laboratory?

3. How might you prevent contaminating your bacterial cultures as you work with them?

Materials:

SDA (Sabouraud Dextrose Agar)—1 plate/pair
TSA (Tryptic Soy Agar)—1 plate/pair
Sterile cotton swab in saline—2 swabs/pair

Procedures:

Day One
Divide into pairs for environmental sampling:

1. Each pair will label one **SDA** plate and then place it open (set lid right side up next to the plate) somewhere inside or outside the lab for 15 min.

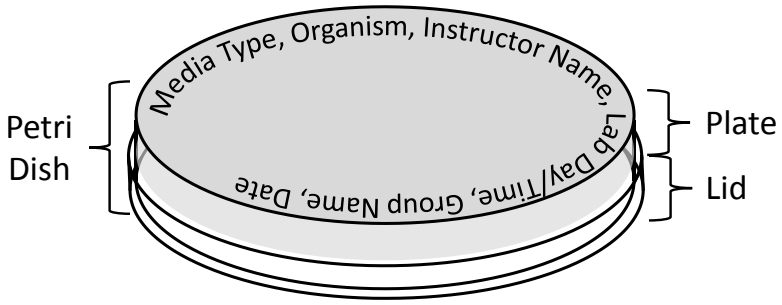
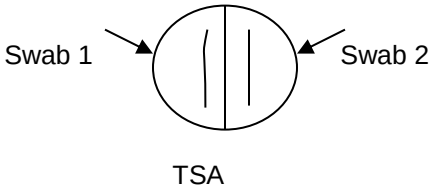


Fig. 1: Petri dish, showing how to label and invert for incubation. (C.S. Ramey)

2. After the exposure, replace the lid and parafilm the petri dish. Place the petri dish, inverted, in the designated receptacle.

3. Label, then divide your **TSA** plate in two. Take the sterile cotton swab in saline and swab a surface, then swab a line down one side of the plate. Label that side of the plate with the surface swabbed. The other partner does the same on the other side of the plate, from a different surface.



TSA

4. Parafilm the TSA plate and incubate it, inverted, <u>in the class incubator</u>.

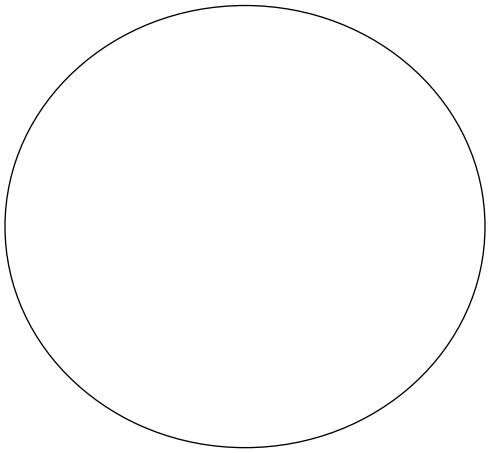
5. <u>Day Two</u> (after several days of incubation)

***SAFETY: Environmental samples are considered BSL-2 once they have been incubated. Wear gloves when handling the plates, make sure the plates are completely sealed, and DO NOT open the plates. Discard in the Biohazard trash container.**

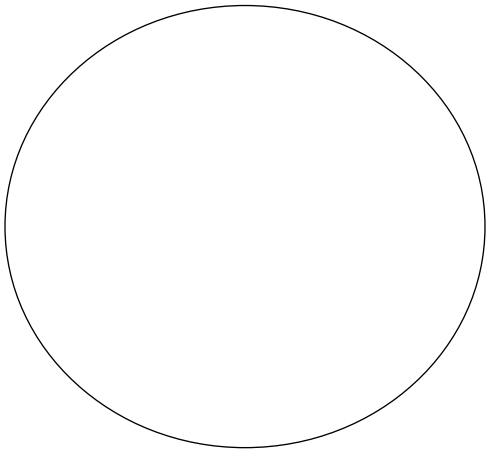
Observe the SDA and TSA plates for growth. Look for fungi and bacterial colonies. Each colony originates from one cell/spore, which landed or was swabbed onto the plate. Over time, the cells multiplied to an extent that is finally visible to see.

Results:

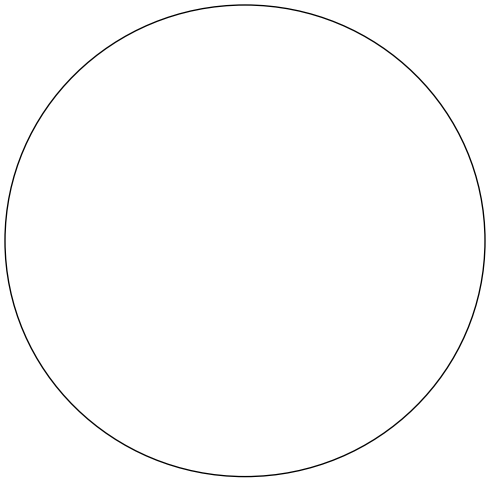
Draw your plates: Note the color and texture of different colonies viewing through the top of the plate and the bottom of the plate. Use a dissecting scope to see finer detail.



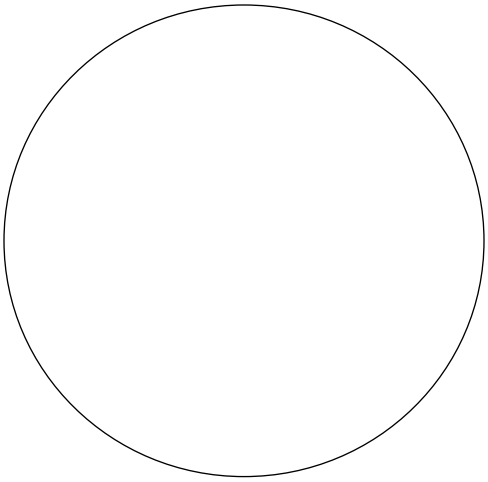
SDA top



SDA bottom



TSA top



TSA bottom

Class Results:

Table 1: SDA Air plates

Pair Names	Location/time	Approx. # of colonies

Table 2: TSA Surface plates

Pair Names	Surfaces Swabbed	Approx. # of Colonies

Questions:

1. Compare and contrast characteristics of fungal colonies vs. bacterial colonies.

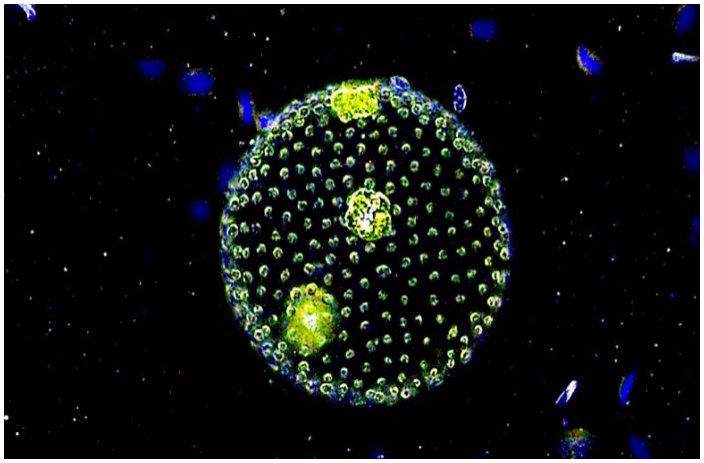
2. What characteristics of fungi account for their distinct type of growth and colors on an agar plate?

3. Compare the class's observations. What locations seemed to have more or less fungal colonies on the SDA plates? Which surfaces seemed to have more or less contamination on the TSA plates? Were there common looking colonies on the plates you observed?

Conclusion:

What did you learn about the importance of aseptic technique and protecting your cultures? How would you apply this to a situation at home or in a hospital?

Survey of Eukaryotic Microorganisms: The Protists
Algae



Volvocales.K Burke. 2012

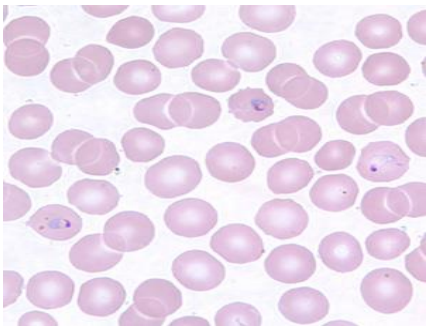
"Algae tend to be outliers in the world of microbiology; but are critical foundations for food chains in aquatic and marine habitats, produce the majority of recycled oxygen in the atmosphere (ahhh… breathing) and were instrumental in establishing an ozone layer that permitted terrestrial evolution, and are responsible for agar – the main substrate used in microbiology labs. These significant contributions and lesser ones as ingredients in ice cream and cake frosting make algae a fascinating microbiological study."

Prof. Don Takeda

Algae are protists that are photosynthetic. These can be microscopic single-celled or colonial organisms, like the *Volvox* above or can be very large organisms like kelp. In this lab you will observe a protist survey that will contain algae and protozoa (the more "animal-like" protists). Most algae are not clinically important, but many produce toxins that can be very serious and cause disease in humans and other animals

Protists of Clinical Importance

Many protozoa are clinically important and may cause serious disease in animals. In this lab you will observe several different protist pathogens, including the protist that causes the disease Malaria. Malaria is one of the most important pathogens, causing serious illness in millions of people each year.



Source: http://www.cdc.gov/dpdx/images/malaria/vivax/Pv_rings_thinC.jp

According to the Centers for Disease Control and Prevention (CDC) https://www.cdc.gov/malaria/about/index.html

- "Malaria parasites are micro-organisms that belong to the genus *Plasmodium*. There are more than 100 species of *Plasmodium*, which can infect many animal species such as reptiles, birds, and various mammals. Four species of *Plasmodium* have long been recognized to infect humans in nature."
- "An experienced laboratory technician or pathologist can distinguish between *P. falciparum*, *P. vivax*, *P. malariae,* and *P. ovale* based on the appearance of the parasites and infected blood cells. Under the microscope, *P. knowlesi* can resemble either *P. falciparum* or *P. malariae*. Increasingly reference diagnostic tools like PCR are employed to confirm malaria infection and to determine definitively which species are involved."

- "Five times, the Nobel Prize in Physiology or Medicine has been awarded for work associated with malaria: to Sir Ronald Ross (1902), Charles Louis Alphonse Laveran (1907), Julius Wagner-Jauregg (1927), Paul Hermann Müller (1948), and Youyou Tu (2015)."

Learning Objectives:

After this lab you should be able to:
1. Observe specimens and recognize characteristics that identify them as Protists.
2. Examine the diversity in single-celled Eukaryotic organisms.
3. Relate the structure, function, and life cycles of the organisms to their ability to cause disease.

Introduction:

Protists are a group of organisms that have traditionally been placed together because they are primarily single-celled eukaryotic organisms. They are often categorized into plant-like, animal-like, and fungal-like groups. However, they are not phylogenetically related as a major group and their taxonomy is in a state of flux. Scientists are studying the genetics of these organisms to more accurately develop a taxonomic scheme that is meaningful, instead of just dumping them all together. In today's lab you will observe live specimens and prepared slides in order to observe the diversity of the group and get a sense of size of these unique and, despite their size, complex organisms. Many Protists are disease-causing organisms. Some terms you will need as you view the prepared slides (hint—always look at the label of the slide for clues about what you will observe on that slide):

Trophozoite: the active feeding stage

Sporozoite: the motile infective stage

Merozoite: the result of schizogony (a trophozoite replicates its organelles and nuclei multiple times, then the cell divides into multiple daughter cells called merozoites)

Cyst: the dormant stage, usually resistant to harsh or changing conditions, also the stage that is often part of transmission from host to host.

Taxonomic Supergroups:

- **Amoebozoa:** use **pseudopodia** for feeding and motility
  - *Entamoeba*, (and slime molds)
- **Excavata:** most have a **flagellum** which aids in feeding
  - *Giardia, Trichomonas, Trypanosoma, Leishmania*
- **Chromalveolata: ciliates and apicomplexans** (and Dinoflagellates, Diatoms, brown algae, golden algae)
  - *Balantidium, Cryptosporidium, Toxoplasma, Plasmodium*
  - **Rhizaria:** (radiolarians, forams)
- **Archaeplastida:** (Green algae)

Pre-lab questions:

1. Define the term "Protist".

2. Explain why the Protist is no longer recognized as a Kingdom (you will need to look this up in your text or online).

3. Compare and contrast Algae and Protozoa.

4. Are all photosynthetic Protists algae?

Materials:

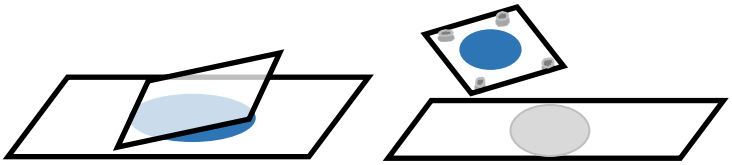
Wet Mount Materials:
Glass slides
Hanging drop slides
Coverslips
Vaseline
Detain/Protoslo

Live Material:
Mixed Protists

Prepared Slides:
Amoeba proteus
Trypanosoma gambiense (West African trypanosomiasis)
Plasmodium vivax (benign tertian malaria)
Paramecium caudatum
Giardia lamblia (giardiasis)
Cryptospiridium parvum (gastrointestinal disease)
Entamoeba histolytica (Amoebic dysentery)
Trichomonas vaginalis (STD)

Procedures:

1. Live material: Observe the live Mixed Protist Survey via a wet mount or hanging drop slide. View the specimen with Bright Field, Phase Contrast, and Dark Field if your microscope is equipped with it. Draw what you observe.



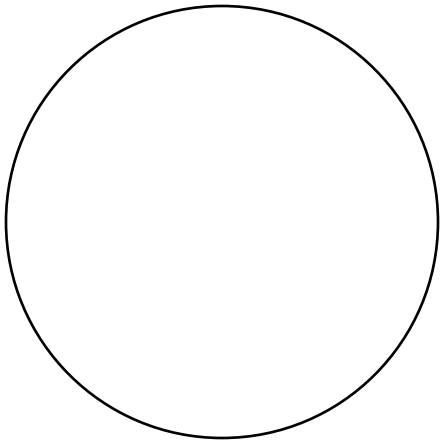
Wet mount Hanging Drop

2. Prepared slides: Examine the prepared slides (use Bright Field only). Refer to images provided in order to help you find the correct organism/structure.

3. Review the life cycles for each disease. Make note of habitat, hosts, transmission routes, and specialized structures.

Results:

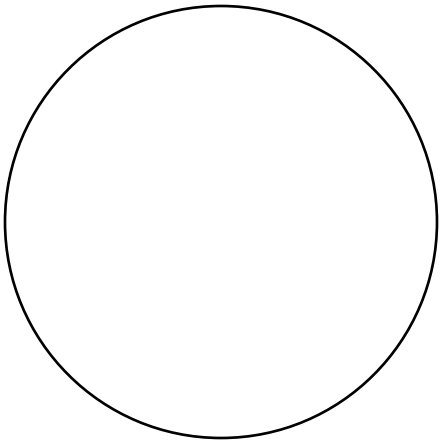
Draw the organisms as you see them through the microscope. Record the total magnification and a measurement. Indicate on the drawing what you measured with a bar symbol: 



Organism _____

Size _____

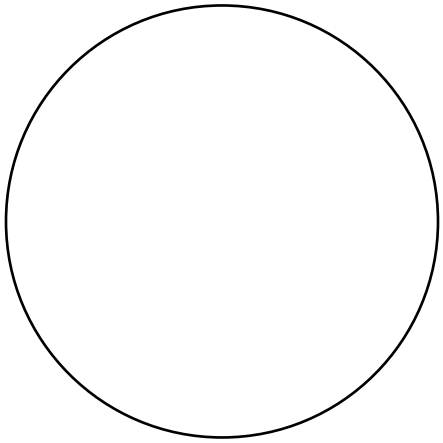
Magnification _____



Organism _____

Size _____

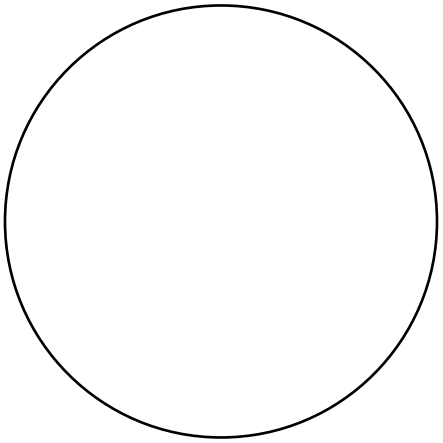
Magnification _____



Organism _____

Size _____

Magnification _____



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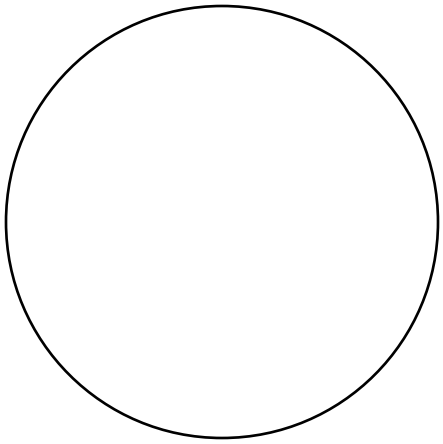

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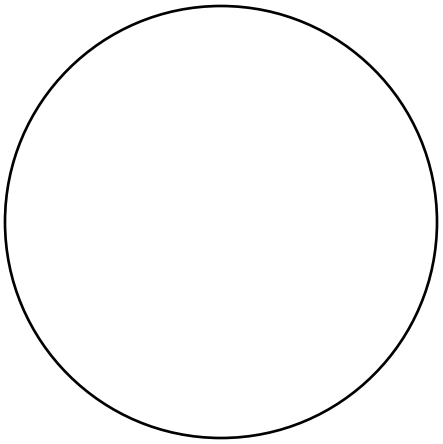

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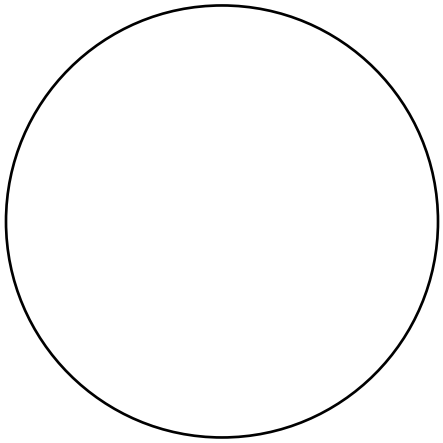
Magnification _____



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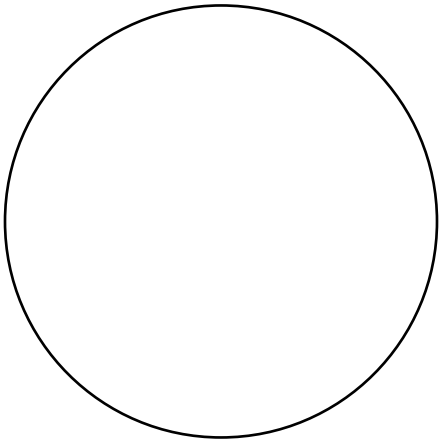

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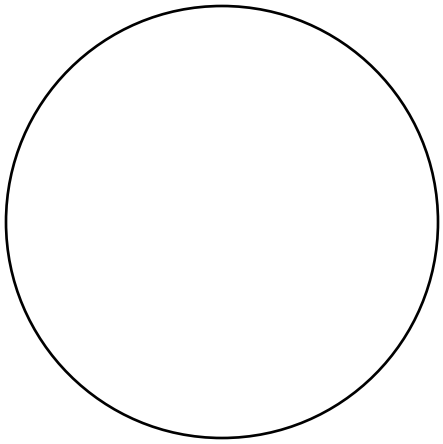

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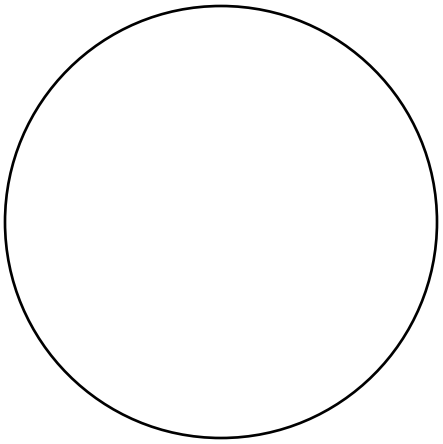

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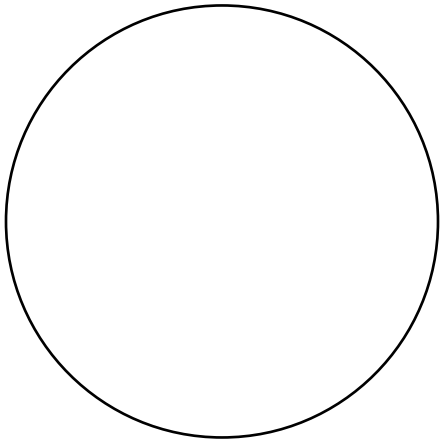
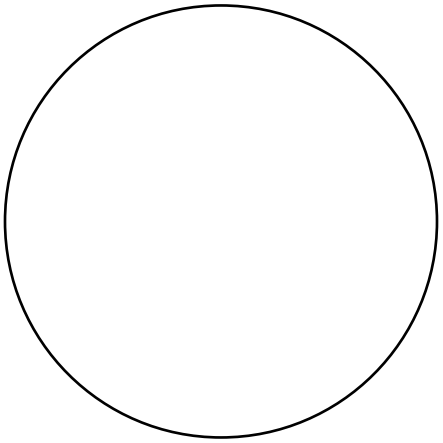


Organism _____

Size _____

Magnification _____



Organism _____

Size _____

Magnification _____

Questions:

1. Compare the algae with the protozoans you observed today.

2. Fill in the chart below with the organisms you observed (both algae and protozoa):

Organism name	Non-pathogen	Pathogen

3. What eukaryotic structures did you observe in different organisms? Give the name of the organism and the structures you observed.

Conclusion: What have you learned?

Reflect on what you observed today in your microscope and the images provided, along with what you know about the organisms' life cycles. Relate what you observed of their structures, size, life stages, etc., to what you know about their ability to cause disease or what you think might help them survive and their potential for causing disease.

Resources:

Protists: http://www.microbeworld.org/types-of-microbes/protista
Malaria: https://www.cdc.gov/malaria/about/index.html
CDC images of parasites: http://www.cdc.gov/dpdx/az.html

Parasitic Helminths

Adult Worm of Necator americanus. Anterior end showing mouth parts with cutting plates.
Source: http://www.cdc.gov/dpdx/hookworm/gallery.html#adults

Learning Objectives:

After this lab you should be able to:
1. Observe specimens and recognize characteristics that identify them as Helminths.
2. Know the taxonomic groups and examine the diversity in the parasitic helminths: Trematoda, Cestoda, Nematoda.
3. Relate the structure, function, and life cycles of the organisms to their ability to cause disease.

Introduction:

Helminths are a group of worms most often referred to as the parasitic worms—as opposed to earthworms, polychaete worms, etc. Worms are multicellular, eukaryotic, invertebrate animals. Most are macroscopic, but have microscopic life stages. They are complex organisms with organs and organ systems. In addition, they usually have complex life-cycles which may include different larval and cyst stages, and multiple hosts.

Terms to know-

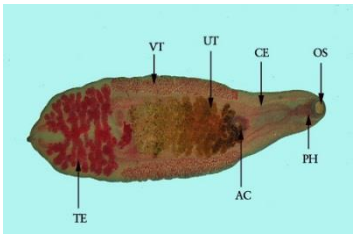
Intermediate host: the host in which larval development occurs

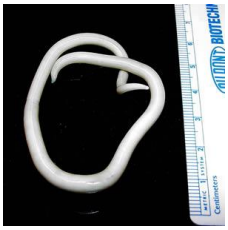
Definitive host: the host in which mature organisms live and undergo sexual reproduction

Hermaphroditic: male and female sex organs occur in the same individual worm (most commonly in cestodes and many trematodes).

Parasite: an organism that lives in or on another organism (the host), to the detriment of the host.

Taxonomy: The taxonomy of organisms is under constant review. We will use the following taxonomy to group the helminthes we will observe.

<table>
<tr><td colspan="2">Phylum Platyhelminthes (flatworms)</td><td>Phylum Nematoda (roundworms)</td></tr>
<tr><td>Class Trematoda</td><td>Class Cestoda</td><td></td></tr>
<tr><td>-Flukes
-Leaf shaped
-Mollusk is usually the intermediate host(s)
-Vertebrate is usually the definitive host
-Digestive system</td><td>-Tapeworms
-Long, ribbon like bodies
-Scolex for attachment, with reproductive segments called proglottids
-Usually also require two or more hosts
-No digestive system</td><td>-Roundworms
-Cylindrical
-Usually only one host
-Complete digestive system
-Very diverse and numerous group.
-Many free-living, predatory, and parasitic types.</td></tr>
<tr><td> 

Adult of C. sinensis stained with carmine. Clearly visible in this image are the oral sucker (OS), pharynx (PH), ceca (CE), acetabulum, or ventral sucker (AC), uterus (UT), vitellaria (VT) and testes (TE). http://www.cdc.gov/dpdx/clonorchiasis/gallery.html</td><td> 
Scolex of T. solium. Note the four large suckers and rostellum containing two rows of hooks. http://www.cdc.gov/dpdx/taeniasis/gallery.html#scoleces</td><td> 
Adult Ascaris lumbricoides http://www.cdc.gov/dpdx/ascariasis/gallery.html#adultsAL</td></tr>
</table>

Pre-lab questions:

In your own words, describe how you will differentiate between and identify examples of each of the three major groups:

Materials:

Wet Mount Materials:
Glass slides
Coverslips

Live Material:
Turbatrix aceti- Vinegar Eels (Nematoda)

Preserved Materials:
Flukes and Tapeworms
Dissected *Ascaris* (Unsegmented Roundworms)

Prepared Slides:
Phylum Platyhelminthes: (on back bench)
Class Trematoda (Flukes)
Fasciola hepatica (liver flukes)
Clonorchis sinensis (Oriental Liver Fluke)
Schistosoma sp. (Blood flukes)
Class Cestoda (segmented Flatworms)
Taenia sp. (proglotids) (Tapeworm)
Dypilidium caninum (Dog tapeworm)
Phylum Nematoda (unsegmented Roundworms):
Ascaris lumbricoides
Enterobius vermicularis (Pinworm)
Necator americanus (Hookworm)
Trichinella spiralis

Procedures:

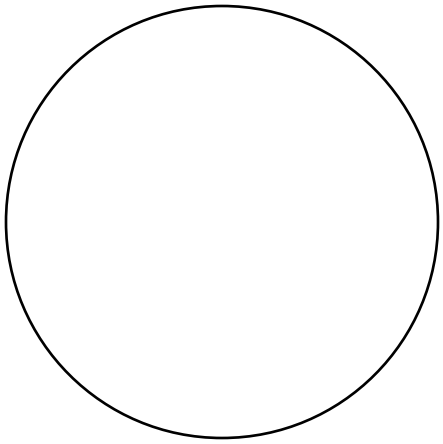
1. Wet Mount of *Turbatrix aceti* (Nematoda)*:*
a. Place one drop of sample onto a cleaned slide. Add a drop of Detain if desired to slow down the movement of the nematode.
b. Carefully place a coverslip over sample.
c. Observe under the microscope (low and high power) with bright-field, dark-field, and phase contrast. Draw some of your observations.
d. Turbatrix can also be observed via a dissecting scope.

2. Prepared specimens: Observe and draw.

3. Prepared slides: Observe, draw, label, and measure the helminths and their structures. Pay close attention to the label on each slide. The slide will give hints as to what you will observe. Terms/abbreviations that you may see on the label:

WM=Whole Mount-the entire organism is on the slide
CS=Cross Section-the organism has been cut across the body in the vertical plane and is mounted on end. You are looking through a cross section of the organism. Think of making orange slices and looking through the cut segments.
TS=Transverse Section and LS=Longitudinal Section-cuts across the horizontal plane.

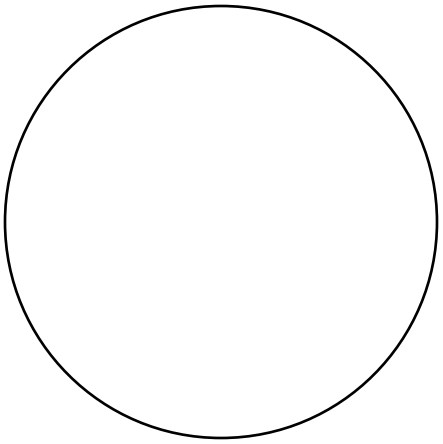
Results:



Organism _____

Size _____

Magnification _____



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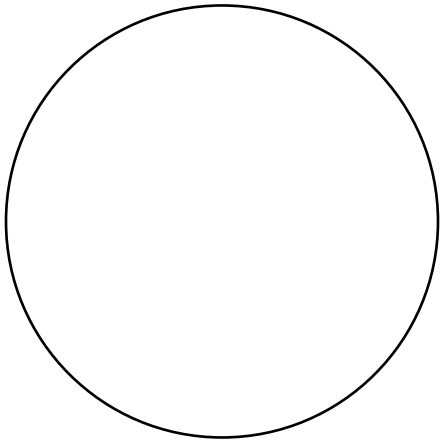

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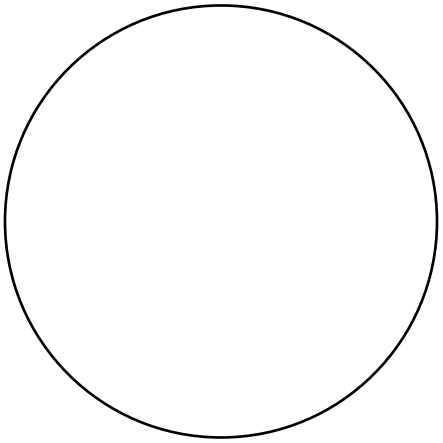

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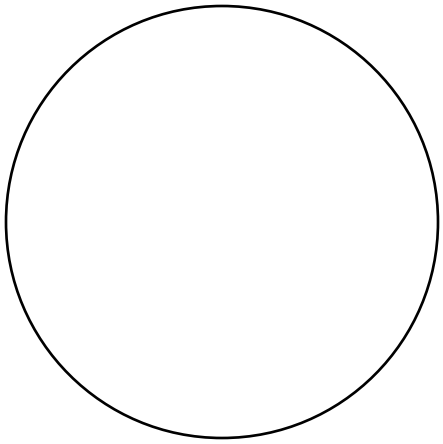

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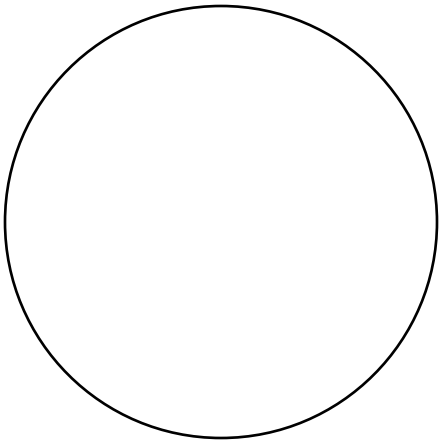

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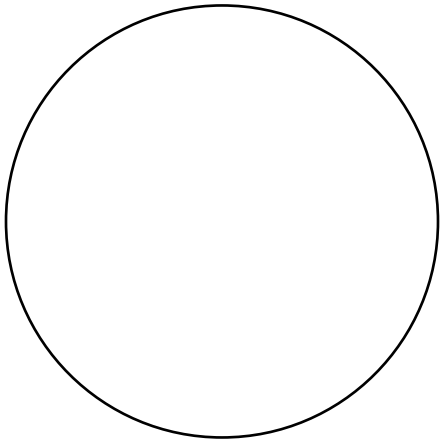

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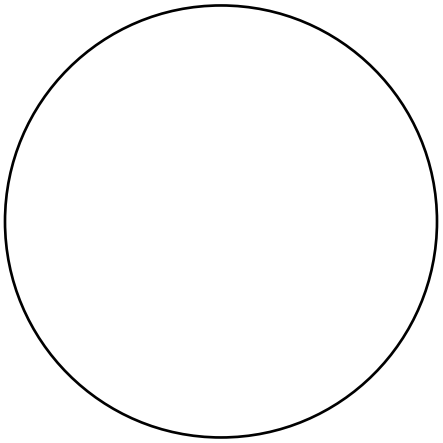

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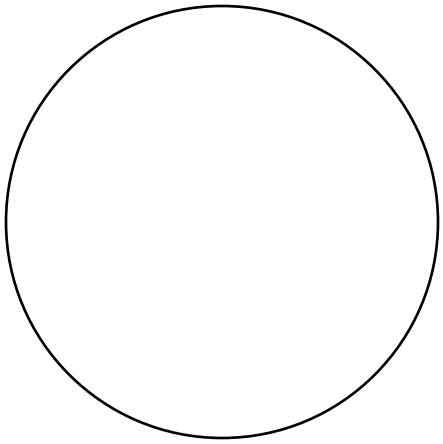

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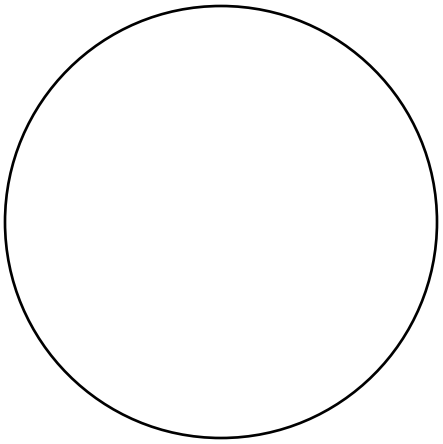

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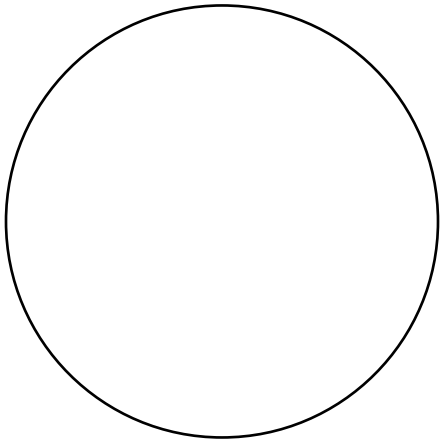

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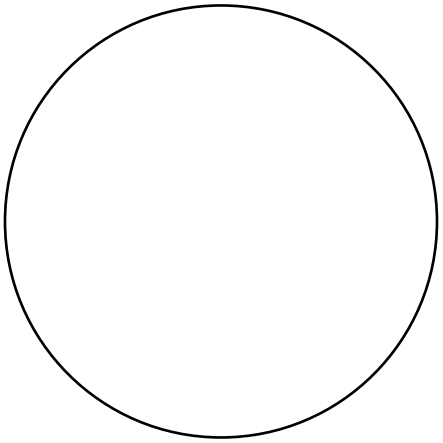


Organism _____

Size _____

Magnification _____

Questions:

1. Fill in the table with the organisms you observed:

Phylum Platyhelminthes		Phylum Nematoda
Class Trematoda	Class Cestoda	

2. Compare and contrast all 3 groups.

Conclusion:

Reflect on what you observed today in your microscope and the images provided, along with what you know about the organisms' life cycles. Relate what you observed of their structures, size, life stages, etc., to what you know about their ability to cause disease or what you think might help them survive and their potential for causing disease.

Resources:

Parasites-
http://www.cdc.gov/parasites/about.html

Fungi

Fungi are everywhere. Even in one's own backyard one can see the progression of development. In the left photo the fruiting body has developed from microscopic hyphae. In the center photo the fruiting body has contracted, splitting open and releasing the spores. There were about ½ cup of spores in this single fungus. The photo on the right shows the fungi many days later as it decomposes. It was covered in a filmy substance that looked wet and like mucous, but was in fact solid and dry like cellophane.



Before

After!

Learning Objectives:

After this lab you should be able to:

1. Observe specimens and recognize characteristics that identify them as different groups of fungi.
2. Define terms relating to structures and life cycles.
2. Examine the diversity in Fungi.
3. Compare and contrast sexual and asexual structures in each group.
4. Diagram or describe the life cycles of the 3 major groups: Zygomycota, Ascomycota, Basidiomycota.
5. Relate the structure, function, and life cycles of the organisms to their ability to cause disease.

Introduction:

Fungi are a group of organisms that can be characterized by their uniqueness. They are non-photosynthetic so they are not plants; they are **heterotrophs** that secrete enzymes into their environment and then absorb the broken down nutrients. However, they aren't animals, though most closely related to them, nor are they protists. They are often **saprophytic** and live on dead and decaying material, or they may be pathogens and parasites that infect plants and animals alike. Fungi have cells walls made of **chitin**, one of the most abundant carbohydrates in the natural world. Chitin is also found in the exoskeletons of crustaceans and arthropods (and is a component of bacterial cell walls). Fungi are most often found as asexual **hyphal** structures that form **fruiting bodies (**like mushrooms, truffles, and the puffball above) during sexual reproduction. Fungi can be **yeasts**-single celled, or **molds**-strands of **hyphae** that form a **mycelium** which can be septate (have walled divisions along the hyphae) or aseptate**.** Some fungi are **dimorphic** and have both a yeast and a mold stage. You will observe both in this lab.

Fungi form both asexual and sexual spores, in groups that reproduce via both methods. Asexual spores come in a huge variety of types and their structures are often used to identify them. In this lab you will observe two of the most common types of asexual spores. *Rhizopus* and other fungi in the group Zygomycota produce **sporangiospores** inside a sporangium. The sporangium is produced at the end of a stalk originating in the hyphal filaments (Fig. 1). *Penicillium* and *Aspergillus*, both in the group Ascomycota, produce asexual spores called **conidia** that are borne naked on a stalk (Figs. 2 and 3). Fungi in the group Basidiomycota do not appear to reproduce asexually.

Photos:

Take notes on labeled diagrams here:

Fig. 1 *Rhizopus* sporangia
By Curtis Clark (Own work) CC BY-SA 3.0

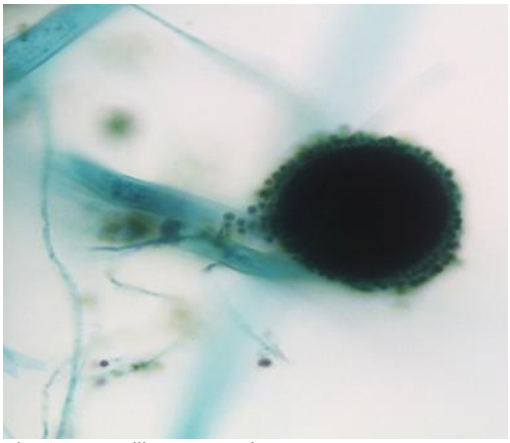


Fig. 2 *Aspergillus* sporangia
K.C. Burke CC BY-NC SA 4.0

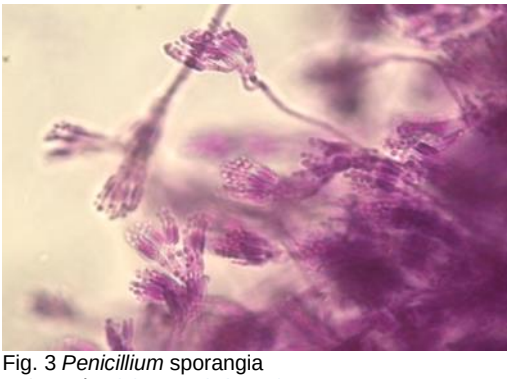


Fig. 3 *Penicillium* sporangia
K.C. Burke CC BY-NC SA 4.0

Sexual spores of Zygomycota are called **zygospores**, and it follows that Ascomycota produce sexual spores called **ascospores** and Basidiomycota produce sexual spores called **basidiospores**. Sexual reproduction occurs when hyphae of opposite mating types fuse and fertilization occurs. Zygospores are a single spore from fused hyphae. Ascopores and basidiospores are produced inside segments

of hyphal filaments that have formed a fruiting body. You will see prepared slides of each type.

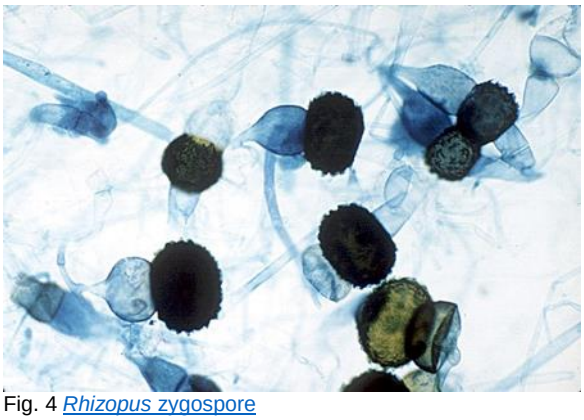


Fig. 4 *Rhizopus* zygospore
Curtis Clark CC BY-SA 3.0

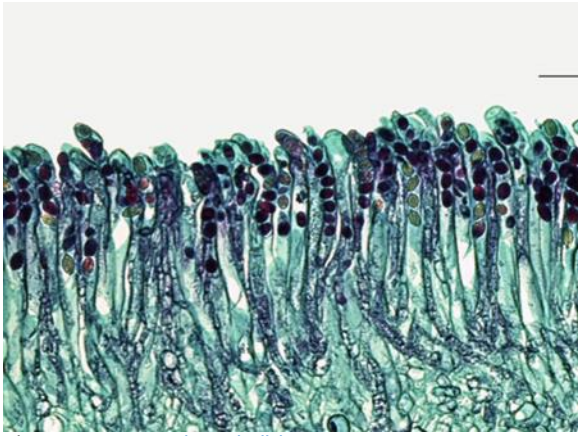


Fig. 5 Ascospores (*Morchella*)
By Jon Houseman CC BY-SA 3.0

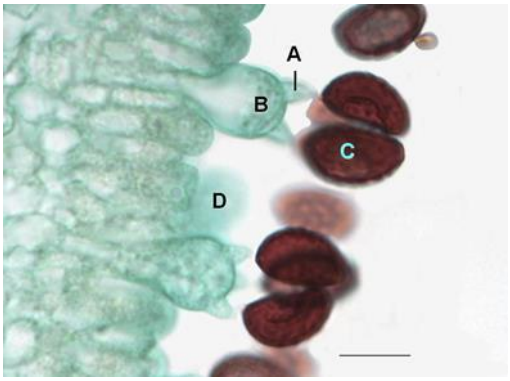


Fig. 6 Basidiospores (*Coprinus*)
By Jon Houseman CC BY-SA 3.0

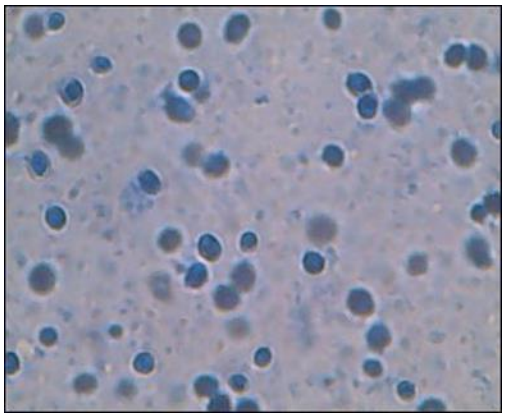


Fig. 6 Yeast cells, *Candida albicans*
Public Domain (Pinoche)

Pre-lab questions:

1. Study the images in your text or the lab atlas and then draw the **asexual** structures of the following fungi:

a. *Rhizopus*

b. *Aspergillus*

c. *Penicillium*

Materials:

Wet Mount Materials:
Glass slides
Coverslips
Lactophenol Cotton Blue Stain

Live materials:
Your environmental plates-DO NOT OPEN
1 SDA plate of *Saccharomyces cerevisiae*
Penicillium notatum – on a plate-demo only, DO NOT OPEN
Rhizopus stolonifer – on a plate -demo only, DO NOT OPEN

Preserved Materials:
Amanita
Other mushrooms- Morchella, Puffball, Bird Nest Fungus
Spore print
Fungal pathogens- Black knot fungus, Corn smut

Prepared Slides:
Rhizopus, Penicillium, Aspergillus (mixed types—all three on one slide)
Rhizopus zygospore
Coccidioides immitis
Candida albicans (yeast)
Coprinus basidiospores
Pezizza ascospores

Procedures:

1. Live specimens:

A. Wet Mount of *Saccharomyces cerevisiae:*

1) Place one drop of Lactophenol Cotton Blue onto a cleaned slide.

2) **Aseptically** pick a **small** amount of growth, with a loop, from the SDA plate and transfer it to the drop of Lactophenol Cotton Blue; mix well and carefully place a coverslip over the stain.

3) Observe under the microscope (low and high power) with bright-field, dark-field, and phase contrast. Draw some of your observations. Look for areas with well-separated cells and for cells that are budding. Keep your eyes on the budding cells and you may see the daughter cells separate!

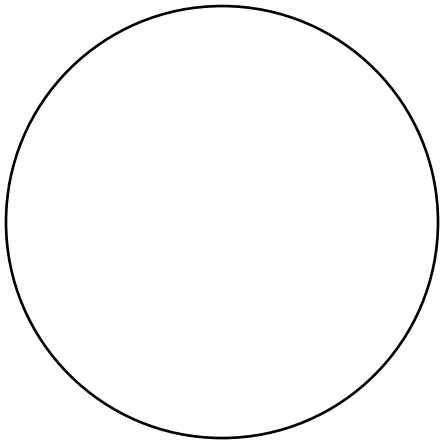
B. Observe under the dissecting scope and then draw the live plates of *Penicillium notatum* and *Rhizopus stolonifer* (DO NOT OPEN the plates). Observe the top and the reverse. Note structure/texture, color, etc.

3. Preserved specimens: Observe and draw.

4. Prepared slides: Observe, draw, label, and measure the fungi and their structures. Refer to images provided in order to help you find the correct organism/structure.

Results:

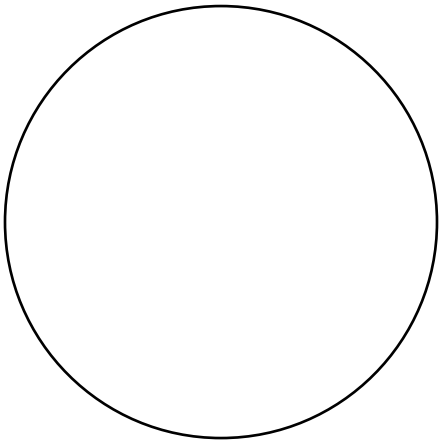
1. Draw the yeast, plates, and prepared slides. Draw the asexual spores (mixed type slide) of each fungi separately.



Organism _____

Size _____

Magnification _____



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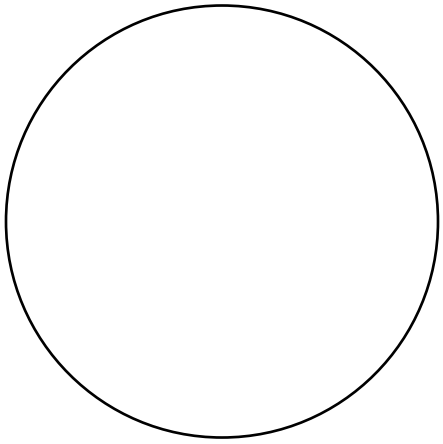

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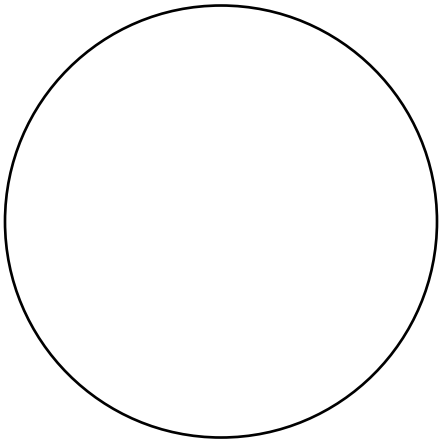

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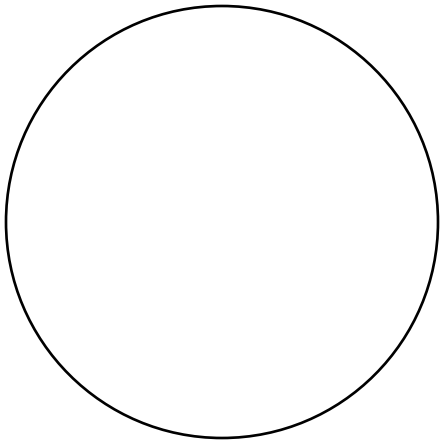

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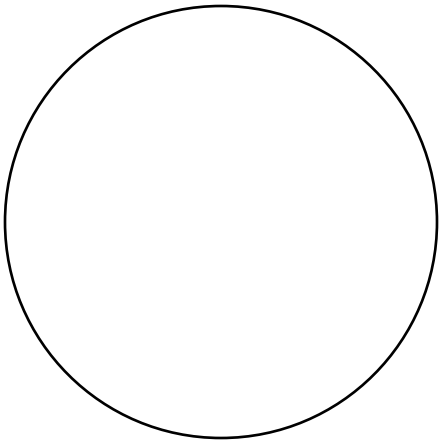

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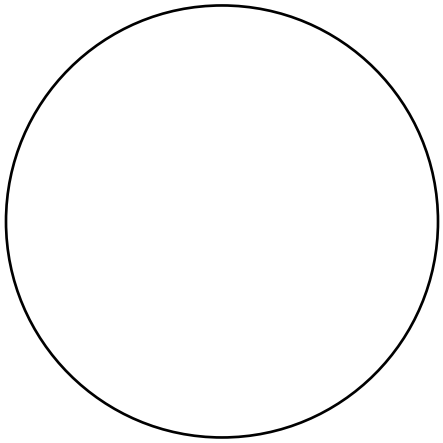

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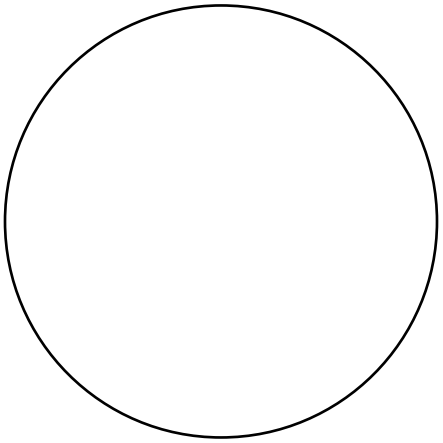

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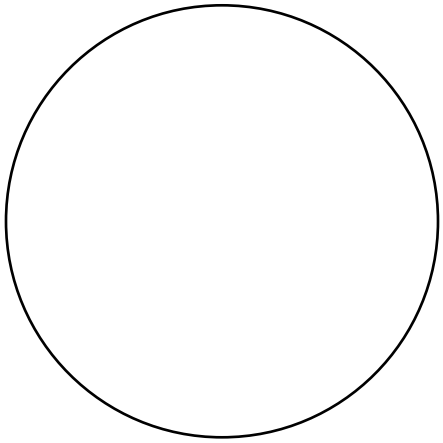

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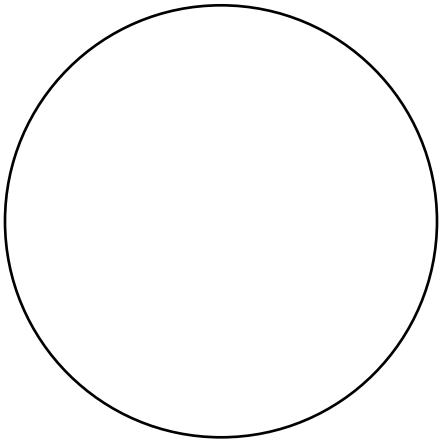

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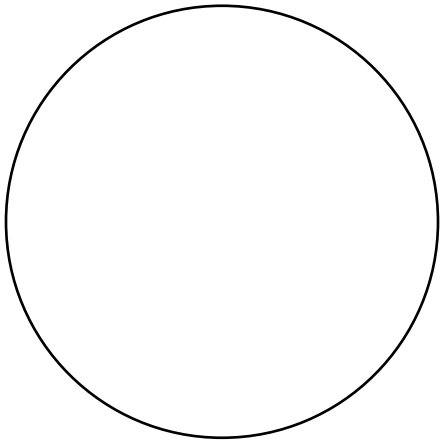

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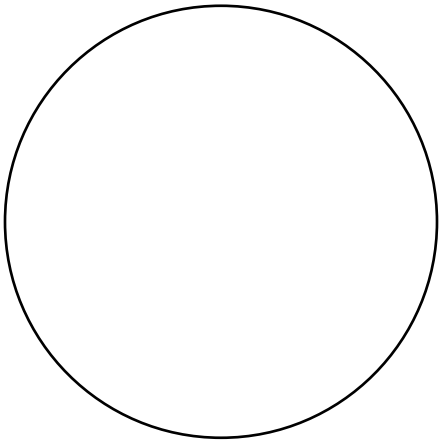


Organism _____

Size _____

Magnification _____

Questions:

1. Work with your lab partners to construct a chart to help you organize the terms in this lab for each group, Zygomycota, Ascomycota, Basidiomycota and examples of each:

Hyphae
Mycelium
Fruiting Body
Yeast
Mold
Asexual spores
Sporangiospores
Conidia
Sexual spores
Zygospores
Ascopores
Basidiospores
Dimorphic

2. Compare and contrast yeasts and molds.

3. Compare and contrast ascospores with basidiospores.

4. Some molds are good and some are not. Explain and give examples.

Conclusion: What have you learned?

Reflect on what you observed today in your microscope and the images provided, along with the organisms' life cycles. Relate what you observed of their structures, size, life stages, etc., to what you know about their ability to cause disease or what you think might help them survive and their potential for causing disease.

Pure Cultures: Aseptic Transfer Techniques and Streak Plates for Isolation

"Out of 110 aseptic opportunities, 45 breaks in aseptic technique were observed. Other results revealed that 80 percent of the time peri-care was omitted, the integrity of the sterile field was contaminated 70 percent of the time, and 20 percent of the time contamination occurred with donning of sterile gloves. When evaluating how well the nurses performed the catheterization procedure as specified by the directions for use (DFU), 30 percent either omitted or incorrectly performed the steps" (1).

Learning Objectives:

After this lab you should be able to:
1. Define and explain the purpose of aseptic technique.
2. Identify aseptic techniques used in our lab, and apply them to clinical and home situations.
3. Perform various transfer techniques and streak plating properly and without contamination.

Introduction:

Protect yourself, Protect others, Protect your cultures.

This is the mantra of **aseptic technique**. Microbes are ubiquitous and one must work purposefully to exclude them. The point of aseptic technique is to prevent the spread of contamination. In our lab classroom we don't want to spread contamination to ourselves, or lab mates, or take it home. This means we work not to spill cultures, create aerosols, set loops down on the table prior to sterilizing them, leave our lab coats in lab, etc. In addition we want to maintain **pure cultures**. A pure culture is a single species/type of bacterium being cultured (grown) in media (a plate, slant, or broth). To maintain pure cultures we enlist a variety of aseptic techniques. For example, one sterilizes their instrument before and after touching bacterial growth when transferring it, keeping cultures protected from environmental contamination, etc.

The following is a short list of things to keep in mind when working with cultures (though it is not meant to be the only things to consider, your instructor will discuss and demonstrate many others, and many are outlined in the lab safety and lab biohazard guidelines so review those).

- Keep workspace clear of personal items; use pens, pencils, etc., provided for you
- Get together with your group to get organized before you gather materials and get started
- Always use a test tube rack when transporting test tubes
- Never pick up or handle a test tube by the cap, they are loose and the test tube may fall, break, and cause a spill
- Prevent aerosols: Do not vigorously mix or vortex cultures, do not put hot loops into a culture-broth or agar, do not use the incinerator to dry stained slides (you will learn to air dry and heat fix them later)
- Never set test tubes or petri plate lids down. When transferring cultures, you will hold onto the lids and never set them down
- Inoculate test tubes as shown, don't try to invent a new method
- Keep petri dish covered with the lid slightly raised when transferring bacteria in and out of the plate.
- Sterilize loops and needles in the incinerator before and after touching bacterial cultures with them.
- Wash hands, wash hands, wash hands!

As stated above, creating and maintaining pure cultures is an important skill to acquire, and along with its general importance it will be critical during the Unknowns, and at some point you will be assessed on your ability to do this. The **streak plate** method is a way to separate bacteria in a mixed culture in order to isolate and **subculture** single species/types of bacteria into a pure culture. Natural samples (soil, water, skin), patient samples, surfaces, etc., will be a mix of bacteria when cultured onto a plate or into a broth. In order to study or identify (ID) one bacterium, it must be separated from the mix.

Streak plates are also used when maintaining pure cultures. One cannot tell if a broth culture is pure or contaminated. But if one streaks the broth onto a plate, one will be able to. A streak plate takes one loopful of bacteria from a broth, or a portion of a colony from a plate, and methodically spreads that amount of bacteria around the entire plate. After the plate is incubated, the first part of the streak there will have a lot of bacterial growth and no separate colonies. As the bacteria are spread around the plate, eventually bacterial cells will be well separated from each other and grow into single colonies during incubation. The individual colonies can then be subcultured separately to make pure cultures.

One colony grows from a single bacteria/bacterial unit.

Quadrant Streak Plate for Isolation:

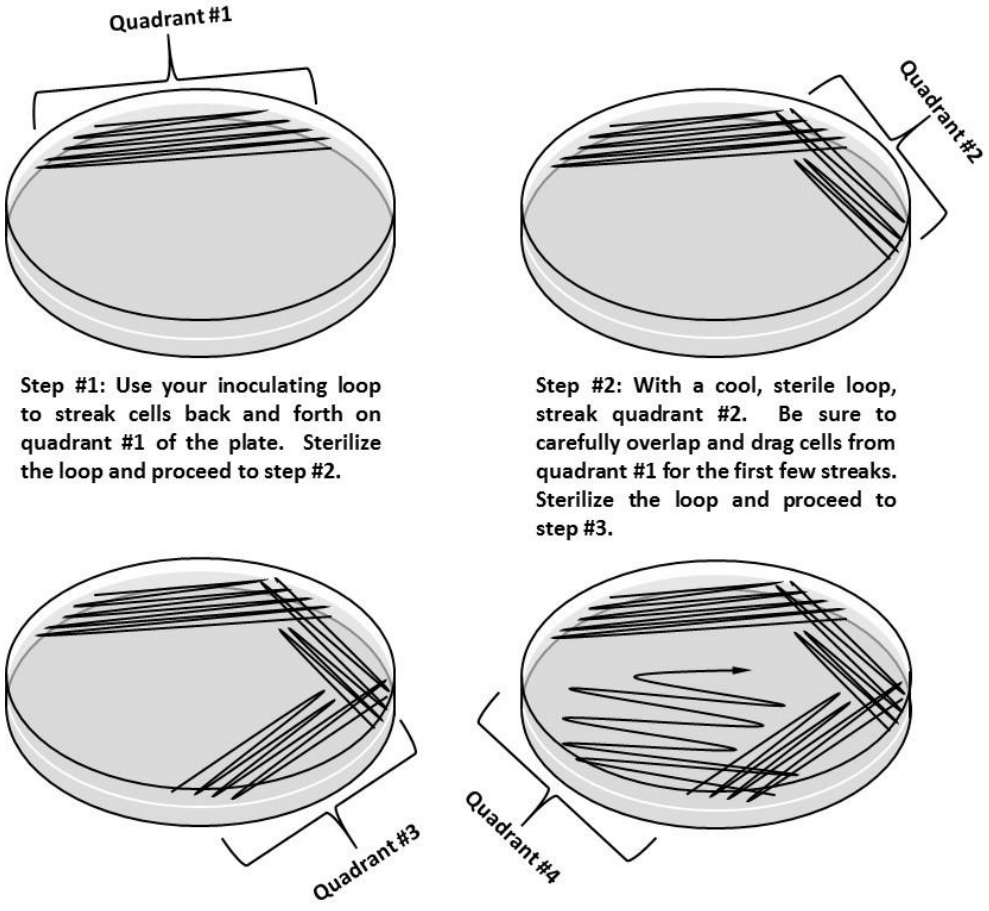


Fig. 1-Quadrant Streak Plate technique

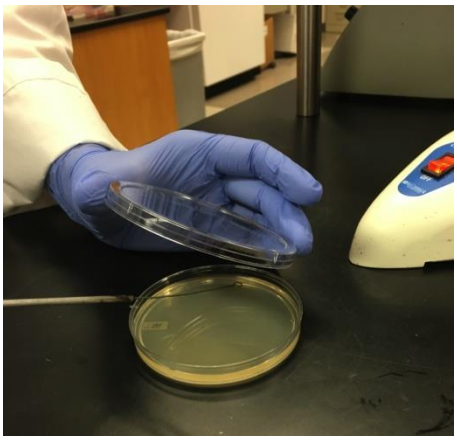


Fig. 2-Protect your plate with the lid as *you streak it*

Practice your own streak here:

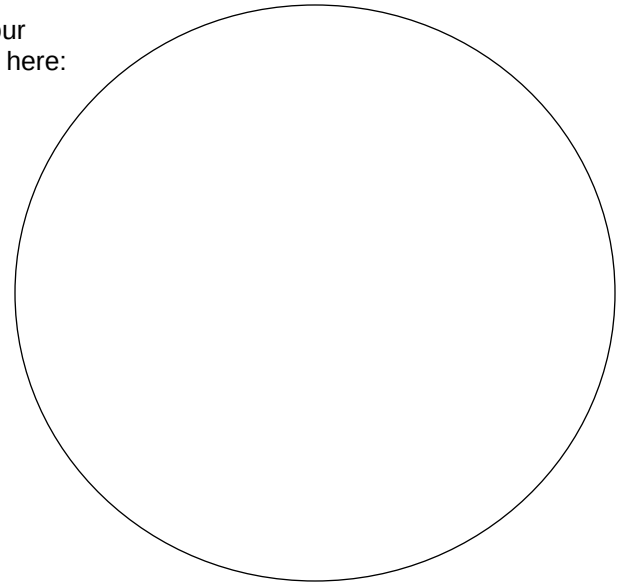


Fig. 2-Protect your plate with the lid as you streak it.

After the plate is incubated, the first part of the streak will have a lot of bacterial growth and no separate colonies. As the bacteria are spread around the plate, eventually bacterial cells will be well separated from each other and grow into single colonies during incubation. The individual colonies can then be subcultured separately to make pure cultures.

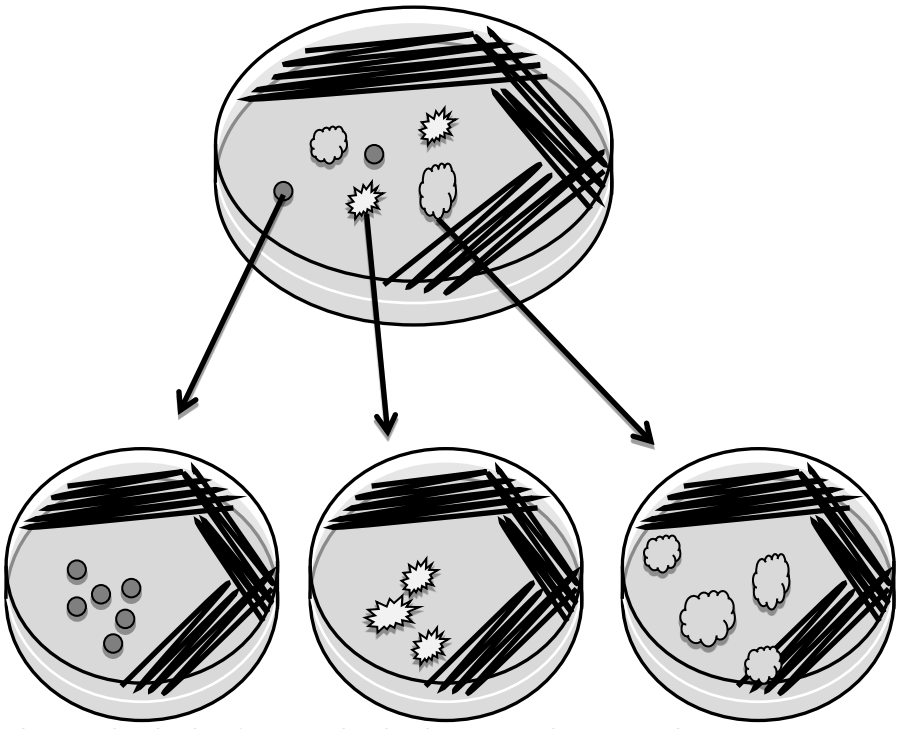


Fig. 3-Subculturing from a mixed culture to make pure cultures.

Pre-lab questions:

1. What is asepsis?

2. What is a pure culture?

3. Why is aseptic technique important?

4. What kind of lab behaviors and actions support contamination control in the lab classroom?

Materials:

Tryptic Soy Agar plates (TSA plates)
Tryptic Soy Broth tubes (TSB)
Tryptic Soy Agar slants (TSA slants)
Bacterial Cultures
Loop
Incinerator

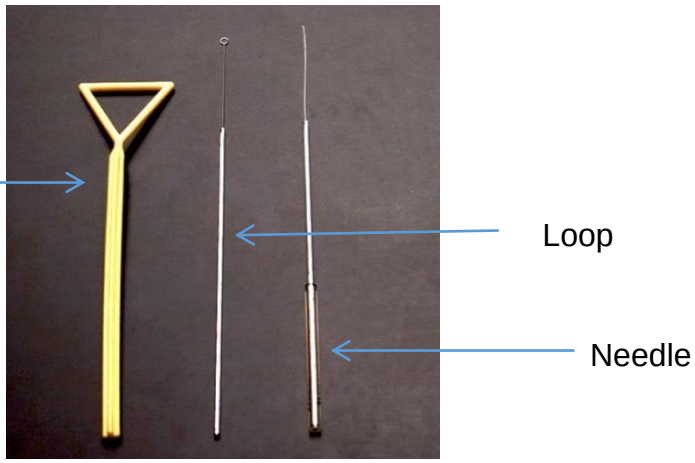


Fig. 4 Inoculation instruments

Fig. 5- Incinerator

Procedures:

Prepare your work area by sanitizing the lab bench, plug in and turn on your incinerator, gather materials and with your group organize the work to be done. Make sure that everyone in your group works together to stay organized and informed. Though you may split up some of the workload, everyone is accountable and must know how to do each technique, procedure, etc.

1. Transfer techniques from one tube to another:

(continued on next page)

a) Broth culture to a new broth:

- Hold loop like a pencil
- Sterilize loop in incinerator for 5-10 seconds (look at the loop in the incinerator for it to become red-hot), insert the full length of the wire into the incinerator.
- While loop is cooling and still holding it like a pencil, remove the cap by gently pulling the tube while holding the cap by curving your pinky finger around it. See Fig. 6.
- Insert the loop into the test tube; do not touch the lip of the tube with the loop or your fingers. Fig. 7. Dip the loop into the liquid and withdraw it from the tube.
- Insert the loop into a new tube, twist the loop gently and withdraw it from the tube.
- Sterilize the loop in the incinerator.

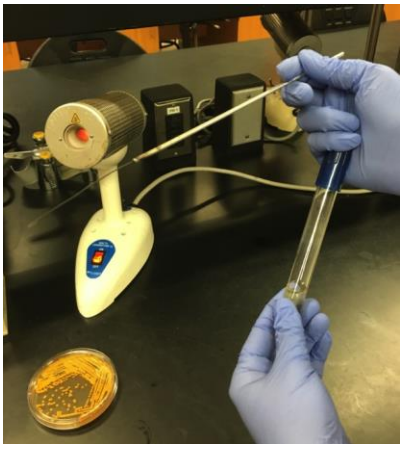


Fig. 6-Open test tube

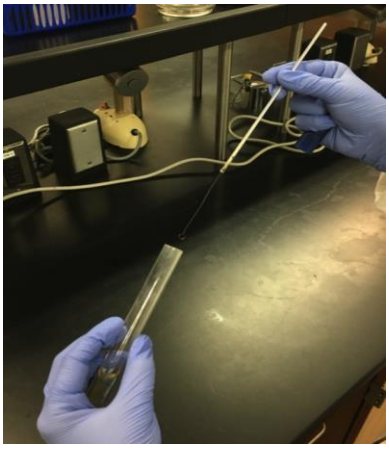


Fig. 7- Inserting the loop into a broth or slant

b) Broth to slant

- Hold loop like a pencil
- Sterilize loop in incinerator for 5-10 seconds (look at the loop in the incinerator for it to become red-hot), insert the full length of the wire into the incinerator.
- While loop is cooling and still holding it like a pencil, remove the cap by gently pulling the tube while holding the cap by curving your pinky finger around it. See Fig. 6.
- Insert the loop into the test tube; do not touch the lip of the tube with the loop or your fingers. Dip the loop into the liquid and withdraw it from the tube.
- Insert the loop into a slant. Inoculate the slant by placing the loop on the surface of the slant, at the bottom, and draw the loop up the surface of the slant; withdraw the loop from the tube.
- Sterilize the loop in the incinerator.

c) Agar plate (solid media) to a broth or slant

- Hold loop like a pencil
- Sterilize loop in incinerator for 5-10 seconds (look at the loop in the incinerator for it to become red-hot), insert the full length of the wire into the incinerator.
- Let the loop cool for a few seconds. Still holding it like a pencil, remove the lid of the plate while keeping it over the plate. The most common way of doing this is to “clamshell” the lid by opening it at an angle of the plate. Fig. 8
- Insert the loop into the plate and “pick” a colony. This means, lightly touch a colony of bacteria to get a small amount on the loop. You should NOT see a big glob of bacteria on the loop. You should be barely able to see any material on the loop—trust us, there will be plenty of bacteria on your loop.
- Inoculate a slant or broth.
- Sterilize the loop in the incinerator.

d) Broth or slant to an agar plate (streak plate)

- Hold loop like a pencil
- Sterilize loop in incinerator for 5-10 seconds (look at the loop in the incinerator for it to become red-hot), insert the full length of the wire into the incinerator.
- While loop is cooling and still holding it like a pencil, remove the cap by gently pulling the tube while holding the cap by curving your pinky finger around it. See Fig. 6.
- Insert the loop into the test tube; do not touch the lip of the tube with the loop or your fingers. Dip the loop into the broth, or pick a colony from a slant and withdraw it from the tube.
- Clamshell the agar plate and inoculate by performing a streak plate, Figs. 1 and 2.
- Sterilize the loop in the incinerator.

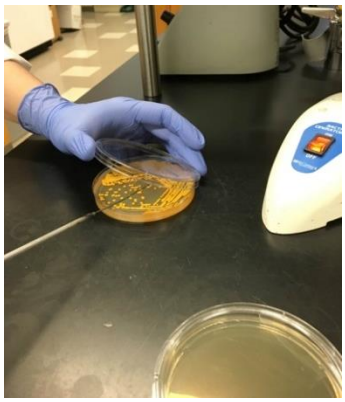


Fig. 8-Partially open the lid of the plate to gain access without exposing the plate to the open air.

2. Perform an isolation streak from a mixed culture

a) Each group inoculates the 2 organisms, *E. coli* and *Sarcina aurantiaca* onto

- 1 TSA plate (Quadrant Streak Plate Method)
- 1 TSA slant
- 1 TSB (5mL)

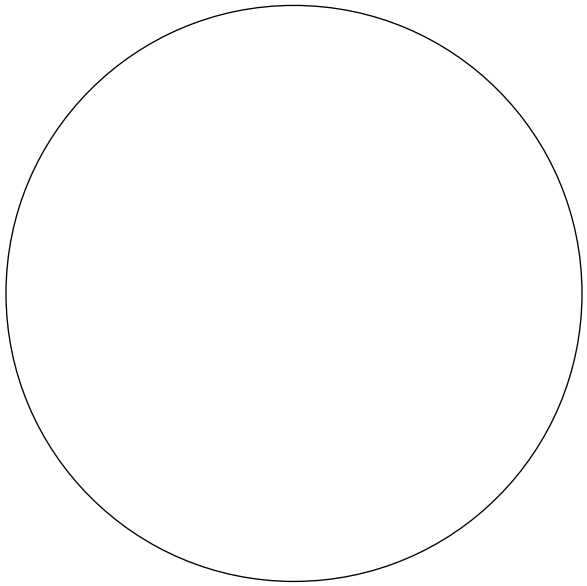
b) Mix the cultures (make 1 test tube of mixed culture per group):

- To the *Sarcina aurantiaca* culture test tube add 0.5ml *E. coli* using the sterile transfer pipette.
- Mix the culture well.
- Put the transfer pipette into the small red biohazard bag on your benchtop.

c) **Each student** will then streak a TSA plate from the mixed culture using the quadrant streak method, Fig. 1.

Results:

After you retrieve your streak plate the next day from the incubator, draw what it looks like below. Indicate differences in colonies.



Questions:

1. List several aseptic techniques you personally performed or complied with today.

2. Why is it important to cool an inoculating loop before touching it to a culture (give more than one reason)?

2. What is the purpose of the isolation streak method?

3. Was your isolation streak successful? Describe why or why not?

4. How do you choose a colony from a plate to subculture and to ensure that it will be a pure culture?

Conclusion:

1. How will you know if your culture is contaminated, and what can you do about it? Comment for each of the following-

Contaminated broth (how will you know and what will you do?):

Contaminated slant (how will you know and what will you do?):

Contaminated plate (how will you know and what will you do?):

2. How confidant are you in your ability to perform a successful streak plate with the correct pattern and isolated colonies? What will you do to improve? What instructor assistance do you need?

Resources:

William, D.C. "When One is Too Many: One Hospital's Strategies to Reduce CAUTI." *Infection Control Online.* 11 July 2016.Web. 27 August 2016.

Bacterial Growth Patterns: Building your Stock Cultures and Observing Culture Characteristics

The Unknowns.

The unknowns seem to strike fear into students when they first hear about them, and even still once they have been fully prepared to participate. The culture characteristics of the bacteria you will work with during the semester are important in the identification of them and will be critical to know when it comes time to ID your unknown bacteria.

Learning Objectives:

After this lab you should be able to:
1. Define the correct terms for describing culture characteristics.
2. Use the correct terminology to describe growth patterns.
3. Subculture bacteria each to a plate, slant, and broth.
4. Assess your cultures (plates, slants, and broths) for growth, purity, and culture characteristics.

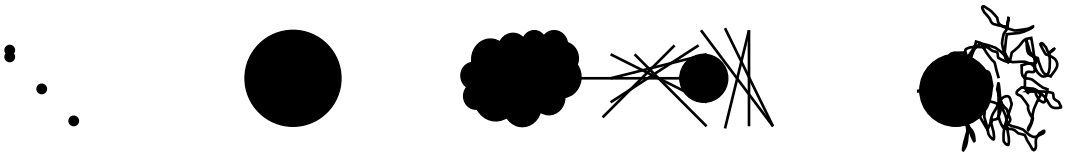
Introduction:

In this lab you will begin to build a culture collection. During each lab your group will subculture several bacteria, each onto a plate, a slant, and into a broth. Your group will use these cultures for many of the subsequent labs and should study them for their growth and physiological characteristics. Eventually, the unknowns will be comprised from instructional stock cultures of these organisms.

Culture Characteristics on plates, slants, and broths.

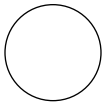
Colony Morphology and characteristics on plates-
Characteristics of **individual colonies** are best observed by looking at **a single isolated colony**. Each colony arises from one cell or group of cells (a tetrad or pair for example). Once colonies grow together in a larger mass or lawn it can be difficult to correctly characterize the colony morphology, especially the margins and elevation. Using the Colony Counters or a dissecting scope can help when evaluating colony characteristics. Be careful to protect your cultures by opening the plates only briefly.

1. Overall Size and Shape-

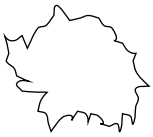


Punctiform Round Irregular Filamentous Rhizoid

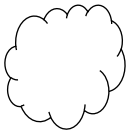
2. Margin-the bacterium may form discrete colonies, or may be a "spreader" which spreads quickly across a plate and colonies merge together as they expand.



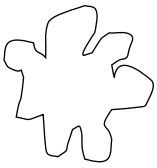
Entire



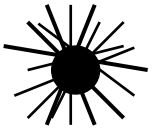
Erose



Undulate



Lobate



Filamentous

Rhizoid

3. Elevation-

Flat

Raised

Convex

Umbonate

Wrinkled

4. Surface-a colony will exhibit particular surface characteristics. It might be smooth, shiny, dull, wrinkled/rough, or mucoid (very glossy).

5. Pigmentation/color-bacteria may produce pigmented colonies in a variety of colors and shades of white/beige. In addition, some bacteria produce diffusible pigments that will affect the color of the media. Compare plates to see if there is a difference in the color of the agar surrounding the colonies in order to assess if a diffusible pigment is being produced.

6. Opacity-

- Transparent-can see through the colony like looking through a glass window.
- Translucent-can see through the colony slightly, like looking through frosted glass.
- Opaque-cannot see through the colony.

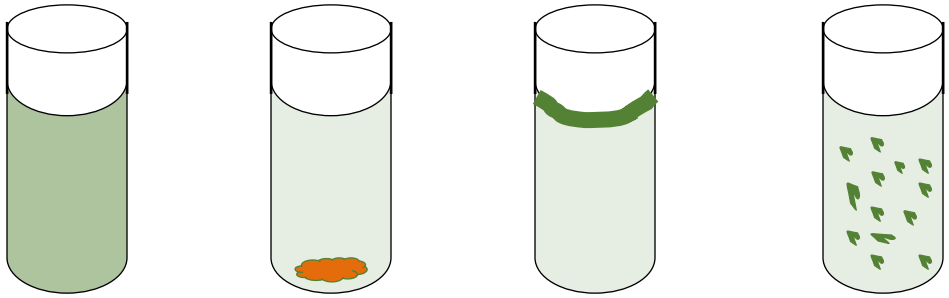
7. Texture-this is evaluated when subculturing a colony from a plate, or when picking a colony for a stain, etc.

- Moist/wet
- Butyrous-like butter!
- Mucoid/viscous-sticky/snotty
- Dry-doesn't stick to the loop; powdery
- Friable/crusty-breaks or clumps; brittle

Culture Characteristics on slants-
Similar growth characteristics to those observed on plates can be seen on slants. There are additional slant characteristics that experienced microbiologists use to evaluate growth. Those characteristics have to do with overall patterns of growth since single colonies are not often observed on a slant. For our purposes, when evaluating slants look for growth, that the culture is not contaminated, and that in general it matches your plate characteristics.

Culture Characteristics in broths-
Broth tubes should be observed before disrupting or shaking, then assessed again after gently mixing the tube. One should also note any pigmentation.

- Turbid-many bacteria will form uniform turbidity (cloudiness) throughout the test tube.
- Sediment-bacteria may settle to the bottom of the test tube. The broth may be very clear or slightly turbid. Upon mixing, one should make note of what happens to the sediment. Some sediments will disperse evenly in the tube, making it appear more turbid. Other sediments can be quite viscous and will not dissolve completely.
- Pellicle-bacteria may form a ring around the tube or a film across the top.
- Flocculence-bacteria may appear in clumps that remain after mixing.



Turbid Sediment Pellicle Flocculence

Pre-lab questions:

1. Why is it important to subculture bacteria?

2. Explain why one should evaluate single colonies of bacteria on plate media.

3. Draw a colony on a plate that is raised, lobate, translucent, and wrinkled. Show whatever perspectives are needed to illustrate those terms.

4. Why should you pay careful attention to the growth characteristics of the culture collection you build?

Materials:

Over time you will build stocks and evaluate growth patterns of the following: Organisms will be posted on the days you are to subculture them.

1 TSA Plate, 1 TSA Slant, 1 TSA Broth for each of the following:

- *Staphylococcus saprophyticus*
- *Staphylococcus epidermidis*
- *Lactococcus lactis*
- *Micrococcus luteus*
- *Sarcina aurantiaca*
- *Sporosarcina ureae*
- *Moraxella catarrhalis*
- *Mycobacterium smegmatis*
- *Corynebacterium pseudodiphtheriticum*
- *Bacillus cereus*
- *Alcaligenes viscolactis*
- *Escherichia coli*
- *Citrobacter freundii*
- *Serratia marcescens*
- *Pseudomonas fluorescens*
- *Enterobacter aerogenes*

Procedures:

For each set of organisms posted:

1. Label all plates, and create labels on tape and label all of the tubes.
2. Do an isolation streak on a TSA plate
3. Inoculate a TSA slant
4. Inoculate a TSA broth
5. Incubate all (NO parafilm in the incubator)
6. After incubation evaluate each culture for presence of growth, its growth characteristics, and absence of contamination. If any cultures are contaminated inform and show them to your instructor as you may need to re-culture.
7. After evaluating your new stocks, parafilm the plates and store the cultures in the student refrigerator.

Results:

As you subculture and evaluate your new stock cultures fill in the following chart:
(Write out the entire name of the bacterium and remember to use the correct binomial nomenclature format!)

Name of Bacterium	Plate Characteristics	Slant Characteristics	Broth Characteristics	Pure Cultures?

Questions:

Answer the following once you have completed your culture collection.

1. Were any of your cultures contaminated? If so, give possible explanations why the culture was contaminated.

2. Compare and contrast the growth characteristics of the following groups of bacteria:

a. *Staphylococcus saprophyticus vs. Staphylococcus epidermidis*

b. *Alcaligenes viscolactis vs. Escherichia coli vs. Citrobacter freundii*

Conclusion:

What factors-environmental, inoculation, etc., might affect how well bacteria grow in a culture?

Bacterial Population Counts: Direct Count, The Standard Plate Count, and Indirect Turbidimetric Methods

Why do we want to know how many bacteria are in a sample or specimen? Do you think it's important to know the number of microbes in food? Quantifying the bacterial level in a sample can be done in several ways. New rapid diagnostic tests can help identify particular bacteria in a sample, but the total count is informative in a manufacturing or diagnostic process.

What about in human disease? Typically, urinary tract infections are determined not by the mere presence of bacteria in a urine sample, but by the magnitude of bacteria (>10^5 CFU/ml) in the sample. [1, 2, 3]

Learning Objectives:

After this lab you should be able to:
1. Perform a serial dilution on a bacterial culture.
2. Design a dilution scheme for a Standard Plate Count.
3. Determine the final dilutions in a serial dilution and the # of CFU/ml (cell density) of the original sample.
4. Utilize the Spectrophotometer to determine the OD of bacterial culture samples.
5. Graph absorbance vs. the dilution of your serial dilutions.
6. Compare and contrast Direct Count, Standard Plate Count, and the Indirect Turbidimetric method of enumeration bacterial counts in a sample.

Introduction:

Quantifying the bacterial level in the sample can be done in several ways. New rapid diagnostic tests can identify particular bacteria in a sample, but the total bacterial count is informative and important in a manufacturing or diagnostic process; there are several ways to do this. Later in the course you will use a statistical technique on water samples called the Most Probably Number (MPN).

A. Direct microscopic counts of cells can be done, visually, and by various types of electronic particle counters.

B. Viable bacterial counts can be determined by the Standard Plate Count (SPC) method. A sample is diluted several times (serial dilution) and then plated over the agar surface of a petri plate. After incubation the number of colonies on the plate can be counted and are directly related to the number of bacteria in original sample, based on the magnitude of the dilutions. Each colony arises from a single bacterium, or group of bacterium depending on the typical arrangement (tetrad, staph, diplo, etc.) and is referred to as a Colony Forming Unit (CFU).

C. Indirect counts of a sample can be performed by using a spectrophotometer and measuring the turbidity (aka absorbance or Optical Density) of the sample. This is called the Turbidimetric method. A standard curve must be created using the known count of a sample, and then counts of various dilutions can be extrapolated.

There are advantages and disadvantages to each method. Direct counts sample small amounts of a larger sample and one can't distinguish between live cells and dead cells. One could however, see a variety of cell types. Likewise, the Turbidimetric method would also count live and dead cells, though one could not distinguish different types of cells. In addition, the Optical Density (OD) of a culture may not always be linear especially at high cell density (twice the number of cells may not cause twice the turbidity). The Standard Plate Count does represent live cells, and sometimes a variety of colony types

indicate the composition of the original sample. The SPC would not account for cells that might not grow well on the type of media being used, or cells that might be inhibited by other bacteria or other environmental factors. This is not a problem in a sample of known bacteria.

Pre-lab questions:

1. What is meant by "serial dilution"?

2. Why would it be important to know the number of bacteria in a food or beverage sample?

3. Write the following in scientific notation:
   a. 1:10

   b. 1:10,000

   c. 234×10^4

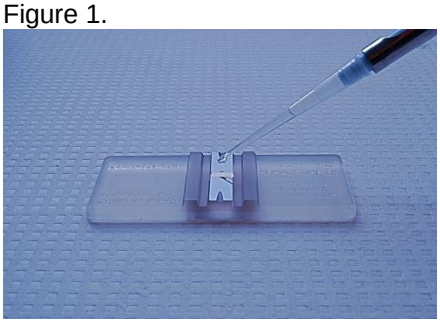
4. What is the inverse of 3.0×10^{-5}?

5. Which methods might over estimate the number of cells in a sample?

A. The Direct Count:

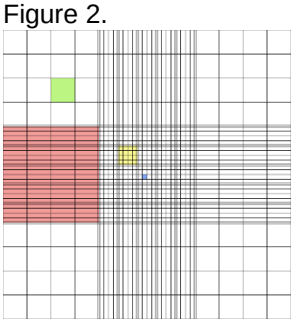
Microbes in a sample can be counted by viewing and counting them through a microscope and utilizing a special slide called a Petroff-Hausser counting chamber. Blood cells can be counted this way via a hemocytometer (see resource section). A known sample volume is placed on the slide and the slide has gridlines marking areas of known volume. The marked areas can be evaluated in a particular pattern. Cells are counted in each area and thus a total count for the sample can be calculated.

Figure 1.



Petroff-Hausser Counting Chamber
Todd. CC-BY-SA-2.0

Figure 2.



Petroff-Hausser Counting Chamber
Zephyris (Richard Wheeler)
CC-BY-SA-3.0

Back in the day, I worked for a juice company in QC. One of the tests on OJ was to count fungal and insect contaminants via a Petroff-Hausser slide.

Prof. Kelly Burke

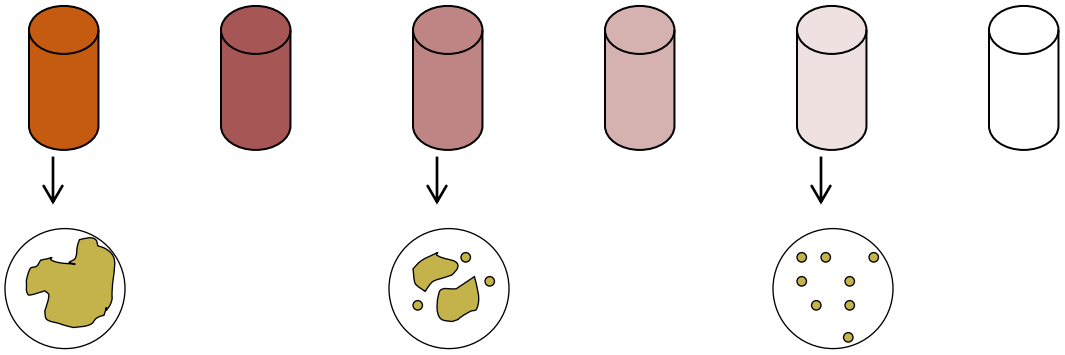
Electronic cell counters also count individual cells, typically as they flow past a laser (flow cytometry). Both of these methods count both live and dead cells and potentially any debris in the solution. You will not be performing a Direct Count in this lab.

B. The Standard Plate Count-- Spread Plate Method of Isolation:

In the SPC one wants to determine the density (number) of bacteria in a broth sample/specimen of bacteria of unknown density (number of cells/unit volume). One could take a small sample from the specimen, perhaps 1.0ml or 0.1ml and spread it across a petri dish to count the resulting colonies (remember that each colony is from one bacterial cell or unit). However, most specimens, based on the turbidity, might have millions of bacteria per ml in the sample. If 1.0ml was plated onto agar the number of bacteria would be so many that it would be impossible to count or even discern individual colonies. Therefore, one must perform a series of dilutions with the specimen to obtain a sample that will produce a plate on which colonies can be counted.

To do this, a known volume is taken from the original sample and placed in a dilution tube of a known volume, usually of sterile water or broth. After mixing, a known volume from the dilution tube is taken and placed in another dilution tube. This series of dilutions can be repeated a number of times resulting in progressively fewer cells in each tube down the line.

Figure 3.



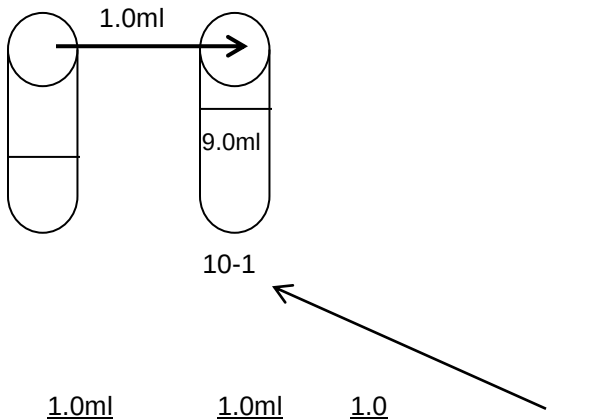
From the diluted mixtures one can then remove 1.0ml or 0.1ml and spread it over a plate of media. Somewhere in the series will be a “countable plate”. A countable plate is one that has between 30-300 CFU/ml (**C**olony **F**orming **U**nits/ml). Fewer than 30 colonies (TFTC) and miscounts on a plate become statistically problematic. More than 300 colonies (TNTC) and counting becomes very difficult and errors are easily made! You’ll see this when you plate your samples.

Once one has counted the number of CFU on a plate, that number must be multiplied back by the amount the sample was diluted—the dilution factor. For example, if you dilute your sample by 100 times (meaning that the sample you used was 100x more dilute than the original specimen), you must multiply your count by 100 in order to determine the density of bacteria in the original specimen. There are several ways to calculate the dilutions in the series and the final dilutions plated. Any type of dilution can be made; however, in a SPC the dilutions are typically 1:10 or 1:100, which become fairly easy to calculate once you practice them and get the patterns. The process is illustrated below:

1. A single dilution can be figured out by the following:

$$\frac{\text{Amount transferred}}{\text{Amount transferred + volume of the dilution tube = DF (dilution factor)}}$$

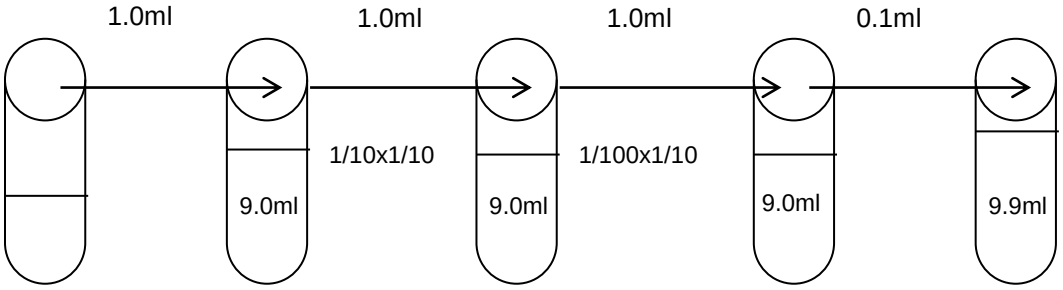
If 1.0ml is transferred to a 9.0ml dilution tube then the dilution is:



$$\frac{1.0\text{ml}}{1.0\text{ml} + 9.0\text{ml}} = \frac{1.0\text{ml}}{10.0\text{ml}} = \frac{1.0}{10.0} \text{ or } 1{:}10 \text{ or } 10^{-1}$$

What would the dilution be if 0.1ml were added to 9.9ml? Show how you would determine this:

2. A series of dilutions is cumulative. Each tube is more dilute than the last, so the dilutions are compounded (multiplied together) as the series progresses:



Undiluted original sample	10^{-1}	10^{-2}	10^{-3}	?

These dilutions can also be calculated with this commonly used equation:

$V_1D_1=V_2D_2$

V= volume
D= dilution

Let's say you transferred 10.0ml from your original undiluted sample to a 90.0ml dilution tube. V_1 is the volume you will transfer (10.0ml). D_1 is always 1 because that is the starting dilution tube. V2 is the total volume in the next dilution tube once you've transferred your sample to it. So in this case,

10.0ml (1) = 100.0ml (D_2), solving for D_2:

10.0ml/100.0ml = D_2

1/10 = D_2 or $10^{-1} = D_2$

Here is a more unusual example. Transfer 3.0ml into a dilution tube with 10.0ml (Why? Because one can! Not that one normally would…)

3.0ml (1) = 10.0ml (D_2) solving for D_2

3.0ml/13.0ml = D_2

0.23 = D_2 or $2.3x10^{-1} = D_2$

3. The next step is to plate your sample. Again, typically you would plate 1.0ml or 0.1ml depending on what you might need for your final dilutions. **Show the Dilution Factor (DF) for each plate (the final dilution value for the plate):**

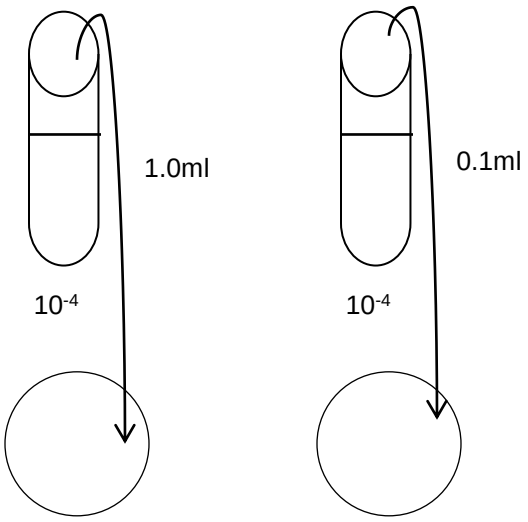


Plate DF **Plate DF**

To determine the number (original cell density) of bacteria in the original sample:

$$\text{OCD} = \frac{\text{Number of CFU}}{\text{Amount plated}} \times \text{dilution factor}$$

If you diluted a plate 100x (1:100, or 10^{-2}), transferred 1.0ml to an agar plate resulting in 65 colonies on the plate:

65 is the number of CFU
1.0 ml is the amount plated
10-2 is the dilution factor

So,

$$\frac{65 \text{ CFU}}{1.0\text{ml} \times 10^{-2}} = 65 \times 10^{2} \text{ CFU/ml} = 6.5 \times 10^{3} \text{ CFU/ml}$$

= the density of bacteria in your original specimen.

Materials:

2 Groups per Table:
5mL test tube culture of *E. coli*
2- 9.9 mL Water Blanks (Yellow Dots)
3- 9.0 mL Water Blanks
4- TSA plates (1 plate for ea. DF 10^{-5} – 10^{-8})
1 mL pipettes
4- Sterile yellow spreaders -1 pkg (dispose into pipette container on your bench)

Procedures:

The Standard Plate Count- Refer to the diagram below. The first thing your group should do is gather all the materials needed and work through the diagram until you are confident you know how to perform the dilution series. Then make a plan and begin the procedure. This will save you time in the long run, and will go a long way to prevent mistakes and poor results.

You will use the spread plate technique. First practice the technique with a non-sterile spreader and petri plate. After transferring the dilution sample to the plate, with one hand, open the petri plate, keeping the lid over the plate. With the other hand move the spreader back and forth across the plate while turning the plate at the same time. Spread and turn several times in order to evenly distribute the sample over the entire plate. Close the plate, let it dry, and then invert and incubate. Dispose of the spreader as instructed.

1. Mix the tube of *E. coli* well.

2. Use a sterile 1.0ml pipette to transfer 0.1ml of the *E. coli* culture to the first 9.9ml dilution tube. Mix well. Discard pipette.

3. Use a new sterile 1.0ml pipette to transfer 0.1ml from the first dilution tube to the second 9.9ml dilution tube. Mix well. Discard pipette.

4. Use a new sterile 1.0ml pipette to remove 1.0ml from the second tube to the third tube, this time a 9.0ml tube. Mix well. Discard pipette. Repeat for the 4th and 5th tubes, mixing each tube along the way.

Now begin to plate your samples. Dispense and spread the sample onto the plates one at a time. Do not dispense the samples and then go back to spread them. The plate will absorb the liquid and thus the sample will not be able to be spread across the plate. This will result in most of the bacteria clumping in the middle of the plate and accurate counts will not be achieved. One person can dispense the sample and another can spread the sample.

1. From the third test tube transfer 0.1ml, with a sterile 1.0ml pipette, to the first plate A. Spread the sample.

2. From the fourth test tube transfer 0.1ml, with a sterile 1.0ml pipette, to the second plate B. Spread the sample.

3. From the fifth test tube transfer 0.1ml, with a sterile 1.0ml pipette, to the third plate C. Spread the sample.
4. From the sixth test tube transfer 0.1ml, with a sterile 1.0ml pipette, to the last plate D. Spread the sample.

Keep your dilutions, and your original *E.c.* broth for the next part of the lab.

4. Set the plates aside to allow the liquid to be absorbed. Invert and incubate.

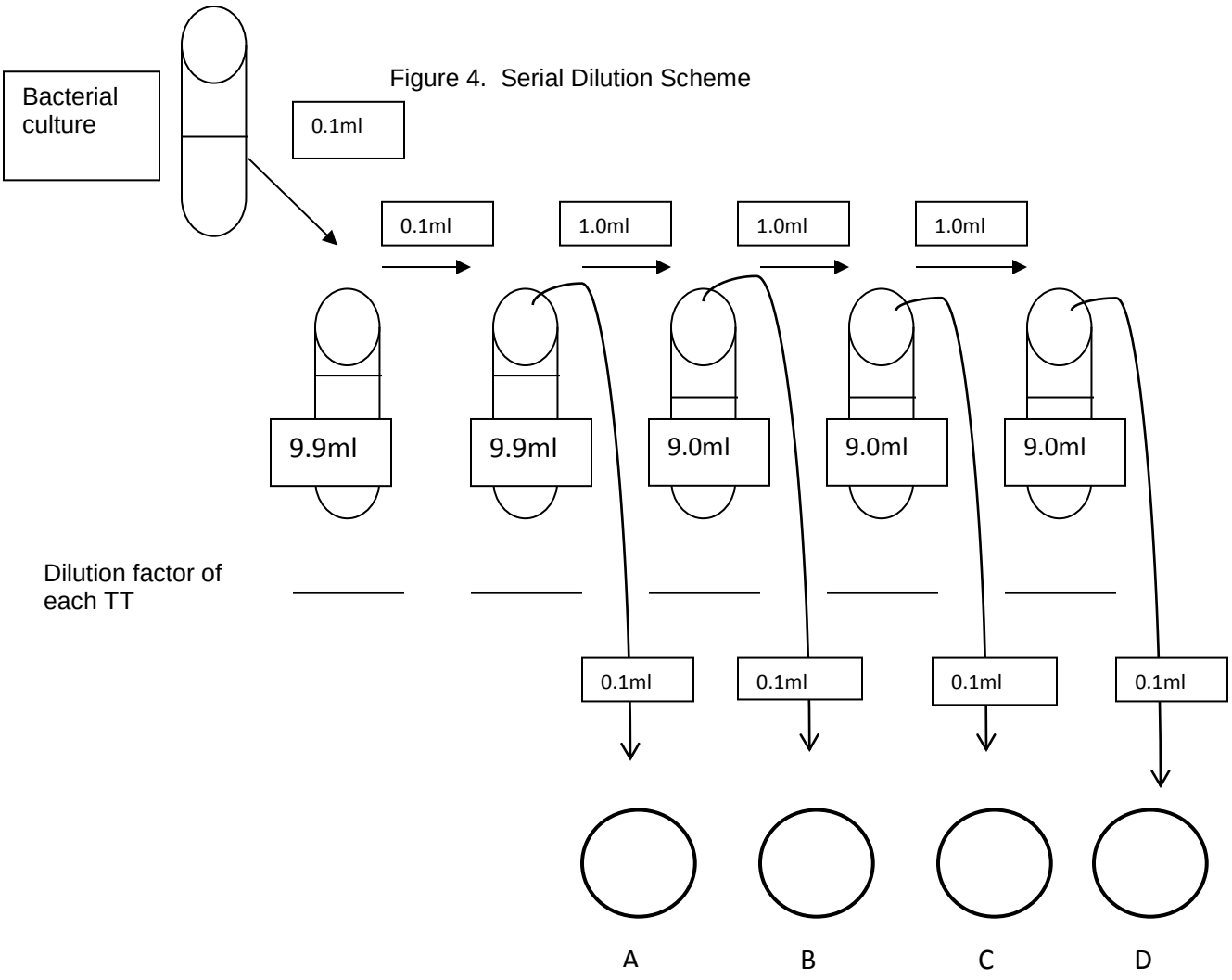


Figure 4. Serial Dilution Scheme

Results:

1. After incubation determine which plate is the countable plate (30-300 CFU). There should only be one, correct? Enter the information in the chart below.

Plate counts and determination of original cell density:

Plate	A	B	C	D
DF of plate				
# of Colonies*				
Original cell Density**				

* Give the colony count on each plate or indicate if plates are TFTC or TNTC
**Calculate based on the countable plate

2. Explain your results and/or issues that you had:

Questions:

Standard Plate Count:

1. Using the formula $V_1D_1=V_2D_2$, find the final dilution of the following (remember that $D_1=1$):

a) 10.0ml of a sample is transferred to 90.0ml of diluent

b) 4.0ml of a sample is transferred to 7.0ml of diluent

2. Using the formula determine the original cell density in the sample:

$$\text{OCD} = \frac{\text{Number of CFU}}{\text{Amount plated} \times \text{dilution factor}}$$

a) You counted 72 colonies on a plate in your dilution series. The plate was inoculated with 1.0ml from a 10^{-4} test tube, what is the OCD?

b) You counted 235 colonies on a plate in your dilution series. The plate was inoculated with 0.1ml from a 10^{-7} test tube, what is the OCD?

3.
a) You must make a 10^{-1} dilution using 5.0ml of your original sample. Show how you would do this. Hint: What volume of diluent do you need to add the 5.0ml to in order to perform and 10^{-1} dilution?

b) You must make a 10^{-2} dilution using 5.0ml of your original sample. Show how you would do this.

c) Show how you would make a 10^{-1} dilution using 4.0ml of your original sample.

d) Show how you would make a 10^{-2} dilution using 6.0ml of your original sample.

4.
a) You are instructed to add 2.0ml of your original sample to 2.0ml of diluent. What is your dilution?

b) You are instructed to add 2.0ml of your original sample to 4.0 ml of diluent. What is your dilution?

c) In your own words, explain the difference between the above dilutions:

5. 1.0ml of sample added to 99.0ml of diluent is a 10^{-2} dilution. What would happen if you added 0.1ml instead of 1.0ml? What would the final dilution be?

6. The colony count of a 10^{-8} plate in a dilution series is 162.
   a. What is the bacterial count in the original sample?

   b. What would the colony count be on the 10^{-7} dilution?

   c. What would the colony count be on the 10^{-9} dilution?

7. Set up a dilution scheme, using the following materials, with a final plating of four plates with dilutions of 10^{-5} through 10^{-8}. You must use exactly all 4 water blanks and plate four plates. Diagram the dilution scheme showing all tubes, plates, and dilutions.

Original sample
3 sterile water blanks (99mL)
1 sterile water blanks (9ml)
4 Petri plates

C. The Turbidimetric Method—Indirect Determination of Growth by Optical Density

Materials: 2 Groups per Table-

Your Dilution Series
Original *E. coli*
DI water (for blank)
TSB (for blank)
8 cuvettes and a cuvette rack (Dispose cuvettes with liquid in Biohazard trash can)
Kimwipes
5mL pipettes

Procedures:

You will measure the absorbance (OD) of **each one of your dilutions, and the original *E. coli* broth**, individually in a spectrophotometer. The spectrophotometer is a machine that shines a light through a liquid sample. A perfectly clear sample will transmit all the light. A sample that is cloudy (turbid) or colored will absorb some of the light and impede the transmission of the light. Therefore there is a direct relationship between turbidity and absorbance and an indirect relationship between turbidity and transmission. Do not write directly on cuvette tubes or cuvette rack. You may label the rack with tape.

1. Follow the directions on the Spectrophotometer for calibrating it. Ask your instructor if you do not know how to use the instrument, do not attempt to guess. It is important to blank the spectrophotometer with the sterile un-inoculated water to zero absorbance.
2. Follow the diagram below to set up the cuvettes.
    a. Using a 5ml pipette, dispense 2ml of sterile water into 1 cuvette as your blank (for the dilution tubes).
    b. Using a 5ml pipette, dispense 2ml of TSB into 1 cuvette as your blank (for the *E.c.* culture).
    c. Using a 5ml pipette, dispense 2ml of the original *E. coli* culture into a cuvette.
    d. Using a separate 5ml pipette for each dilution tube, dispense 2ml of each dilution into a corresponding cuvette.
    e. Use the blank cuvette (water only) to blank the spectrophotometer. Place the cuvettes sequentially into the spectrophotometer and read the optical density of the original culture and each of your dilutions. Record the O.D values into the table, plot them on a graph, and answer the questions.

Results:

1. Fill out the following table:

Tube/cuvette	Dilution	O.D.
1. Original broth	1:1	
2.		
3.		
4.		

5.		
6.		

2. Graph the results on the attached graph. And answer the questions below. Be careful to set the axes correctly and space the dilutions correctly.

Questions:

1. Why is it important to have a plate count along with the absorbance measurement?

2. What is the relationship between the O.D. values graphed and the cell number from the SPC for your culture?

Conclusion:

Compare and contrast the advantages and disadvantages of the 3 main procedures for determining bacterial counts in a sample. (SPC, OD, Direct count)

Resources:

1. Bryan C, "Infectious Disease, Ch.7, Urinary Tract Infections." *Microbiology and Immunology Online*, Univ of S. Carolina School of Medicine. 17 Feb 2015. Web. 20 Oct. 2015.
http://www.microbiologybook.org/Infectious%20Disease/Urinary%20Tract%20Infections.htm
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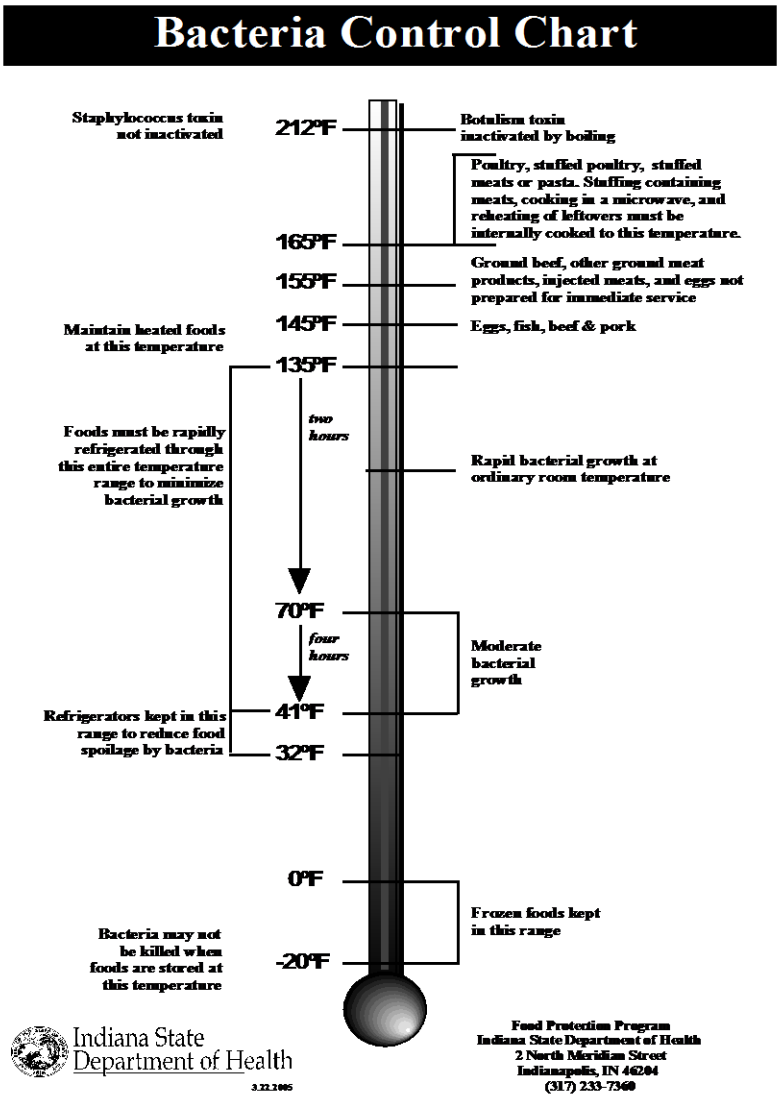
3. "Urine Culture." *Lab Tests Online.* 24 Feb. 2015. Web. 28 Oct. 2015.
https://labtestsonline.org/understanding/analytes/urine-culture/tab/test/
4. "Cell Counting with a hemocytometer." *Celeromics.* n.d. Web. 20 Oct. 2015.
http://celeromics.com/en/resources/docs/Articles/Cell-counting-Neubauer-chamber.pdf
5. Reynolds J. "Serial Dilution Protocols." *ASM Microbelibrary.* 1 April 2013. Web. 5 Nov. 2015.
http://www.microbelibrary.org/component/resource/laboratory-test/2884-serial-dilution-protocols

Environmental Effects on Growth: Temperature



Source: http://www.in.gov/isdh/files/bac_chart_vert.pdf

This chart from the Indiana State Dept. of Health shows the relationship of various temperatures on the growth of foodborne bacteria. What temperatures do most bacteria grow in? How does this relate to things like food storage, medication storage, etc.?

Learning Objectives:

After this lab you should be able to:

1. Define the terms: Cardinal temperatures, psychrophiles, psychrotrophs, mesophiles, thermophiles, hyperthermophiles.

2. Know several relative temperatures in centigrade.

3. Relate temperature to survival of bacteria in food, water, etc.

4. Describe the effects temperature can have on a cell.

5. Briefly relate production of prodigiosin to gene regulation in *Serratia marcescens*.

Introduction:

Heat is a common method of controlling the growth of microorganisms. But what temperatures are sufficient? We can freeze, refrigerate, pasteurize, boil, autoclave, incinerate, etc. Why do we need so many different methods?

Different bacteria flourish or survive at different temperatures. Some microbes live in glaciers, some in the guts of animals, others in hot springs. The range that a particular bacterium grows in is called the cardinal temperatures: the minimum, optimum, and maximum temperatures. Outside of that range the bacterium may not survive. Bacteria can be categorized based on their temperature requirements for optimal growth:

Psychrophiles: 0°C-20°C

Psychrotrophs: 0°C-35°C

Mesophiles: 15°C-45°C

Thermophiles: 45°C-80°C

Hyperthermophiles: 65°C-113°C

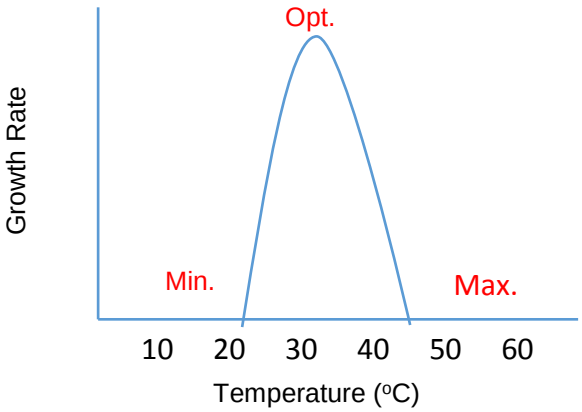


Figure 1.
This graph shows the cardinal temperatures range for a bacterium. The minimum temperature is 20°C, the optimum is 32°C, and the maximum is 45°C. We would characterize this bacterium as a Mesophile. Why? You will need to refer back to this graph when you work on the Questions section at the end of the lab.

Temperature may have several different effects on the cell that can inhibit growth, decrease metabolic function, or cause cell death. Metabolic reactions and efficiency in a cell are dependent on enzymes, and on membrane activity and transport. Enzymes are proteins; therefore, they can be denatured by both high and low temperatures. Enzyme activity can be sped up by higher temperatures and slowed by lower temperatures. The optimal temperature is that which provides for efficient enzyme/metabolic activity within the cell. In addition, high temperatures can disrupt membranes and transport systems by destroying ("melting") lipids in the membrane. Low temperatures can slow membrane transport and decrease ("solidify') fluidity of the membrane. Microorganisms that live at the extremes have structural and metabolic adaptations that help them survive.

In this exercise you will grow different bacteria at a range of temperatures to examine their growth patterns. You will observe the range and optimal temperature for each. In addition you will examine the effect of temperature on gene expression and metabolism by observing production of the pigment prodigiosin by *Serrratia marcescens*

Pre-lab questions:

1. Utilizing this conversion equation, determine what the following Fahrenheit temperatures would be in Centigrade and label these on the chart above:

$$(°C) = [(°F) - 32] \times 5/9$$

250°F is _____ °C (autoclave)

212°F (boiling) is _____ °C

135°F is _____ °C

107°F is _____ °C

98.6 °F (body temp.) is _____°C

77°F (RT) is _____°C

41°F is _____ °C

32°F (freezing) is _____ °C

2. What is the primary effect temperature has on living cells?

Materials:

Temperature growth ranges

Student stock TSB cultures-
Pseudomonas fluorescens
Bacillus cereus
Escherichia coli

Instructor provided TSB culture-
Streptococcus mutans

16- TSB tubes

4-Sterile transfer pipettes

Buckets labeled according to incubation temperature

Prodigiosin production

Student stock TSA plate culture-
Serratia marcescens

2- TSA Plates

Procedures:

2 Groups per Table:

Temperature growth ranges

1. Obtain the 16 test tubes of TSB and label the tubes with the organism and the temperature. Each organism will be inoculated into 4 tubes of TSB. These four tubes will then be inoculated at 4 different temperatures.

2. So, there will be 4 test tubes for each of the 4 organisms:

P. fluorescens → 5ºC, 25ºC, 37ºC, 42ºC

B. cereus → 5ºC, 25ºC, 37ºC, 42ºC

E. coli → 5ºC, 25ºC, 37ºC, 42ºC

S. mutans → 5ºC, 25ºC, 37ºC, 42ºC

3. Using a sterile transfer pipette inoculate 1 drop of the organism into the 4 separate tubes of TSB. Place the correctly labeled and inoculated test tube of each organism into each temperature bucket on the front table:

5ºC
25ºC
37ºC
42ºC

Prodigiosin production

1. Streak 2 TSA plates with *Serratia marcescens.* Label one for incubation at 25ºC and one for incubation at 42ºC.
2. Place, inverted, on the correct side (25ºC or 42ºC) of the pan labeled for incubation.

Results:

Temperature growth ranges

1. After incubation, retrieve your group's TSB tubes from the labeled buckets and mix them gently and evenly distribute the bacteria in the tube.
2. Compare all the tubes of the same species to each other (all the *E. coli* to each other, etc.). Score them according to how turbid the tubes are and record the results in the table:

0 = no growth (clear)
1 = slightly turbid
2 = moderately turbid
3 = very turbid

(Note that you can also compare all the species to each other at a given temperature.)

Enter your ratings for each bacterium/temperature and give the temperature classification for bacterium.

Organism	5ºC	25ºC	37ºC	42ºC	Optimum Temperature	Temperature Classification

Prodigiosin production

1. Retrieve the *Serratia marcescens* TSA plates. Observe and compare the differences in growth at the two temperatures, 25ºC and 42ºC.
2. Record your observations in the table.

Enter your observations on the growth characteristics of *Serratia marcescens*

Temperature	Growth Characteristics
25ºC	
42ºC	

Questions:

1. Organisms found in hot springs are generally not human pathogens. Explain why.

2. What are the major effects that heat may have on a cell?

3. Why is the turbidity of different bacteria at the various temperatures different?

4. How does the difference in prodigiosin pigment production of *Serratia marcescens* relate to gene expression?

5. How might you use temperature to help you distinguish *Serratia marcescens* from *E. coli*?

6. Refer back to Figure 1., the graph of a mesophilic bacterium. Utilize the graph paper to draw the curves of each temperature category. Compare your results to the graph and label your organisms to the category they most closely match.

Conclusion:

Reflect on the lab and your results and discuss why freezing, refrigerating, and even boiling may not be sufficient to kill all bacteria.

Resources:

Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2014). *Prescott's Microbiology* (9th ed.) (pp. 141-149). New York, New York: McGraw-Hill.

Indiana State Dept. of Health: http://www.in.gov/isdh/files/bac_chart_vert.pdf

Environmental Effects on Growth: pH

"Growth of the bacterium *Clostridium botulinum* in canned food may cause botulism—a deadly form of food poisoning. These bacteria exist either as spores or as vegetative cells. The spores, which are comparable to plant seeds, can survive harmlessly in soil and water for many years. When ideal conditions exist for growth, the spores produce vegetative cells, which multiply rapidly and may produce a deadly toxin within 3 to 4 days of growth.

Acidity may be natural, as in most fruits, or added, as in pickled food. Low-acid canned foods are not acidic enough to prevent the growth of these bacteria. Acid foods contain enough acid to block their growth, or destroy them more rapidly when heated. The term "pH" is a measure of acidity; the lower its value, the more acid the food. The acidity level in foods can be increased by adding lemon juice, citric acid, or vinegar."

National Center for Home Food Preservation
http://nchfp.uga.edu/how/general/ensuring_safe_canned_foods.html

Learning Objectives:

After this lab you should be able to:

1. Define pH.

2. Explain the quantitative difference between two different pH values, for example, pH 4 and pH 6.

3. Describe the major effects pH changes can have on a cell.

Introduction:

The pH of an environment can affect the growth and survival of the microbes in that environment. pH is a measure of the hydrogen ion concentration or activity, in a solution, expressed as:

$$\mathbf{-\log_{10}[H+]}$$

Every organism has an optimal pH range at which it will grow. If the pH is too high or too low it can affect things like the solubility of molecules, denaturation of proteins/enzymes, membrane transport, proton-motive force/membrane potential, etc. These changes can be devastating to a bacterial population. We have exploited this in the production of foods, for example, pickling is a common method of food preservation and compounds like citric acid are often added to food products to lower the pH and prevent spoilage or toxin formation.

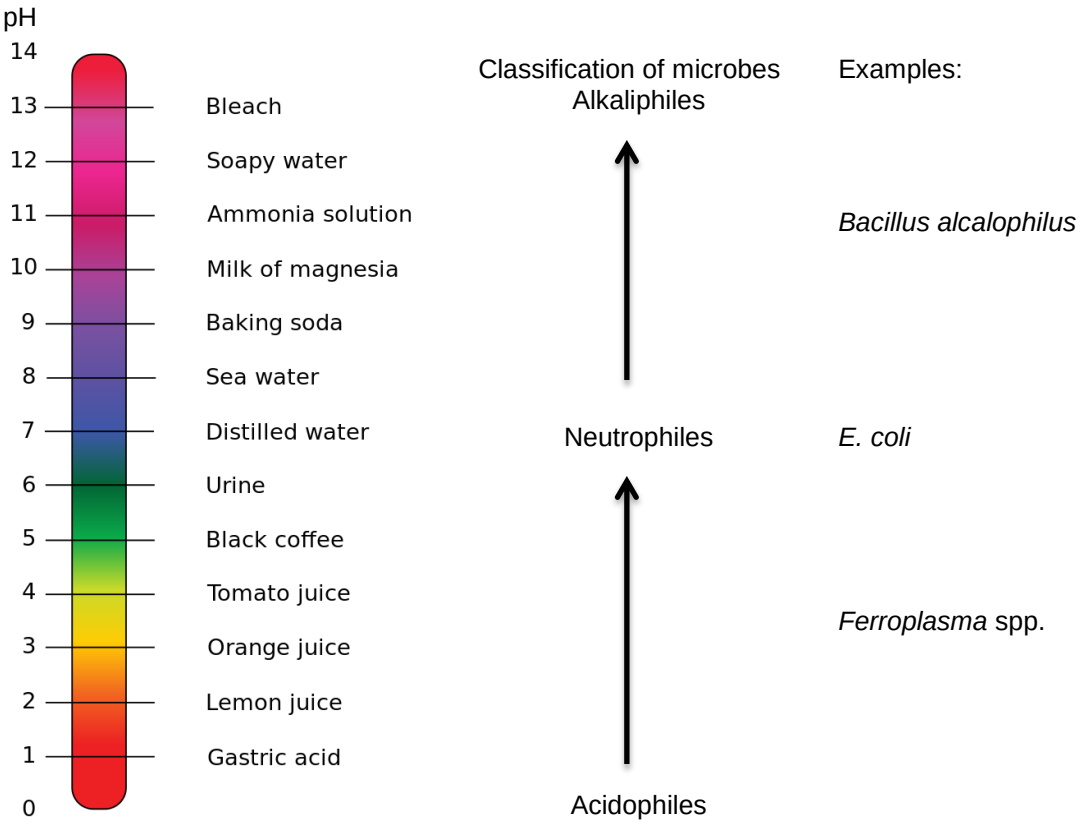
Most bacteria are neutrophils while fungi prefer a more acidic environment from pH 4-6. Archaea are found in all ranges. Nonetheless, all microbes keep their internal environment near neutral and some have developed mechanisms to allow them to survive in the more extreme ranges.

In microbiology we also use a variety of biochemical tests based on pH changes in the media as diagnostic tests. Many bacteria produce acids that are released into the environment as metabolic products of fermentation. These acids can have a big effect on the pH of a closed system like a test tube or agar plate. You will utilize some of these biochemical tests in the future to help you identify bacteria and key characteristics of them.

The pH scale is a tool to measure the relative acidity of solutions, food, natural environments like lakes (water), and as stated, biochemical reactions. The scale is logarithmic reflecting the definition of pH and actually making the scale easy to use. pH 0, which is the most acidic level, has a hydrogen ion concentration of 10^0 moles/L; pH 14, the most alkaline, has a hydrogen ion concentration of 10^{-14} moles/L. Pure water is neutral and therefore has a pH of 7 (10^{-7} moles/L). Each pH level represents then a tenfold decrease or increase in hydrogen ion concentration. So, pH 6 is ten times more acidic than pH 7, and ten times more alkaline than pH 5.

There are 3 main groups of microbes classified by the pH range they can survive in:

Acidophiles: pH 0-5.5
Neutrophiles: pH 5.5-8.0
Alkaliphiles: pH 8.0-11.5



Pre-lab questions:

1. Quantify the difference between:

a. pH 2 and pH 3

b. pH 6 and pH 8

c. pH 5 and pH 9

2. Explain pH in your own words:

3. Explain why we used a selective media, Sabouraud Dextrose Agar (SDA) earlier in the course to inhibit bacteria and select for fungi.

Materials:

2 Groups per Table:
Organisms used from the Student Stock Cultures(TSB):
Staphylococcus saprophyticus
Escherichia coli
Alcaligenes viscolactis
Lactococcus lactis

4 tubes: TSB pH 4.0
4 tubes: TSB pH 6.0
4 tubes: TSB pH 8.0
4 tubes: TSB pH 10.0

Procedures:

Each Group:
1. Use a sterile transfer pipette to inoculate each of the 4 organisms into each pH tube: add 1 drop of broth culture to each pH broth tube.

Staphylococcus saprophyticus → TSB pH 4.0, pH 6.0, pH 8.0, pH 10.0

Escherichia coli → TSB pH 4.0, pH 6.0, pH 8.0, pH 10.0

Alcaligenes viscolactis → TSB pH 4.0, pH 6.0, pH 8.0, pH 10.0

Lactococcus lactis → TSB pH 4.0, pH 6.0, pH 8.0, pH 10.0

2. Place the tubes in buckets and incubate in your classroom incubator.

Results:

Enter your ratings for each bacterium/pH and give the pH classification for bacterium.

Organism	pH 4	pH 6	pH 8	pH 10	Optimum pH	pH Classification

Questions:

1. A buffer is a chemical often added to growth media to prevent shifts in the pH of the media. A buffering system will absorb or release H^+ ions to prevent the media from becoming too acidic or alkaline, respectively. Why would it be important to add a buffer to growth media?

2. Based on your results, is there overlap in the pH ranges of the organisms? Why might this be?

3. The pH of seawater is around 8.3. What factors or media adjustments would you have to make when trying to culture marine organisms?

Conclusion:

Later in course you will use different media to help characterize and identify bacteria. Several tests use fermentation of sugars to differentiate bacteria.

1. How does fermentation of sugars relate to pH?

2. How might you use pH changes to help you identify different bacteria?

Resources:

Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2014). *Prescott's Microbiology* (9th ed.) (pp. 141-149). New York, New York: McGraw-Hill.

Environmental Effects on Growth: Osmotic Pressure

Gather the family/friends/roommates around and try this at home:

Place a piece of lettuce in a bowl of super salty water. Let is sit for a half hour or so. Prediction? Observations? Next, place the same piece of lettuce in a bowl of plain tap water. Let it sit again—this may take a bit longer. Prediction? Observations?

Try to explain what happened, or (should you be out of lettuce) what you think would happen.

Learning Objectives:

After this lab you should be able to:

1. Define osmosis, hypertonic, hypotonic, and isotonic.

2. Analyze scenarios with regard to net water movement in and out of a cell and discuss the effect of osmotic pressure on microorganisms.

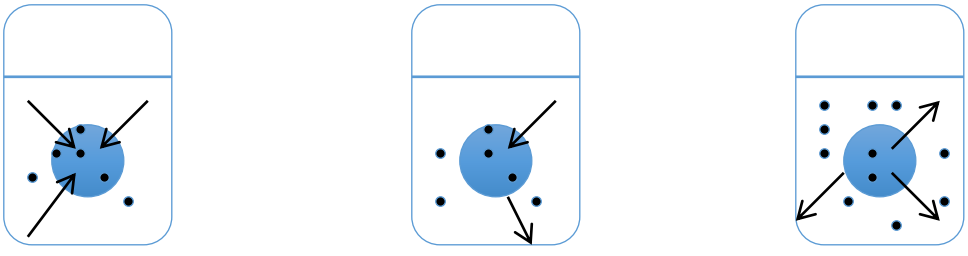
3. Classify organisms based on their salinity preferences.

Introduction:

Like other cells, the osmotic environment surrounding microorganisms can affect them. **Osmosis** is the net movement of water molecules across a semi-permeable membrane, from an area of low solute (high water activity) concentration to higher solute concentration (lower water activity). Water molecules want to balance the solute concentration on either side of the membrane to a more **isotonic** state. Osmotic pressure is the physical force to draw water to one side of the membrane or other. In a **hypertonic** solution (more solutes outside of the cell, higher osmotic pressure) there will be net movement of water out of the cell (in order to create equal osmotic pressure on both sides of the membrane). **Plasmolysis**, cell shrinkage, may occur. In a **hypotonic** solution the opposite will happen. There are more solutes inside the cell relative to the solution and the net movement of water will be into the cell, which may result in **lysis** of the cell.

It's important to remember that osmosis is relative to the two side of the membrane. When considering a microorganism and its environment, one should consider the inside of the cell relative to the environment. Is the environment hypertonic (higher solutes) or hypotonic (lower solutes) relative to the cell? This will determine the net movement of water.

Net movement of water:



Hypotonic Isotonic Hypertonic

The bacterial cell wall helps those (with a cell wall) maintain their osmotic pressure in a fairly stable environment so that neither plasmolysis nor lysis will occur. Most bacteria live in a narrow range of

salinity, usually less than 3% NaCl. **Halophiles** will grow in higher than 3% NaCl. **Extreme halophiles** require a very high salt concentration (up to 30%) and can be found in places like the Dead Sea, Great Salt Lake, etc. These organisms have proteins, enzymes, membranes, etc., that function in the high osmotic environment. In addition, some bacteria have adapted mechanisms to survive in a wider range of osmotic environments and are **osmotolerant**.

Pre-lab questions:

1. What is the primary effect of osmotic pressure on a cell?
2. **Look up the salinity and pH of seawater. What challenges would you have, and what would you do to overcome them, with culturing marine bacteria in the lab?**

Materials:

2 Groups per Table:
Organisms:
Staphylococcus saprophyticus
Escherichia coli
Lactococcus lactis

1 plate of each Sodium Chloride (NaCl) concentration:
TSA with 2.0% NaCl
TSA with 5.0% NaCl
TSA with 10.0% NaCl

Procedures:

Inoculate each plate with the following organisms: *Staphylococcus saprophyticus*, *Escherichia coli, Lactococcus lactis*

1. Use a single streak on ⅓ of the plate for each organism; make sure to label each streak line with the correct organism:

Staphylococcus saprophyticus, *E. coli, Lactococcus lactis* → TSA with 2.0% NaCl
Staphylococcus saprophyticus, *E. coli, Lactococcus lactis* → TSA with 5.0% NaCl
Staphylococcus saprophyticus, *E. coli, Lactococcus lactis* → TSA with 10.0% NaCl

2. Invert and incubate.

Results:

1. After incubation assess the growth of each bacteria on each plate.

2. Fill out the chart below:

Enter your ratings for each bacterium/salinity and give the salinity classification for bacterium.

Organism	2.0% NaCl	5.0% NaCl	10.0% NaCl	Optimum salinity	Salinity Classification

Questions:

1. How do hypotonic and hypertonic environments generally affect most bacteria?

2. What difference between bacterial cells and animal cells helps to protect bacteria against osmotic changes in their environment?

3. How would you characterize marine bacteria in relationship to osmotic pressure?

4. Define water activity.

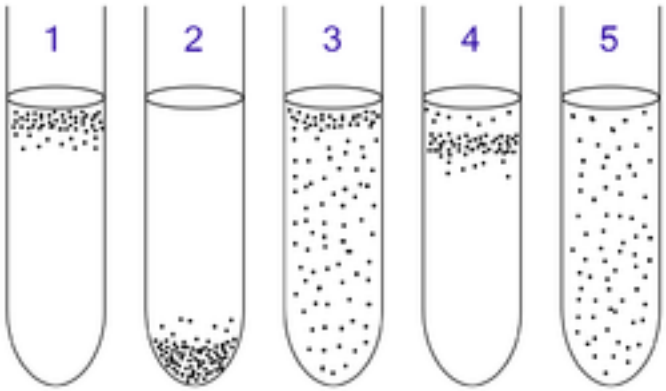
Conclusion:

Reflect on your results and discuss how osmotic pressure might relate to food preservation.

Resources:

Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2014). *Prescott's Microbiology* (9th ed.) (pp. 141-149). New York, New York: McGraw

Oxygen Requirements: FTM and the Anaerobe Jar



By Pixie (Own work) [Public domain], via Wikimedia Commons
https://commons.wikimedia.org/wiki/File%3AAnaerobic.png

Learning Objectives:

After this lab you should be able to:
1. Differentiate between the terms aerobe, anaerobe, and facultative anaerobe
2. Determine the oxygen requirements of a bacterium via Fluid Thioglycollate Medium (FTM) and the GasPak System.
3. Describe how a GasPak jar is used to culture anaerobes

Introduction:

It's hard to imagine living without oxygen. And it's hard to imagine that oxygen respiration can result in many toxic products. Animals do require oxygen and have enzymes to break down those toxic products. Bacteria on the other hand are not all the same when it comes to oxygen requirements. There are many categories regarding oxygen utilization in bacteria.

Aerobes require oxygen, **anaerobes** cannot survive in the presence of oxygen because they do not have the enzymes to break down toxic by-products and will quickly die in the presence of oxygen. **Facultative anaerobes** do have the necessary enzymes and can grow with or without oxygen. Two other categories are the **microaerophiles**, which prefer a reduced oxygen atmosphere, and **aerotolerant** organisms, which do not use oxygen, but are not killed by it.

Take a look at the drawing above, which test tube illustrates each of the categories just listed?

In this lab you will compare two methods for growing and observing oxygen requirements of a few different bacteria. The first is a piece of equipment called a GasPak jar. The other is a medium called Fluid Thioglycollate Medium (FTM). For the GasPak system you will inoculate 2 plates of TSA with the same bacteria and incubate one aerobically in your incubator and one anaerobically in the GasPak jar. You will inoculate tubes of FTM with the given bacteria and incubate them as normal. Then you will compare the growth patterns of the same bacteria in each.

GasPak System:

Purpose: Permits the growth of anaerobic and facultative bacteria.

Reagents/Indicators: Oxygen indicator

Mechanism/reactions: The Gas Generating Sachet reduces oxygen in the container and produces carbon dioxide.

Directions: Inoculated plates are inserted into the jar. The sachets are opened and packet is removed from the foil. An oxygen indicator is placed in the jar and the jar is immediately closed.

Interpretation: Growth--Microaerophilic, Facultative anaerobe, Anaerobic

Fluid Thioglycollate: Oxygen Requirements:

Purpose: The use of thioglycollate broth permits growth of anaerobic bacteria. Growth patterns can help distinguish aerotolerance and oxygen requirements of bacteria.

Media: Contains glucose, cysteine, and sodium thioglycollate (oxygen reducing agent)

Reagents/Indicators: Resazurin

Mechanism/Reactions: This is a nutritive medium with a reducing agent (sodium thioglycollate), which, due to a chemical reaction, removes oxygen from the broth. A chemical indicator is included in the broth-resazurin. The pinkish color indicates the presence of oxygen.

Directions: Deep stab needle inoculation.

Interpretation: Aerobic, Microaerophilic, Facultative anaerobe, Anaerobic

Pre-lab questions:

1. Define the terms aerobe, anaerobe, and facultative anaerobe.

2. Compare microaerophiles with aerotolerant organisms.

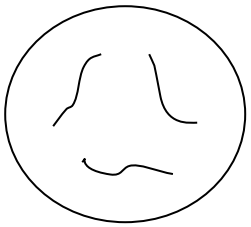
3. How will you compare the FTM tubes with the GasPak plates, in other words, how are they related? What do you expect to observe?

Materials:

3 FTM tubes
2 TSA plates
GasPak jar, sachets, indicator
Bacterial cultures:
Staphylococcus saprophyticus (Use student stock organism)
Pseudomonas fluorescens (Use student stock organism)
Clostridium sporogenes (Use slant on front table, one per table only)

Procedures:

1. Quickly streak each of the 3 organisms onto 2 TSA plates:



2. Immediately place 1 plate in the anaerobe jar. Incubate the other plate aerobically in your class incubator.

3. Inoculate each organism into a separate Fluid Thioglycolate Medium(FTM):
Staphylococcus saprophyticus, Pseudomonas fluorescens , Clostridium sporogenes

4. Incubate FTM tubes in your class incubator.

Results:

1. **GasPak:** Compare the GasPak plate to the plate incubated aerobically and fill in the following chart.

Organism	GasPak growth (+/-)	Aerobic growth (+/-)	Oxygen requirement type

2. FTM: **Draw your FTM tubes and label each with the appropriate oxygen requirement type**

Questions:

1. List the oxygen requirements for each of the three bacteria you used.

2. Why did the anaerobe not grow on the aerobic plate?

3. What is the role of the resazurin in the FTM?

Conclusion:

Explain the relationship of the GasPak system to the FTM tubes.

Environmental Effects on Growth: Antimicrobial Sensitivity Testing

Learning Objectives:

After this lab you should be able to:

1. Describe the purpose and use of the Antibiotic Susceptibility test.
2. Describe the implications that the test is an "in vitro" test and not actually a test in a patient.
3. Explain the need for and the ways in which this protocol is standardized.
4. Determine if the bacteria tested are Sensitive, Resistant, Or considered Intermediate, for each antibiotic used.
5. Perform the Kirby-Bauer method.

Introduction:

Antibiotic susceptibility testing can be done in many ways, including new genomic tests, the MIC broth dilution, and the Kirby-Bauer (K-B) disk diffusion method that you will perform in lab today. Identifying the correct antibiotic to use is critically important, especially in today's situation with the increasing number of antibiotic resistant bacteria emerging in all populations. Delay of using the appropriate antibiotic, one that the bacterial disease agent is sensitive to, can greatly reduce successful patient outcomes at an alarming rate and can be the difference between life and death. All antibiotic susceptibility tests have limitations. One of the biggest drawbacks of the K-B test is that it is an in vitro test. What happens in a petri dish is not always the same thing that will happen in an actual patient. Why do you think there could be differences (think about this, you will be asked again!)?

In order for the K-B test to be valid across institutions and be accurate for each patient, it must be standardized. This includes the media used, Muellar-Hinton agar, the depth the media is poured, age of the media, etc. As the media ages, or if the depth or moisture content are not accurate, the antibiotics in the disks will not diffuse through the media the same from batch to batch. So consistency in media is essential. The antibiotics are prepared specifically for this test by infusing known amounts into standard paper disks. During the test the antibiotics will diffuse out into the media, based on the molecular size of the antibiotic. The amount of bacteria placed on the plate must also be standardized. This can be done in a variety of ways—usually by prepping a broth culture via a spectrophotometer (how does this work? Think about what you did when you measured the absorbance of your dilutions in the SPC procedure), or by using a McFarland standard. The MacFarland standard method employs a prepared test tube of a

specific turbidity with which one can prepare a broth of the bacterium to be tested to the same turbidity. This helps ensure that a consistent amount of bacteria is plated. Incubation temperature and time are also critical, as well as accurate measurement and interpretation of the zone of inhibition. As bacteria begin to grow on a plate, the antibiotics are diffusing out into the agar. If the bacteria are sensitive to the antibiotic, at some point their growth will be inhibited and a zone of no growth (zone of inhibition) will be apparent around the disk. The diameter of the zone is measured and the size of the zone determines whether a bacterium is determined Sensitive (S), Resistant (R), or Intermediate (I) to the antibiotic. The size and interpretation of the zone is specific to each bacteria-antibiotic pair and is referenced in a chart.

Pre-lab questions:

1. What is an antibiotic and how, generally, do they work? (You will likely need to look this up!)

2. Define the terms “in vitro” and “in vivo”.

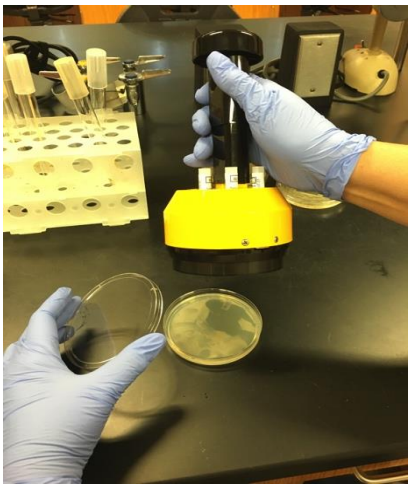
3. Based on the Introduction and question #2; what happens in a petri dish is not always the same thing that will happen in an actual patient. Why do you think there could be differences?

Materials:

Per group-*Staphylococcus saprophyticus*
Staphylococcus epidermidis
Escherichia coli (all from the Student Stocks)
3-Mueller Hinton Agar plates
3-TSB tubes
McFarland Standards and lined cards
Sterile swabs
Antibiotic disk dispenser with Chloramphenicol 30µg (C30), Penicillin 10µg (P10), Trimethoprim 5µg (TMP5), Ciprofloxacin 5µg (CIP5), Novobiocin 30µg (NB30)
Rulers (mm)

The lab exercise on antimicrobial sensitivity is one of my favorites because it highlights antibiotics, one of the most important medical discoveries of the 20th Century. Few alive today can remember a time before antibiotics existed: when 1 in 7 people died of tuberculosis and 41 years was the average lifespan in the U.S. Now that antibiotic resistant “superbugs” are becoming more prevalent, I feel it is valuable for you to observe first-hand, the proper use and testing protocols for antibiotics. In this lab, students visualize the effectiveness of 5 different antibiotics against both Gram-positive and Gram-negative bacteria, and draw conclusions about how antibiotics are matched to certain bacterial infections. By replicating a protocol used in clinical labs, I hope you will also gain an appreciation for the strict quality control measures used in the assessment of antibiotics.

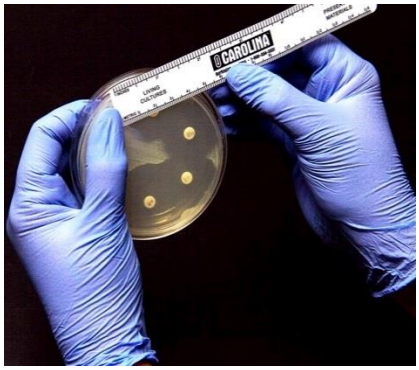
~Professor Kelly Cude



Procedures:

1. Make a suspension of each organism using a sterile swab and compare with the McFarland 0.5 Turbidity standard (use the striped card to help see the turbidity of the standard and the inoculated broth).
2. Use another sterile swab for each inoculated TSB to streak a Mueller Hinton plate to create a “confluent lawn” of growth.

3. Dispense antibiotic disks on top of the inoculated plates using the disk dispenser. Antibiotics to be tested- Chloramphenicol 30µg (C30), Penicillin 10µg (P10), Trimethoprim 5µg (TMP5), Ciprofloxacin 5µg (CIP5), Novobiocin 30µg (NB30).
4. Let disks sit and adsorb onto the agar for a few minutes, then invert and incubate at 35C for 24h-48h (when comparing the Novobiocin results to the unknowns later in the course, make sure that you note the incubation time so that you have an accurate reference point).
5. After incubation, measure the zones and compare to the reference chart.



Results:

1. Enter the size of the zones of inhibition in the chart below:

Bacterium/Gram reaction	C30 (mm)	P10 (mm)	TMP5 (mm)	CIP5 (mm)	NB30 (mm)
Staphylococcus saprophyticus					
Staphylococcus epidermidis					
Escherichia coli					

2. Based on the Zone of Inhibition Chart interpret your results and record below:

Bacterium/Gram reaction	C30 R, I, S	P10 R, I, S	TMP5 R, I, S	CIP5 R, I, S	NB30 R, I, S
Staphylococcus saprophyticus					
Staphylococcus epidermidis					
Escherichia coli					

Questions:

1. What is the purpose of the Kirby-Bauer test?

2. Where you able to measure and interpret each bacterial-antibiotic pair? If not, what were some of the issues and why do you think they occurred?

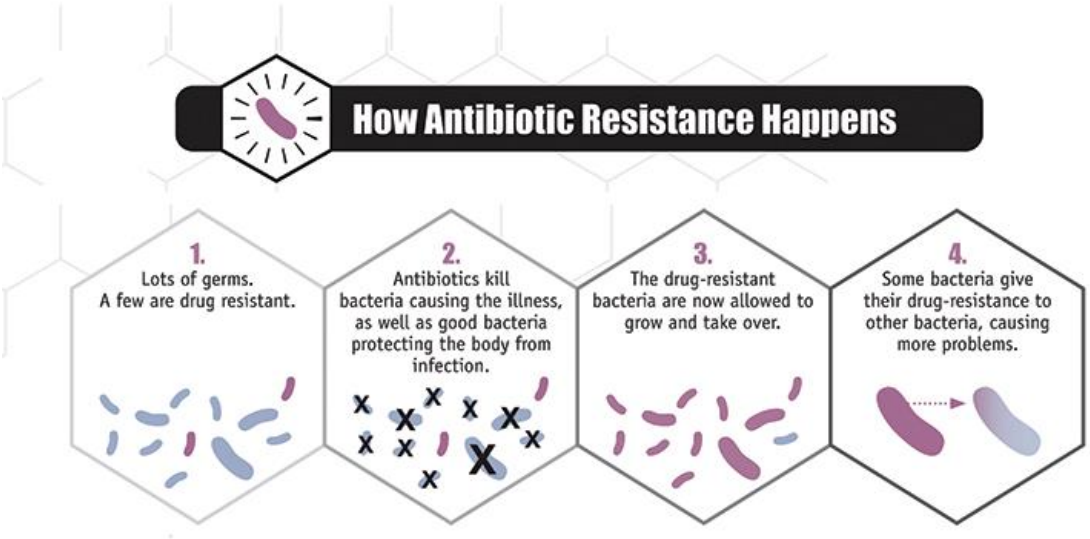
3. How might the amount of bacteria—too much, or too little--affect the results of the test?

4. Compare the results of the Gram negative bacteria tested, with the Gram-positive bacteria tested. You may need to look up which are G+ and G-. What are the implications of this?

Conclusion:

How can you apply what you have learned to your future career?

Transformation [1]



Source: **http://www.cdc.gov/drugresistance/about.html**

Antibiotic resistance is a critical problem in healthcare today. How do bacteria become resistant to antibiotics? In this lab we will focus on #4 from the CDC infographic above. Some bacteria can transfer genes from one bacterium to another in a process called "conjugation" or may pick up genes from the surrounding environment (from dead bacteria), via "transformation". These genes, often on a plasmid, may code for antibiotic resistance mechanisms.

Learning Objectives:

After this lab you should be able to:
1. Define the vocabulary related to transformation, recombinant plasmid, selectable marker, vector, and competent cell, genetic engineering, gene regulation, etc.
2. Relate the mechanism of genetic recombination, specifically transformation, to the problem of antibiotic resistant bacterial infections.
3. Describe how gene regulation can be affected by a bacterium's environment.
4. Outline the process of transformation in bacteria.
5. Evaluate the outcomes of the bacterial transformation activity.

Introduction:

This lab will demonstrate principles of
1. Bacterial reproduction
2. Genetic recombination in bacteria
3. Transformation as a method of genetic exchange and recombination
4. Transformation and genetic exchange as a vehicle of antibiotic resistance
5. Gene regulation in bacteria
6. Genetic engineering and recombinant organisms

Fred Griffith, a British microbiologist, discovered in 1928 that virulence could be transferred from killed pathogenic bacteria to live non-pathogenic bacteria.[2] He called this process "transformation". In 1944, Oswald Avery and his research group discovered that DNA was the factor being transferred.[3] These scientists had discovered and defined transformation, one of the mechanisms of bacterial recombination (Joshua Lederberg and others discovered the other two methods, conjugation and transduction). This was also the beginning of molecular biology and genetic engineering.

Today we know that genetic recombination in bacteria can be a source of antibiotic resistance. In addition, genes that code for antibiotic resistance mechanisms—like enzymes that break down the antibiotic in the bacterium—are carried on plasmids.

In this lab you will demonstrate the processes of transformation and gene regulation in bacteria, and genetic engineering, by forcing a bacterium to take up plasmid DNA from its environment (transformation) and express a gene (gene regulation) it would not normally have (genetic engineering). Typically, a very low percentage of bacterial cells will actually be transformed during this process. Some will successfully take up the plasmid; others may reject the plasmid in one of a few ways. But remember, most bacteria can grow so fast that, even if only one in a million are transformed with a plasmid that gives the cell resistance to an antibiotic, one cell can be millions in a matter of hours!

There are two sides to the process in the lab you will do. First of all, you will insert a plasmid (called pAMP) that contains a gene (*amp*^{R}) that confers resistance to the antibiotic Ampicillin into *E. coli*. Therefore, the *E. coli* is transformed and if subsequently exposed to Ampicillin it will not be affected and will grow as usual. Transformation happens in nature and is a vehicle for the spread of antibiotic resistance in bacteria. In a lab, antibiotic resistance genes can be paired with other genes on the same plasmid. These are called recombinant plasmids because they have been constructed from genes of different organisms. One could insert a plasmid that contains a specific gene for a desirable protein. But, it might be difficult to know which bacteria have successfully taken up the plasmid and the gene(s) it contains. To be efficient, one would want to select the bacteria that can make protein (called transformants--the bacteria that have been successfully transformed), and not end up with bacteria that cannot. Therefore, if an antibiotic resistance gene is paired with the other gene (the gene of interest), then the acquisition of resistance, which is easily tested, indicates the acquisition of the gene of interest. The cells resistant to Ampicillin are then "selectable markers" for the other gene.

When I was a grad student at UCLA, I engineered a custom recombinant plasmid to express genes that I had mutated. The plasmid had the same amp^R *gene as pAMP and pGLO, so I was able to select for transformants by plating the cells on media that contained ampicillin. After inserting the mutated gene into the plasmid, I transformed E. coli cells and conducted growth experiments. My plasmid eventually became very popular with other researchers in the Microbiology, Immunology and Molecular Genetics Department, who nicknamed it 'pUC-Shane'!*

Dr. C. Shane Ramey

The second aspect of today's process, then, is that the plasmid with Ampicillin resistance also carries the gene (the gene of interest) to produce green fluorescent protein (called pGLO which has the *AMP*^{R} and *gfp* genes-see Fig. 1), which glows when exposed to UV light. Cells that have pGLO will fluoresce only when under the correct environmental conditions, specifically the presence of the sugar Arabinose. You will use the antibiotic resistance property to select for cells that, potentially, will fluoresce. Then, we'll feed the transformed bacteria Arabinose in order to "turn on" the genes for fluorescence. The presence of the Arabinose sugar is the environmental switch that helps regulate the expression/production of the green fluorescent protein. As you will observe in today's lab, bacteria plated onto media that contains Ampicillin will grow, and if the media also contains Arabinose, they will glow! You will have successfully selected for a bacterium that you actually engineered to fluoresce.

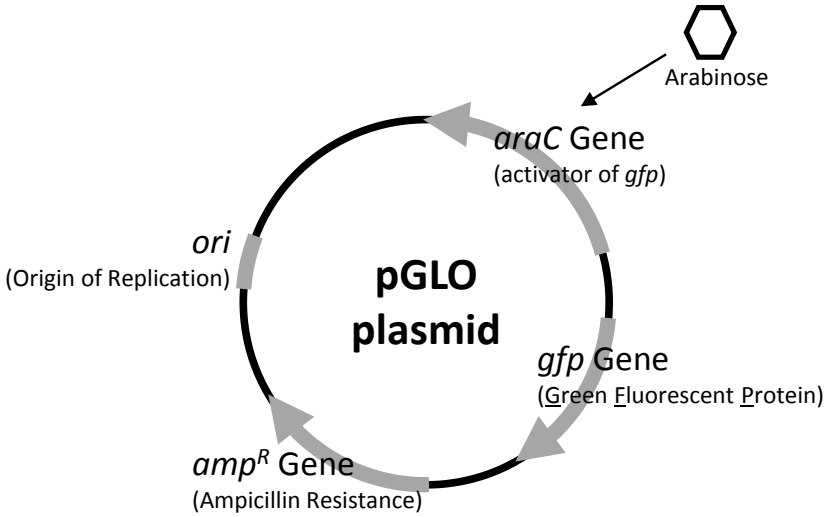


Figure 1. Components of the pGLO plasmid (C.S. Ramey)

In nature, not all bacterial cells can undergo transformation. Those that are able are called "competent" cells. In the laboratory, cells can be encouraged to undergo transformation by altering their environment and cells temporarily so that they will uptake DNA; they become competent due to lab manipulation. The method you will use is the calcium chloride/ heat shock procedure (see Figure 2). The positive Ca ions released in the solution will neutralize the negative charge on the DNA molecule (a plasmid in our case), which reduces the charge barrier for the DNA entering the cell (remember that cells have a net negative charge). Heating the cells increases the permeability of the bacterial membrane. Thus, DNA will more readily enter the bacterial cells and transformation may proceed. These steps must be done quickly in order not to damage or kill the cells. Later, you will calculate the "Transformation Efficiency" obtained by your bacterial culture. Not all cells will become competent, nor will all competent cells complete transformation.

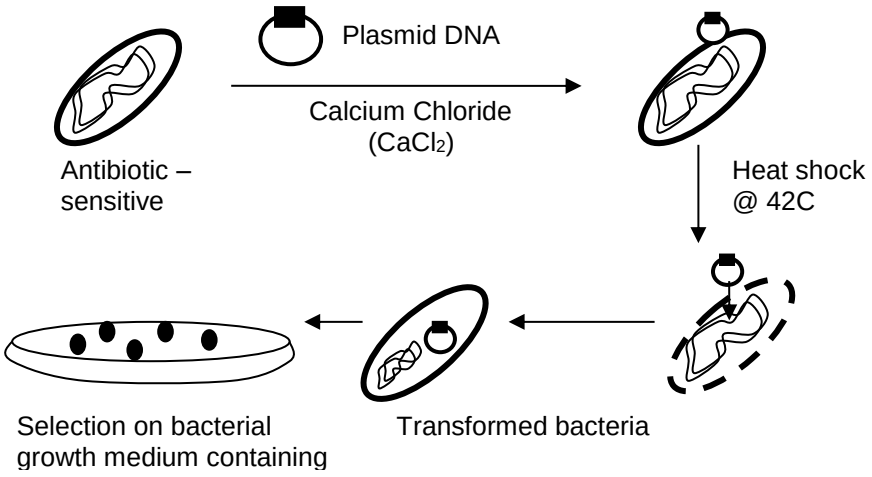


Figure 2: Bacterial Transformation. (K. Cude)

Pre-lab questions:

1. Define the terms transformation, recombinant plasmid, and competent cell.

2. Investigate green fluorescent protein, online, and describe what it means to "fluoresce". Did you find any unusual applications of genetically modifying organisms with this protein?

3. Explain the use of antibiotic resistance as a "selectable marker".

4. Predict what will happen if transformed cells are fed only:
a. Glucose

b. Glucose and arabinose

c. Glucose and sucrose

Materials:

Day 1-
200 ul micropipette (P200)
1000 ul micropipette (P1000)
Pipette tips
Microfuge tubes
E. coli culture (HB101 strain)
Ice bath
42C water bath
37C water bath
50mM $CaCl_2$ (keep ice-cold)
10 ul pGLO (plasmid concentration 0.2 µg/ µl)
Sterile LB broth
1 LB agar plates
2 LB+AMP
1 LB+AMP+Arabinose agar plates
2 Sterile Spreaders-small

Day 2-
Your inoculated plates from Day 1
UV lights

SAFETY NOTE: Wear gloves while handling the transformed bacteria. You are genetically engineering an antibiotic resistant bacterium.

Procedures:

1. Label two microfuge tubes with the following: one as P+ (plasmid) and the other, P- (no plasmid, control).

2. Using a P1000 micropipette, add 500 ul of ice-cold 50mM $CaCl_2$ to one of the tubes; you will split this volume into both tubes later so it doesn't matter which tube you start with.

3. Using a sterile inoculating loop, transfer one to three isolated colonies of the starter *E.coli* colonies (a colony approximately the size of an o) into the tube.

4. Using a P200 micropipette and a clean pipette tip, suspend the cells by gently pipetting the solution in an out several times. Hold the tube up to the light to check for any cell clumps.

5. Now you will split this volume, half in each tube. Using a P1000 micropipette, remove 250 ul of the suspension and place it in the second tube so that you now have 250 ul of the suspension in both the P+ and P- tubes.

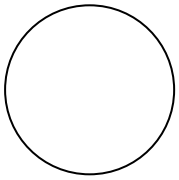
6. Replace the cap on the tubes and return them to the ice bath.

7. Incubate both tubes (P+ and P-) in ice for at least one minute.

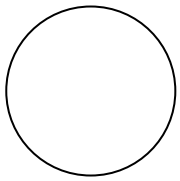
8. Add **10 ul of pGLO (plasmid concentration 0.2ug/ ul)** to the re-suspended cells in the **P+ tube only**. Mix the cells with the plasmid by gently and slowly pumping the solution in and out using a clean pipette tip. Return the P+ tube to the ice bath.

9. Incubate both tubes in ice for **20 minutes.**

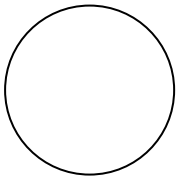
10. While the tubes are incubating obtain 1 LB, 2 LB+AMP, and 1 LB+AMP+Arabinose agar plates and label them:
    a. Label the **bottom of the plates** of one set LB and LB+AMP with P-
    b. Label the **bottom of the plates** of the other set LB+AMP and LB+AMP+Arabinose with P+



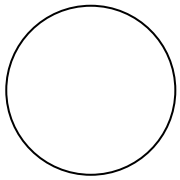
LB **(P-)**



LB +AMP **(P-)**



LB +AMP **(P+)**



LB +AMP+Arabinose **(P+)**

11. Following the 20-minute incubation in ice, carry your ice bath with the cells to the 42C water bath. Take the tubes directly from the ice bath and place them in the 42C water bath for 90 seconds to heat-shock the cells. Immediately afterward place the cells back into the ice bath for 2 more minutes.

12. Using a P1000 micropipette, and a clean pipette tip add 500 ul of sterile LB to the P+ and then with a new pipette tip, add 500ul of sterile LB to the P- tube. Gently, finger vortex (flick the tube with your finger) the tubes to mix the cells and broth together. Incubate the cells in the 37C water bath for 10 minutes.

13. After the 37C incubation period the cells are ready to be plated out onto the agars.
-Line up the two P- plates as shown in the above picture. Using a P200 micropipette, transfer 50 ul of cells from the P- tube to each of the two plates. Make sure to evenly spread the drops across the agar surface
-Use a sterile cell spreader and rotate the plates to evenly distribute the culture across the agar. *Place the contaminated cell spreaders in the pipette disposal trays.*

14. Using a new micropipette tips and a sterile cell spreader, repeat the above procedure for the P+ plates by inoculating with the cells from the P+ tube.

15. Allow plates to dry, right side up, for about 5 minutes. Then place inverted plates in your incubator. Discard tubes in the biohazard containers.

Results:

1. After incubation, count and record the number of colonies on each plate.

2. While wearing safety goggles and in a dark place, illuminate the plates with the hand held UV light to determine which colonies are producing the Green Fluorescent Protein. Record results.

Table 1: Number of Transformed Colonies.

CELLS	PLATE	Growth ? Y/N	Glowing ? Y/N	AGAR PLATE Growth?	Growth ? Y/N	Glowing? Y/N
Plasmid - (P-)	LB # of colonies:			LB +Amp # of colonies:		
Plasmid + (P+)	LB +AMP # of colonies:			LB +Amp + Arabinose # of colonies:		

3. Transformation Efficiency:

Show all your work. Include word equations, show substitutions, and include units!

a) What was the total mass (µg) of plasmid DNA added to the cell suspensions? Note in the experiment you used 10 µl of plasmid (step 8) that had a concentration of 0.2 µg /ul.

b) Using the information from a) and your colony count of transformed cells, calculate the number of colonies transformed per microgram of plasmid DNA. This is the **Transformation Efficiency** of your transformation event.

TE=# of transformed colonies
ug DNA

Questions:

1. Explain how the bacterial cells, normally not competent, were made competent during the procedure.

2. Differentiate between the purposes of each of the 4 plates used in the exercise.

3. Contrast the uses of the antibiotic and arabinose in the exercise.

4. Why did the P- bacteria grow on the LB agar without ampicillin and not in the Petri dish with the LB agar with the ampicillin?

5. For the P+ plates, why did colonies "glow" on one plate and not the other?

6. How is the use of Arabinose a means of gene regulation?

7. *E. coli* cells can double about every 20 minutes with the proper environmental conditions. How long would it take a single transformed cell to become 100 million cells, all with resistance to ampicillin?

Conclusion:

Reflect on why transformation is a potential method of increasing antibiotic resistance in populations of bacteria and on how this, and the lab exercise, reinforces the principles of aseptic technique in a hospital, home, lab, etc.

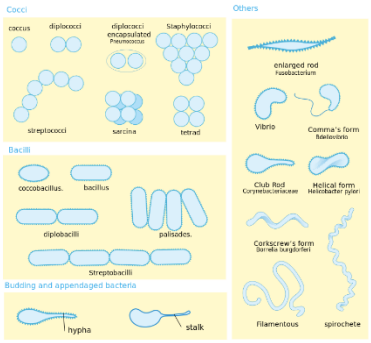
Resources:

1. Cude, K., Golbert, M. (2015). Transformation. In *Bio 107: Molecular and Cellular Biology Laboratory Manual*. 14th Ed.: (89-99). College of the Canyons. Adapted and used with permission.

2. Griffith, F. "The Significance of Pneumococcal Types." *The Journal of Hygiene* 27.2 (1928): 113–159. Print. Web. 12 November 2015
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2167760/

3. Avery, O. T., et al. "Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolation from pneumococcus type III." *The Journal of Experimental Medicine* 79.2 (1944): 137–158. Print. Web. 12 November 2015
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2135445/

Smear Prep and Simple Stains



"Bacterial morphology diagram" by Mariana Ruiz LadyofHats. Licensed under Public Domain via Commons - https://commons.wikimedia.org/wiki/File:Bacterial_morphology_diagram.svg#/media/File:Bacterial_morphology_diagram.svg

Bacteria are usually colorless. Many stain techniques have been developed to add contrast so that we can see them more easily via a light microscope. Staining helps us discover important information about bacteria. Although genetic methods have advanced and at times streamlined bacterial identification, it is still sometimes necessary, and is still common practice to stain a patient sample.

Learning Objectives:

After this lab you should be able to:

1. Prepare a bacterial sample for staining.
2. Perform a simple (single) stain on a slide you prepared.
3. Identify the bacterial cell morphology and arrangement of the sample you stained.
4. Define and distinguish between basic and acid stains.
5. Properly use oil immersion, explain its use, and successfully clean the microscope after its use.

Introduction:

Bacterial Cell Size, Morphology, and Arrangement

Bacteria come in many different sizes and shapes (morphology=shape). The sizes of bacteria range from <1.0um to >250um. However, most bacteria, and the ones you will observe in lab range from about 1um-15um. The most common shapes of bacteria, and the ones you will observe are cocci, bacilli, and spirilli.

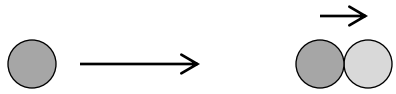
Cocci 

Bacilli 

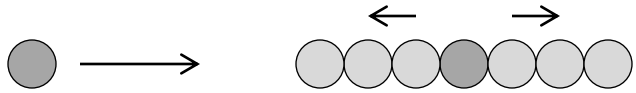
Spirilli 

Bacteria also often grow into different arrangements as the cells divide. Cocci have the most variety in their arrangements, some bacilli may stay in pairs or chains as they divide, but spirilli are found singly. Remember that organisms, and their arrangements, are three-dimensional. Note that sarcina is essentially cuboidal and staphylococci are a cluster formed by cells dividing in an irregular pattern.

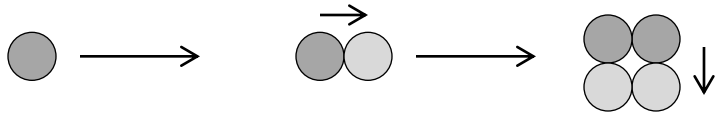
Diplococci



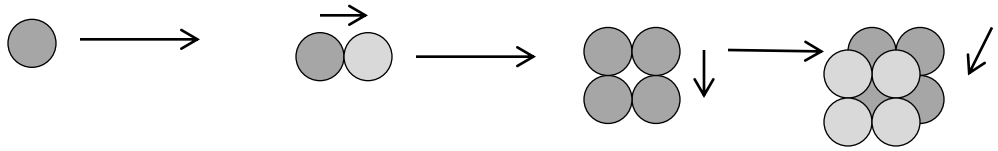
Streptococci



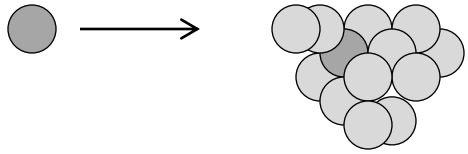
Tetrad



Sarcina



Staphylococci



Stains

Stains are solutions containing a pigmented molecule. The part of the molecule that is colored is called the chromophore. The chromophores carry either a net + or – charge, therefore are attracted to the opposite charge. Stains with a net + charge are called "basic stains", those with a net – charge are called "acid stains". Bacterial cells have a net – charge. Thus, basic stains will attach to the cells, while the cell will repel acid stains.

Basic stains (+ charge)-
- Methylene blue
- Basic fuchsin
- Crystal violet
- Safranin
- Malachite green

Acid stains (- charge)-
- Eosin
- Acid fuchsin

100x lens and Oil Immersion

Bacteria are very small of course, so it is necessary to view them at the highest magnification possible with the best resolution possible. In a light microscope this is about 1000x due to optical limitations. The Oil immersion technique is used in order to enhance resolution. This requires a special 100x objective. The 100x lens is immersed in a drop of oil placed on the slide in order to eliminate any air gaps and loss

of light due to refraction (bending of the light) as the light passes from glass (slide)→air→glass (objective lens). Immersion oil has the same refractive index of glass. When used, light passes through glass→oil→glass without loss due to refraction.

When the oil immersion lens is in place there are a few things to be aware of and very careful about-

- The working distance is VERY small.
- Depth of focus is also VERY small.
- Use only the fine focus, and focus slowly.
- Increase the light, as the lens opening is very small.
- Adjust the condenser and iris diaphragm as needed.
- Do not drag the 40x lens through the oil, if you need to go to lower power-4x or 10x, rotate the nosepiece in the opposite direction to avoid the 40x lens. Dragging the 40x lens through the oil will damage the lens!
- The immersion oil has the same refractive index as glass, so you may go back to low power (again, not 40x) and still see your specimen.
- Do not make any decisions or assessments about your bacterium until you are focused at 100x.
- If you see the cells clearly with the 4x, 10x, 40x, but don't see it with 100x, your specimen may be upside down. Ask your instructor for help.

Viewing with the oil immersion 100x objective lens-
1. Place a stained slide on the stage in the clips.
2. View the slide at low power (4x) and look for staining; for very small cells look for a pattern of regular tiny specks. Once in focus, move to the next objective (10X), focus with fine focus only. Move to 40x, fine focus only (if the 40x objective is not very clear—due to it being repeatedly dragged through the oil, skip it). Make sure that the area you want to see is dead center in the field of view.
3. Rotate the objective aside so that you are between it and the 100x objective and let a small drop of oil fall onto the slide where your specimen is. Carefully rotate the 100x objective into place, immersing it into the drop of oil.
4. While looking through the oculars, very carefully and slowly use the fine focus to bring the image into sharp focus. If you can't get it into focus, or have lost what you are looking for you may go back to 4x or 10x and start over (do not remove the oil, it's fine).
5. When finished with your slides wipe the oil from the 100x objective with lens paper (DO NOT use anything else to clean the lens!). The lens retracts into the lens housing. Be sure to push up gently to remove oil that has moved up in the housing (this happens more when one is viewing a slide and over focuses and pushes the lens up into the housing). Then, use lens cleaner and lens paper to completely remove all oil from the lens. Keep the lens paper flat and use a circular motion. Do not wad up the lens paper. Finally, use lens paper to check all the other objectives and parts of the microscope—focus knobs, stage, drips onto the condenser, etc., and clean up any oil on them.

Pre-lab questions:

1. Review and define these terms:
-Working distance

-Parfocal

-Refractive index

-Resolution

-Field of view

-Depth of Focus

2. What is the total magnification of an image viewed with the 100x objective lens?

3. Does oil immersion increase magnification? Why or why not?

4. What value does each space in the ocular micrometer represent at 100x (refer back to your notes from the Microscopy lab)

Materials:

Student Stock Cultures

Each group should have all Stock Culture Organisms on a TSA plate and TSA slant, and in a TSB broth

- *Staphylococcus saprophyticus*
- *Staphylococcus epidermidis*
- *Lactococcus lactis*
- *Kocuria rosea*
- *Sarcina aurantiaca*
- *Sporosarcina ureae*
- *Moraxella catarrhalis*
- *Mycobacterium smegmatis*
- *Corynebacterium pseudodiphtheriticum*
- *Bacillus cereus*
- *Alcaligenes viscolactis*
- *Escherichia coli*
- *Citrobacter freundii*
- *Serratia marcescens*
- *Pseudomonas fluorescens*
- *Enterobacter aerogenes*

Glass bowls with Sanisol
Glass Slides
Stain trays
Water bottles (partially fill with DI water)
Slide holders or clothespins
Stains: Crystal violet, methylene blue, malachite green, safranin
Lens cleaner/lens cleaning kits
Streptococcus mutans culture
Rhodospirillum rubrum culture

Procedures:

Suggested bacteria for staining (all from Student Stock, except *Streptococcus mutans*—1 broth culture per table), however, with your group, stain and observe as many as possible from your entire stocks:

Moraxella catarrhalis
Bacillus cereus
Streptococcus mutans
Staphylococcus epidermidis
Esherichia coli
Micrococcus luteus
Rhodospirillum rubrum
Smear prep

From a broth culture-
1. Aseptically place a loopful of culture onto a cleaned slide, and spread the liquid around in an area about the size of a quarter. You may do more than one organism on a slide; just make sure they don't touch.
2. Set the slide aside to **completely air dry**. DO NOT heat at all during this time, nor wave the slide around to speed up the drying process. Heating the slide prior to air-drying will cause production of aerosols, which is a biosafety hazard, and will also dry the cells too quickly and distort their shape and size.
3. Heat-fix the slide by holding it (use a slide holder) over a hot plate or in front of the incinerator for 5-10 seconds, until it is warm to the touch. Overheating will distort the cells and may cause aerosol formation. Heat-fixing kills the cells and adheres them to the slide so that they don't come off during the stain procedures.

From solid media (a plate or slant)-
1. Place a small drop of DI water, from your water bottle, onto the edge of your staining tray. Take your loop a transfer a loopful of the water to a cleaned slide.
2. Aseptically pick a small amount of bacteria (do not pick a big glob, just lightly touch a colony, it will be plenty) from the media and place it into the drop of water, and spread around in an area about the size of a quarter to suspend and spread the bacteria evenly around the slide. Avoid clumpy or thick, pasty smears! You may do more than one organism on a slide; just make sure they don't touch.
3. Set the slide aside to **completely air dry**. DO NOT heat at all during this time, nor wave the slide around to speed up the drying process. Heating the slide prior to air-drying will cause production of aerosols, which is a biosafety hazard, and will also dry the cells too quickly and distort their shape and size.
4. Heat-fix the slide by holding it (use a slide holder) over a hot plate or in front of the incinerator for 5-10 seconds, until it is warm to the touch. Overheating will distort the cells and may cause aerosol formation. Heat-fixing kills the cells and adheres them to the slide so that they don't come off during the stain procedures.

Cautions:

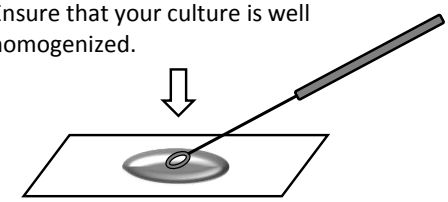
A thick smear will prevent you from seeing individual cells easily and determine the cell morphology, arrangement, and size. Conversely, too few cells will be difficult to find. It will take practice to have just the right amount, but in general, less is better!

Make sure slides are completely air dried, and do not over heat-fix.

Bacterial Smear (Emulsion) Prep

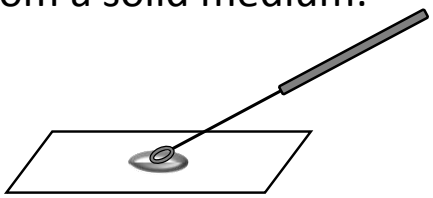
From a broth culture:

Ensure that your culture is well homogenized.

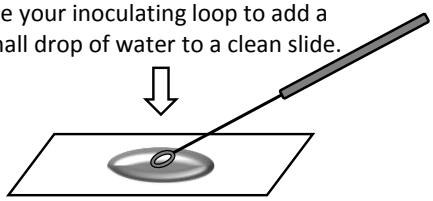


Use your inoculating loop to aseptically transfer bacteria to a clean slide and spread the cells out.

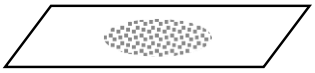
From a solid medium:



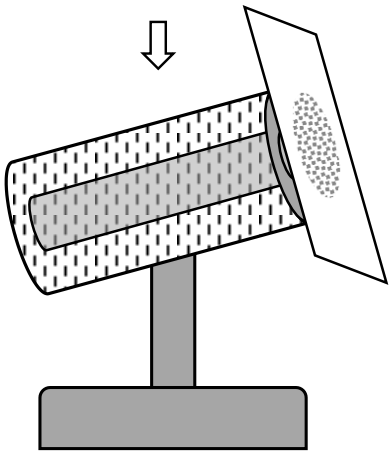
Use your inoculating loop to add a small drop of water to a clean slide.



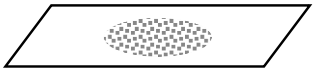
From a plate or slant, aseptically transfer bacteria to the water. Mix in the cells and spread the drop out.



Allow the smear to air dry. It should be slightly cloudy.



Use a slide holder to place the smear near the opening of a bactincinerator (with the cells facing away from the opening) for 5-10 seconds. This heat-fixes the cells.



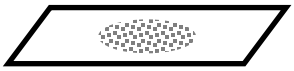
Allow the slide to cool, then continue with the staining protocol.

C.S.Ramey

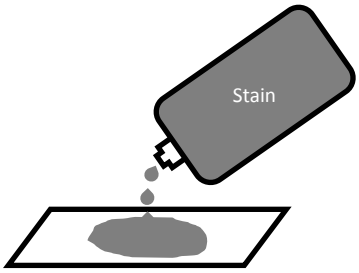
Simple Stain

1. Use an air-dried, heat-fixed smear and flood the smear with one of the basic stains above for 1 minute.
2. Rinse the slide with DI water.
3. Blot slide gently with bibulous paper. Tap gently; do not rub the paper as this may smear your prep.
4. Observe at under oil immersion, 100x objective.

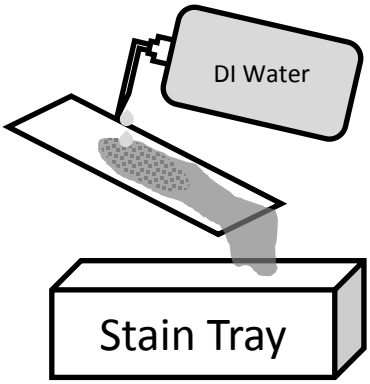
Simple Stain:



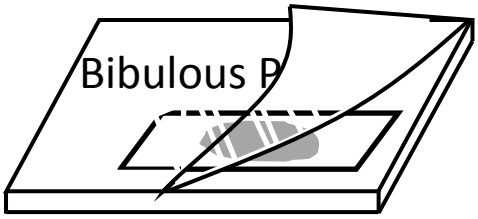
Step #1: Begin with a heat-fixed emulsion.



Step #2: Cover the smear with stain for 1 minute. Use a staining tray to catch excess stain. Be sure to wear gloves.



Step #3: Grasp the slide with a slide holder. Gently rinse the slide with distilled water.

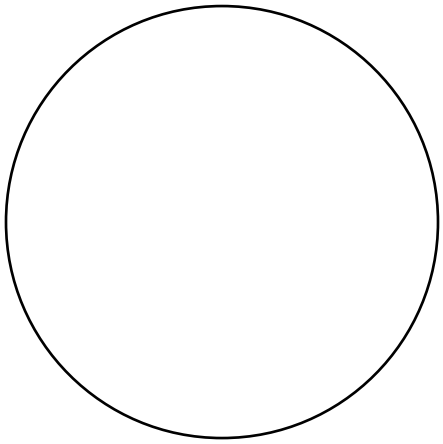


Step #4: Gently blot dry in a tablet of bibulous paper. Do not rub. When dry observe under oil immersion.

C.S. Ramey/K.C. Burke

Results:

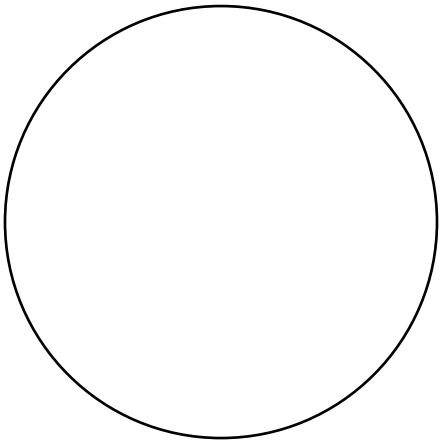
Draw the bacteria you observed, including cell size, use additional paper if needed.



Organism _____

Size _____

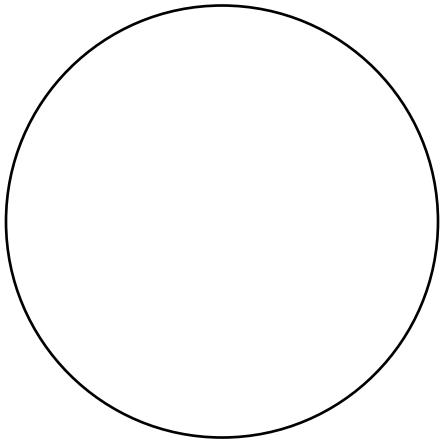
Magnification _____



Organism _____

Size _____

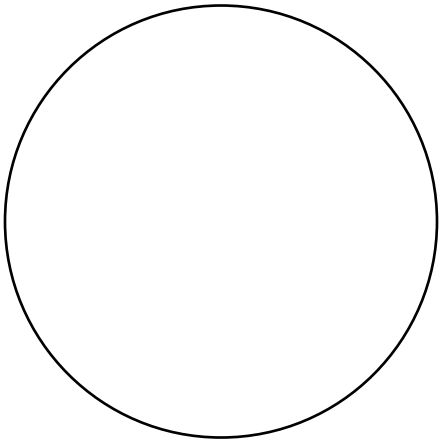
Magnification _____



Organism _____

Size _____

Magnification _____



Organism _____

Size _____

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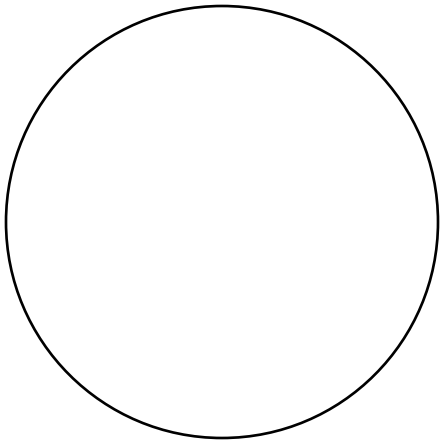
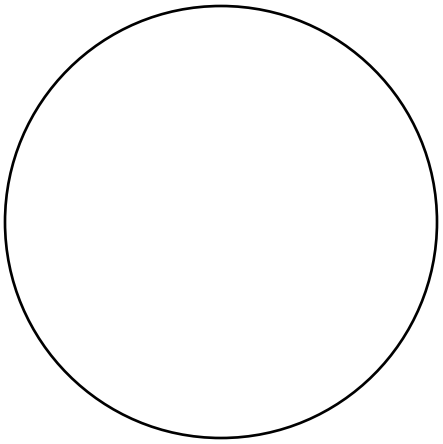


Organism _____

Size _____

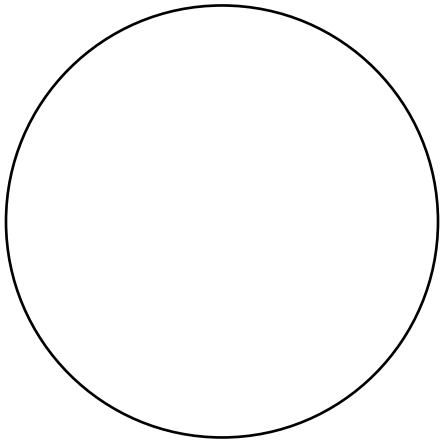
Magnification _____



Organism _____

Size _____

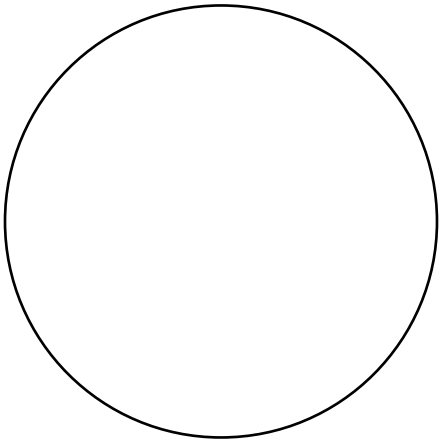
Magnification _____



Organism _____

Size _____

Magnification _____



Organism _____

Size _____

Magnification _____

Table 1. Stain Results

Organism	Stain used	Cell Morphology	Cell Arrangement	Size (um)

Questions:

1. Why do we use oil immersion/100x for viewing bacteria?

2. Explain the importance of a good smear preparation and the pitfalls of a poor smear prep.

3. Why is the staining time important?

4. Go back to our lab on Protozoa. Compare, in quantitative terms, the size of an RBC, a protozoan, and a bacterium.

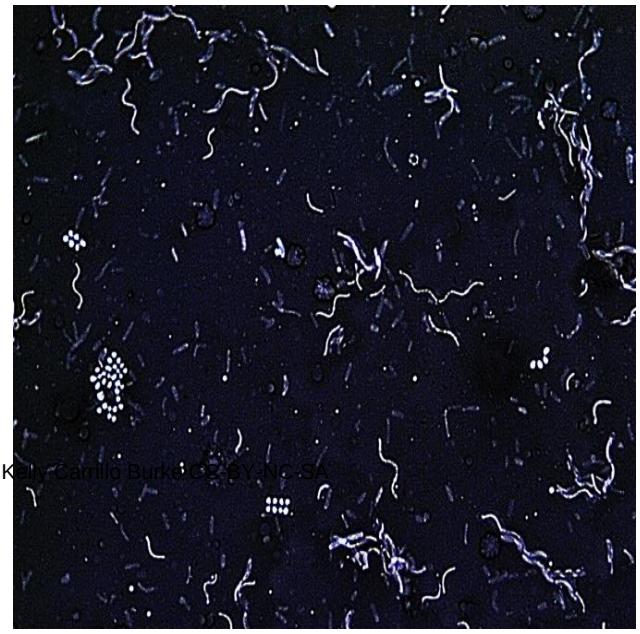
5. Explain why you could use crystal violet but not eosin in the simple stain procedure?

Conclusion:

Reflect on your success with the smear prep. Did you produce a thin smear with visible, well separated cells, etc.? Provide a complete analysis of your smear prep technique (problems, success, improvements to be made, etc.).

Reflect on your success with the simple stain—did you produce stains with which you were able to see morphology, arrangement, and take measurements, etc.? Provide a complete analysis of your staining technique (problems, success, improvements to be made, etc.).

Negative Stain



This image is a Negative stain of a mixed culture. Look closely and see if you identify different cell types by morphology and arrangement. By the end of the semester you will be able to make some very accurate predictions about which bacteria from our course are in this image.

Learning Objectives:

After this lab you should be able to:
1. Perform a Negative stain on a variety of bacterial cultures.
2. Explain the principles of how the Negative stain works.
3. Evaluate and interpret your Negative stain results correctly.
4. Interpret and troubleshoot poor stain results.
5. Compare and contrast the principles of Negative and Simple stains.

Introduction:

Nigrosin is an acid stain. The negative charge of the acid stain and the negative charge of the cell will repel each other. In a Negative stain with Nigrosin, the cells appear clear against a dark (stained) background. When performed well this can be a beautiful stain and can give you a feeling of accomplishment and even wonder when you first see the cells pop against the background. The staining procedure is very different from the other stains done in the course. First of all, the normal smear prep is not performed, and the slide is NOT heat-fixed. The specimen is mixed with a small drop of the Nigrosin, spread across the slide via a second slide, and then air-dried. This is a very mild treatment of the cells, which serves to preserve their shape and size. Thus, the Negative stain is very good for observing cell morphology, arrangement, and size. This can be very helpful when it is difficult to determine these characteristics with other stains. However, because the slide is not heat-fixed, the bacterial can remain viable and it is important to be careful about handling and disposal. It is very important that you do not touch the slide to the objective of the microscope; therefore, you must be very careful with focusing while using oil immersion since the working distance is so small.

Pre-lab questions:

1. What is the purpose/advantage of the Negative stain?

2. Compare the principle of the Negative stain with a Simple stain.

3. How is the procedure different in a Negative stain than a Simple stain?

4. How will the bacterial cells appear?

Materials:

1 broth culture per table:
Streptococcus mutans
Student Stock Cultures-use broths
Glass Slides
Stain tray and slide holder
Nigrosin
Glass bowls with Sanisol
Lens cleaner/lens cleaning kits

Procedures:

Suggested bacteria for staining (any broth from the Stock Cultures may be used)—

Moraxella catarrhalis
Bacillus cereus
Streptococcus mutans
Staphylococcus epidermidis
Escherichia coli
Micrococcus luteus
Rhodospirillum rubrum

1. Clean all slides with lens cleaner prior to doing the stain. The Nigrosin will spread more readily and evenly on cleaned slides; it may be repelled by dirt and fingerprints and thus bead up on the slide.
2. Refer to Figure 1. when preparing for and performing the stain.
3. Place a small drop of Nigrosin at one end of the slide.
   Caution: do not touch the slide with the eyedropper; this can contaminate the stain container.
4. Using your loop aseptically transfer a loopful of culture into the drop of Nigrosin and mix gently without spreading the stain. Do not let the slide dry at this step.
5. Back up the end of a second slide to the mixture so that the drop grabs onto the back of the slide. Gently PULL the drop forward with the second slide to produce a smear across the entire slide. The smear will appear very dark at the starting end and should thin out towards the other end.
6. Let the slide air-dry.

7. Observe dried slides using oil-immersion.

Cautions:

a. Remember that the working distance is very small with oil-immersion and that the cells may be viable. Do not run the slide into the objective as this can contaminate the lens with bacteria. Focus very carefully.

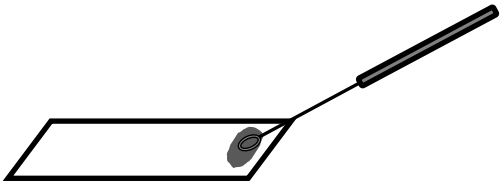
b. When viewing the slide, where the stain is very dark and thick, you may see star shaped cracks. The stain may crackle as it dries in very thick areas. If you see this, simply scan to another area with a lighter gray background and look for cells.

c. Do not mistake air bubbles, which may form, for the bacterial cells. Practice and experience will help you with this; however, air bubbles are usually very round, very clear, and larger than cocci.

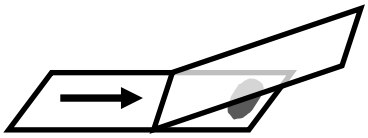
c. If you are observing known rod shaped bacteria, you may see some that look like cocci. These could be cells that are "on end", and short cocco-baccilli could be young recently divided cells. Or, they could be air bubbles.

8. Place used slides into the glass bowl with Sanisol.

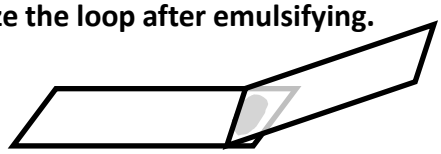
Step #1: Begin with a SMALL drop of acidic stain (nigrosin) at one end of a clean slide. Be sure to wear gloves.



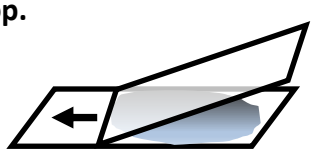
Step #2: Aseptically add organisms and emulsify with a loop. Do not over-inoculate.
Avoid splattering the mixture. Sterilize the loop after emulsifying.



Step #3: Take a second clean slide, place it on the surface of the first slide, and draw it back into the drop.



Step #4: When the drop flows across the width of the spreader slide…



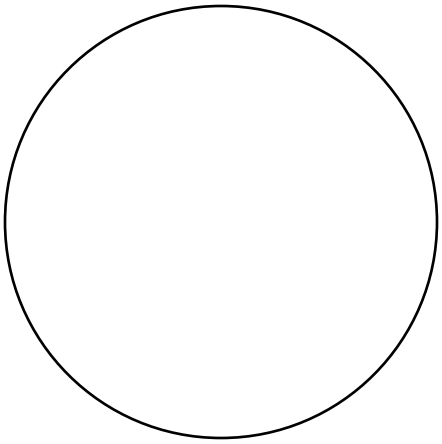
Step #5: …push the spreader slide to the other end. Dispose of the spreader slide in a jar of disinfectant or sharps container.

Step #6: Air dry COMPLETELY and observe under the microscope. Do NOT heat fix.

Figure 1. Negative Stain Procedure

Results:

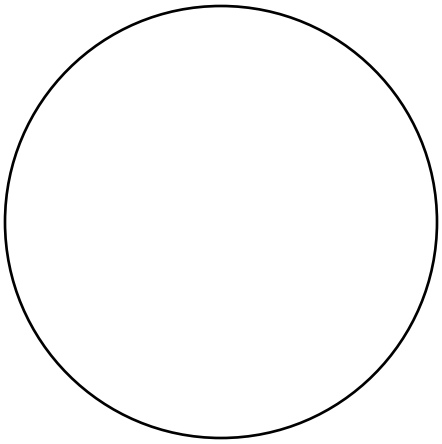
1. Draw your observations in the circles below:



Organism _____

Size _____

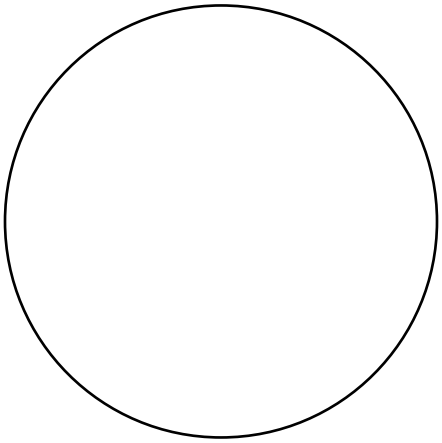
Magnification _____



Organism _____

Size _____

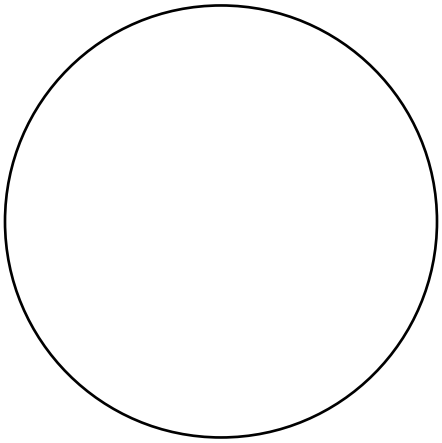
Magnification _____



Organism _____

Size _____

Magnification _____



Organism _____

Size _____

Magnification _____

2. Fill in the table with the organisms you stained:

Table 1. Negative stained organisms

Organism	Cell Morphology	Cell Arrangement	Size (um)

.

Questions:

1. Differentiate between acid and basic stains and give an example of each

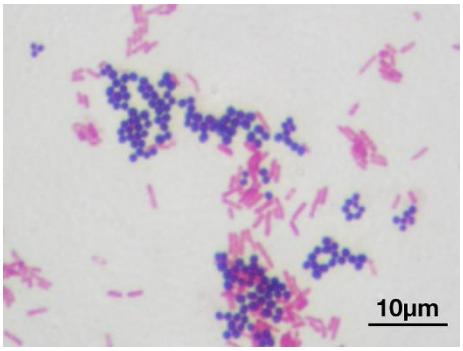
2. Why are size, morphology, and arrangement sometimes more accurate with a Negative stain?

3. What other stains (you may have to look this up!) could be used in place of Nigrosin in a Negative stain? Explain why they would work.

Conclusion:

Reflect on your success with the negative stain. Provide a complete analysis of your stain technique and results. Did you have success with several different bacteria? If you had problems discuss them and your plan to improve your success next time.

Gram Stain



"A Gram stain of mixed *Staphylococcus aureus* (Staphylococcus aureus ATCC 25923, Gram-positive cocci, in purple) and *Escherichia coli*(Escherichia coli ATCC 11775, Gram-negative bacilli, in red), the most common Gram stain reference bacteria." 1000x

Gram stain 01" by Y tambe - Y tambe's file. CC BY-SA 3.0
https://commons.wikimedia.org/wiki/File:Gram_stain_01.jpg#/media/File:Gram_stain_01.jpg

The stain you will perform in this lab was developed in 1884, yet is still the most commonly used stain in bacteriology, and one of the most important fundamental stains in diagnostic microbiology. In developing staining procedures using Crystal Violet, Christian Gram discovered that one procedure would differentiate bacteria into two groups. Some bacteria (no called Gram Positive) retain Crystal Violet throughout the procedure and stain purple. Others (now called Gram Negative) lose the Crystal Violet during the decolorizing step. These cells can be counterstained with a pink stain, Safranin. [1, 2]

Learning Objectives:

After this lab you should be able to:
1. Perform a Gram Stain on a variety of bacterial cultures.
2. Explain the principles of how the Gram Stain works.
3. Evaluate and interpret your Gram Stain results correctly.
4. Interpret and troubleshoot poor stain results.

Introduction:

The Gram Stain is a differential stain because it separates bacteria into two groups based on differences in their cell wall structure. The protocol involves more steps than a simple stain, but is still performed on air-dried, heat-fixed smear preps. The smear prep is critical. If the smear is too thick the bacteria will not stain evenly, nor will they decolorize evenly. This can be a major source of error in evaluating the Gram reaction of a culture.

Bacteria are differentiated into two groups called Gram Positive bacteria and Gram Negative bacteria. The stain complexes with the peptidoglycan (PG) layer of bacterial cell walls and is not effective, nor utilized diagnostically on non-bacterial cells. Bacteria without cell walls, or those with unusual cell wall structures do not Gram Stain well. The cell walls of Gram Positive bacteria have a very thick, highly cross-linked PG layer, while Gram Negative bacteria have a thin PG layer.

- During the procedure the first, or primary, stain is Crystal Violet. Crystal Violet will attach to the PG layer staining the cells purple.
- This action is enhanced with the use of a mordant, Iodine, in the second step. A mordant is a substance that helps fix a stain to its target, in this case the PG in the cell wall. At this point in the procedure all cells, both Gram + and Gram -, will appear purple due to the Crystal Violet/Iodine complex.
- The next step is the most critical in the Gram Stain procedure because it is the step responsible for differentiating the two groups. If not done carefully, incorrect or ambiguous results will occur.

A decolorizer, acetone/alcohol solution, is dripped over the slide to remove Crystal Violet from Gram – cells. Gram + cells will retain the Crystal Violet/Iodine complex due to the many layers of the PG. Gram – cells will lose the Crystal Violet stain and would appear unstained once more. This allows them to be stained a different color with another stain.

- The secondary, or, counterstain used is Safranin and colors the cells pink. Some safranin will also attach to the Gram + cells, but the dark purple color from the Crystal Violet obscures any additional pink staining.

Pre-lab questions:

1. What is the purpose and the advantage of Gram Staining bacteria?

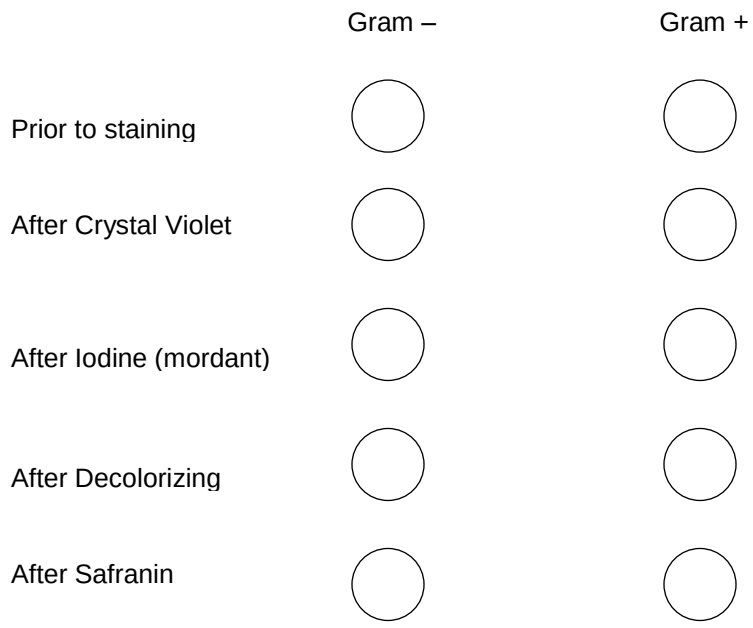
2. Name the chemical and give the purpose of each of the following in the Gram stain:

-Primary stain

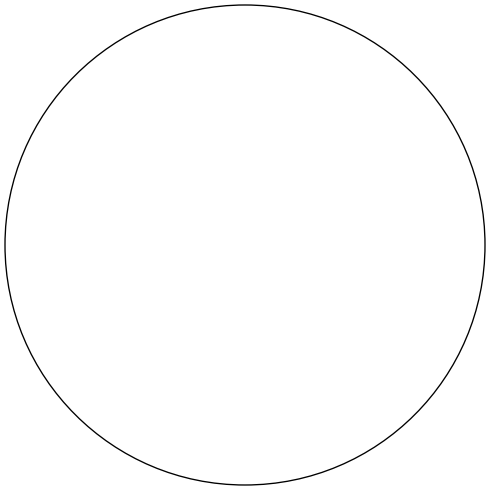
-Mordant

-Secondary stain

3. Color in the following diagram of bacteria (clear, pink, or purple) during the various stain steps:



4. Draw and color a picture with Gram + cocci, Gram + rods, and Gram – rods.



Materials:

Student Stock Cultures
Glass bowls with Sanisol
Glass Slides
Stain trays
Water bottles (partially fill with DI water)
Slide holder or clothespin
Gram Stain kit
Lens cleaner/lens cleaning kits
Streptococcus mutans culture

Procedures:

Suggested bacteria for staining, however, your group should perform stains on and share as many different organisms as possible (all from Student Stock Collection, except *Streptococcus mutans*—1 broth culture per table):

Moraxella catarrhalis
Streptococcus mutans
Escherichia coli
Bacillus subtilis
Staphylococcus saprophyticus
Mycobacterium smegmatis

One person at each table should do a mixed smear of *E. coli* and *S. saprophyticus* which should be observed by everyone. This combination will help you distinguish the colors of G+ versus G-.

Refer to Fig. 1 as you prepare and while you stain your slides.

1. Prepare an air-dried, heat-fixed smear. Tip: Be very careful to prepare a thin smear. A smear that is too thick will not stain correctly! This can cause a fair amount of grief and frustration when evaluating your stain, and may cause you to miss-identify the gram reaction. Prepare several slides at a time.
2. Cover the prepared slide with Crystal Violet for 1 minute.
3. Gently, but completely, rinse the slide with DI water.
4. Cover the slide with Gram's Iodine for 1 minute.
5. Gently, but completely, rinse the slide with DI water.
6. Decolorize the slide with Ethanol/Acetone. Slowly DRIP the decolorizer down the slide until it runs clear of the slide. This should not take more than about 10 seconds. Do not flush the slide with a lot of the ethanol/acetone as this may over decolorize your specimen and cause you to misinterpret the results.
7. Immediately after decolorizing, gently rinse the slide with DI water.
8. Counterstain with the secondary stain, Safranin for 2 minutes.
9. Gently, but completely, rinse the slide with DI water.
10. Blot slide gently with bibulous paper. Tap gently; do not rub the paper as this may smear your prep.
11. Observe at under oil immersion, 100x objective.

Cautions:

Thick smear-

Over heat fixing-

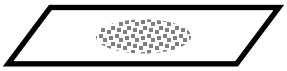
Under decolorizing-

Over decolorizing-

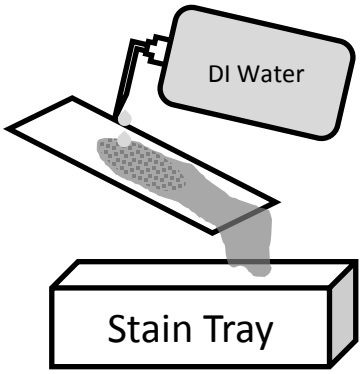
Old cultures-

Gram variable bacteria-

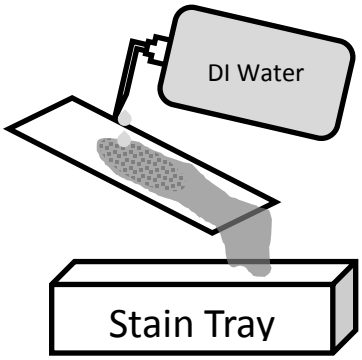
Acid-Fast bacteria-



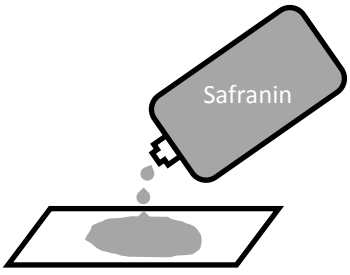
Step #1: Begin with a heat-fixed emulsion.



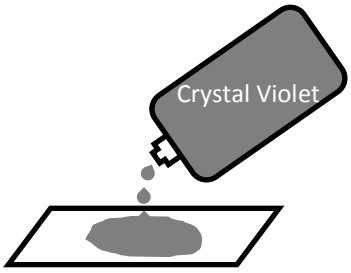
Step #3: Grasp the slide with a slide holder. Gently rinse the slide with distilled water.



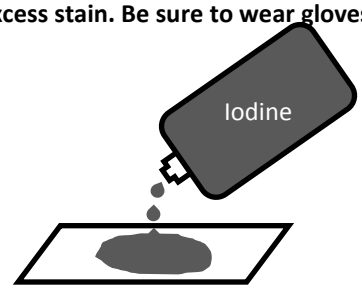
Step #5: Grasp the slide with a slide holder. Gently rinse the slide with distilled water.



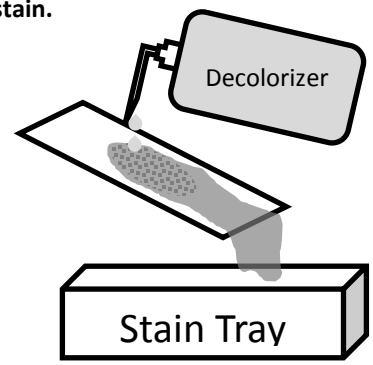
Step #7: Counterstain with safranin stain for 2 minutes. Rinse with distilled water.



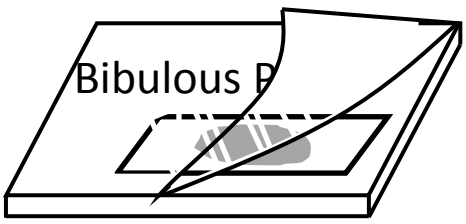
Step #2: Cover the smear with crystal violet stain for 1 minute. Use a staining tray to catch excess stain. Be sure to wear gloves.



Step #4: Cover the smear with iodine stain for 1 minute. Use a staining tray to catch excess stain.



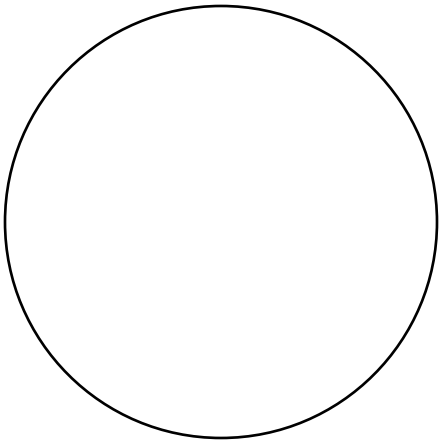
Step #6: Decolorize with acetone/alcohol by allowing it to trickle down the slide until the run-off is clear (<10s). Gently rinse the slide with distilled water immediately.



Step #8: Gently blot dry in a tablet of bibulous paper. Do not rub. When dry observe under oil immersion.

Results:

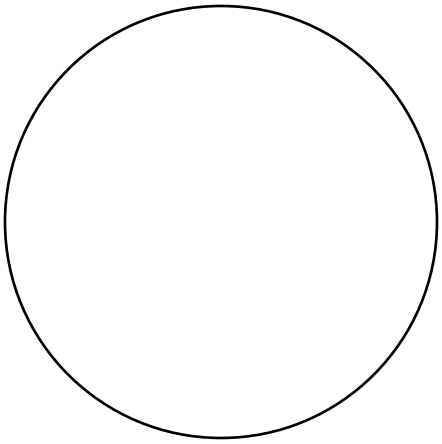
1. Draw and color (or indicate colors of) the bacteria you Gram stained:



Organism _____

Size _____

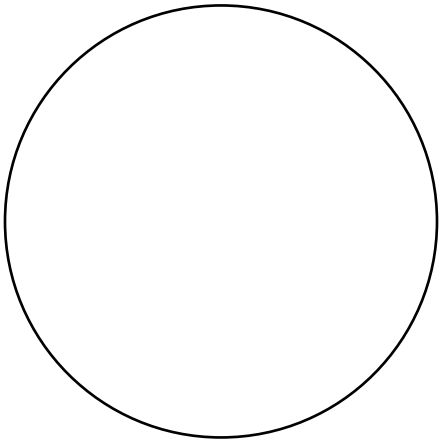
Magnification _____



Organism _____

Size _____

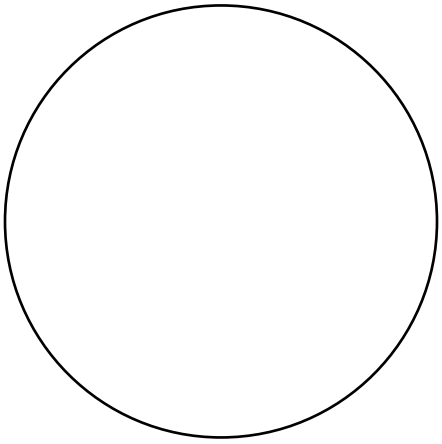
Magnification _____



Organism _____

Size _____

Magnification _____



Organism _____

Size _____

Magnification _____

2. Fill in the table with the organisms you stained, including the mixed slide.

Table 1. Gram stained bacteria

Organism	Color of cells	Gram Reaction (+ or -)	Cell Morphology	Cell Arrangement	Size (um)

Questions:

1. Explain why gram + bacteria stain purple, what happens during the decolorization step, and why Gram- cells stain pink.

2. What is the function of the Iodine step?

3. Were you able to distinguish between G+ cells and G- cells? Give the gram reactions of *E. coli* and *S. saprophyticus.*

4. Why is the acetone/alcohol step the most critical step in the process?

5. If you observed bacteria from a single colony that were gram variable,
a. what other characteristics could you use to help you identify the organism?

b. How might the age of the culture affect the results?

6. What would happen if you Gram stained *E. coli*, but forgot the decolorizing step?

7. What would happen if you Gram stained *S. saprophyticus* and forgot the decolorizing step?

8. Explain, in your own words and experience, whether or not your smear prep was done appropriately for this stain.

Conclusion:

Reflect on your success with the gram stain. Provide a complete analysis of your stain technique and results. Did you have success with several different bacteria? If you had problems discuss them and your plan to improve your success next time.

Rate your confidence from 1-5 (1=completely confident, 5=not very confident) on being able to accurately perform and assess a gram stain. If you are not very confident (3, 4, or 5), please see your instructor for help and a pep talk!

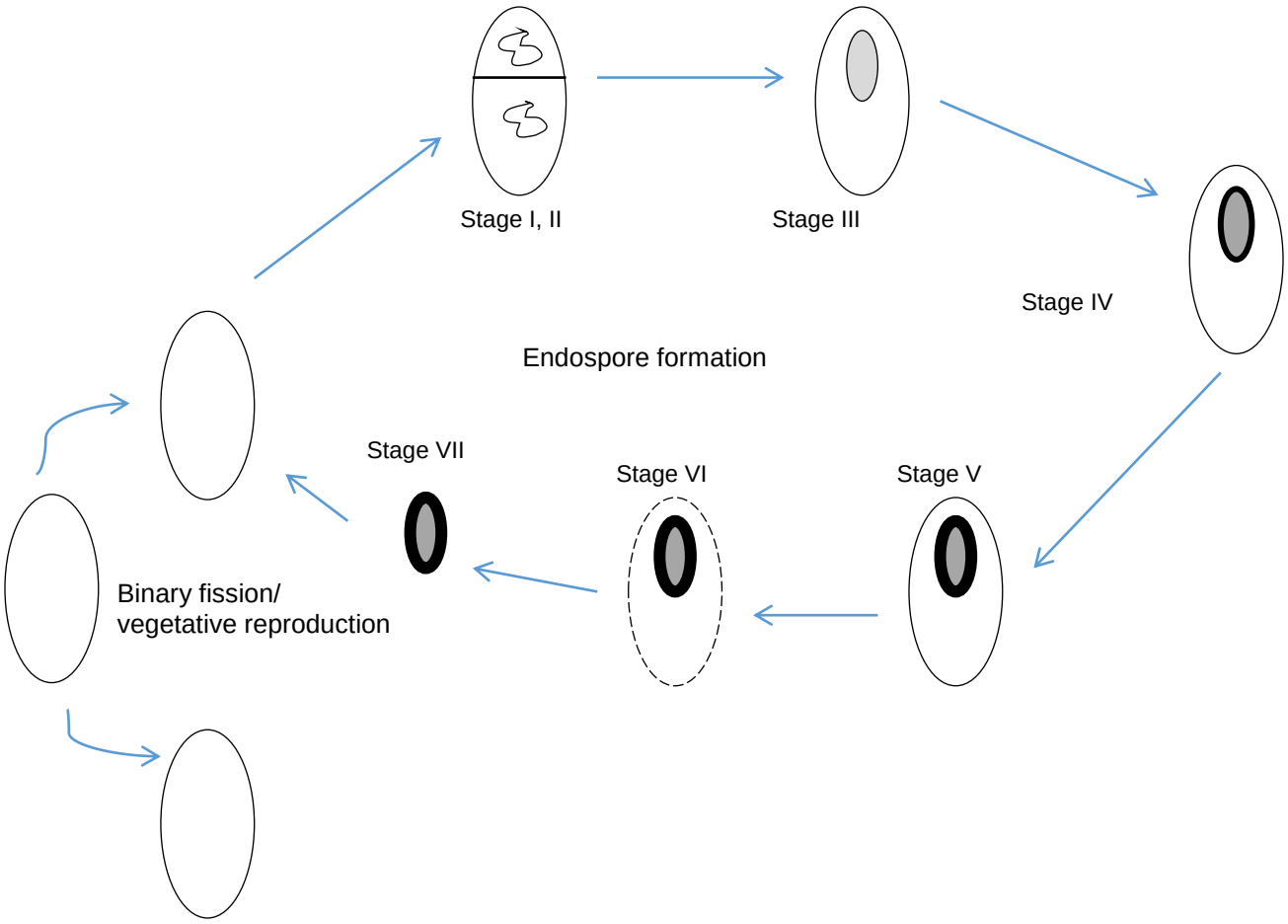
Resources:

1. Smith, Ann C., Hussey, Marise A. The Gram Stain, in Laboratory Protocols. ASM Microbe Library. July 2013 http://www.microbelibrary.org/component/resource/gram-stain/2886-gram-stain-protocols. Accessed 11/25/15

2. Gram, Christian, The differential staining of Schizomycetes in tissue sections and in dried preparations. 1884. http://www.microbelibrary.org/images/stories/ML_2.0/1884p215.pdf. Accessed 12/11/15

Endospore Stain

Endospore formation



Stage I, II: Axial filament and septum formation
Stage III: Engulfment of the forespore
Stage IV: Cortex formation
Stage V: Spore coat synthesis, and exosporium synthesis if present
Stage VI: Breakdown/lysis of the vegetative cell and release of the spore
Stage VII: Germination of the spore into a new vegetative cell

Learning Objectives:

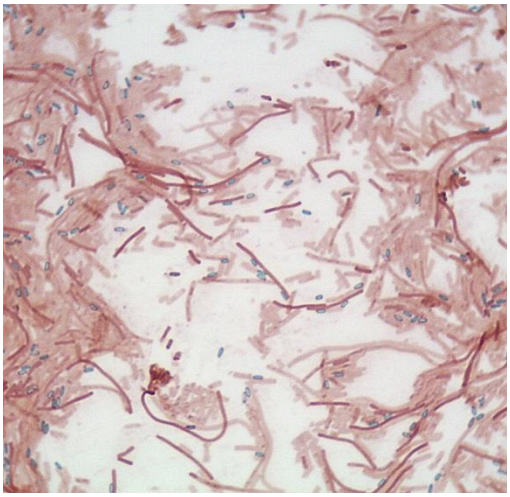
After this lab you should be able to:
1. Perform an Endospore Stain.
2. Explain the principles of how the Endospore Stain works.
3. Evaluate and interpret your Endospore Stain results correctly.
4. Interpret and troubleshoot poor stain results.

Introduction:

The Endospore stain is considered a structural stain. Some bacteria, notably the genera *Bacillus*, *Clostridium*, and *Sporosarcina* produce an internal spore during periods of environmental stress. In the presence of plenty of water and nutrients these bacteria reproduce vegetative cells via binary fission and may not produce spores. As they age the bacteria may readily produce spores as a survival mechanism, especially in a closed system like a petri dish or test tube where nutrients are depleted over time. Endospore production is not a part of reproduction. The spore is a structure for the preservation of the DNA while conditions are poor; once conditions improve the spores will germinate back into vegetative cells, which will then divide normally. The endospore is released as the vegetative cell breaks down, and can survive long periods of time-- some claim millions of years! The spore has a very thick cortex and spore coat that enables it to be resistant to heat, chemicals, UV radiation, desiccation, etc. This can include disinfectants, which makes pathogens that produce endospores to be very problematic. You may have heard of the disease "C-diff" which causes repeated episodes of diarrhea and is transmitted in feces. C-diff is caused by the bacterium *Clostridium difficile*, an endospore former and thus not readily killed, and easily transmitted in healthcare facilities.

The Endospore stain was developed because of the difficulty, due to the structural resistance, in staining spores with traditional techniques. An endospore can be seen in simple and Gram stains, as a clear or empty space in a vegetative cell. In addition, endospores can be seen in wet mounts of live cells using phase contrast microscopy where they appear as bright refractile bodies inside vegetative cells. However, the endospore stain is important for confirmation of the presence of spores.

The procedure requires heating of the primary stain, Malachite Green, while it covers the smear. As the stain heats up it will dry out and the stain can precipitate onto the smear. To prevent this, a small piece of filter paper is placed over the smear and is kept moist with additional stain during the heating process (5-10 minutes). The heating process makes the spore coat more permeable to the stain. Once stained the smear is rinsed with DI water. This removes the stain from any vegetative cells, but is not enough of a decolorizer to remove the stain from the spore (as the spore cools it becomes less permeable and the Malachite Green remains "locked" in the spore). The vegetative cells can be counterstained with Safranin (pink). The Safranin cannot enter the spore. Therefore the spores appear green, while vegetative cells appear pink.



Endospore stain of Bacillus cereus, 1000x
Kelly Carrillo Burke CC-BY-NC-SA

Pre-lab questions:

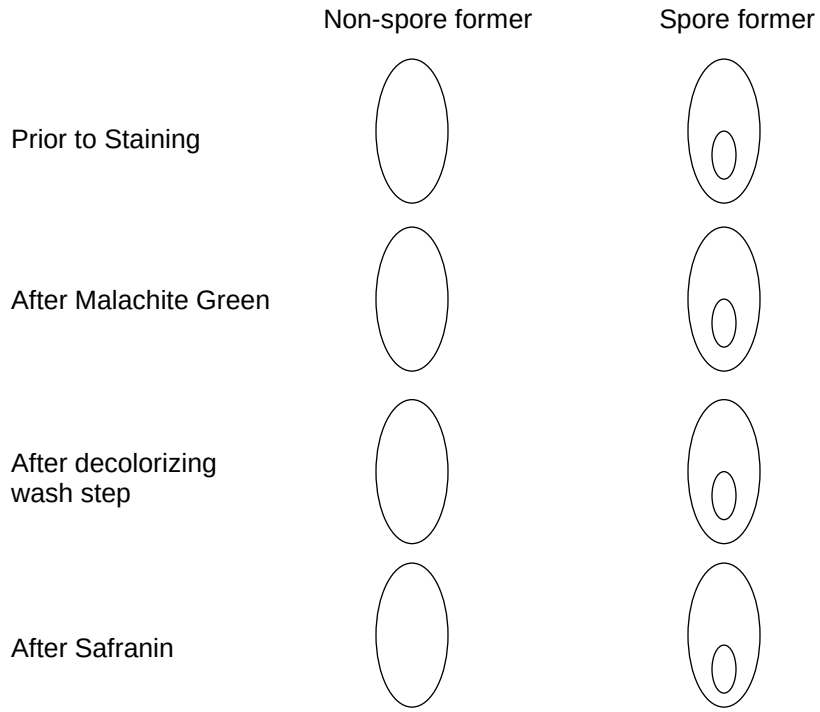
1. What is the purpose of an endospore?

2. How would a disease like C-diff spread so easily through a healthcare facility like a nursing home? What precautions should be taken to prevent spread of the disease from one patient to another?

3. What diseases are some *Bacillus* and *Clostridium* species known for? (Hint: you will have to look this up)

4. Why is it necessary to heat the smear with the stain?

5. Color in the following diagram of bacteria (clear, green, or pink) during the various stain steps:



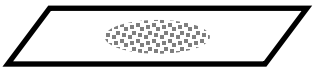
Materials:

Student Stock Cultures and saline spore solutions:
Bacillus cereus
Bacillus subtilis
Sporosarcina ureae
Glass Slides
Stain tray and slide holder

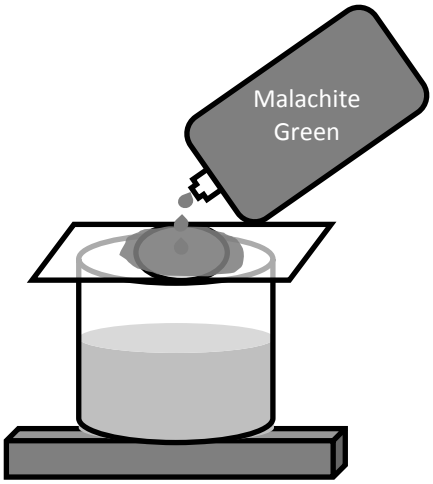
Stain kit:
Malachite Green
Safranin
Filter paper rounds
Hot plate set-up: small beakers of DI water with boiling stones
Glass bowls with Sanisol

Procedures:

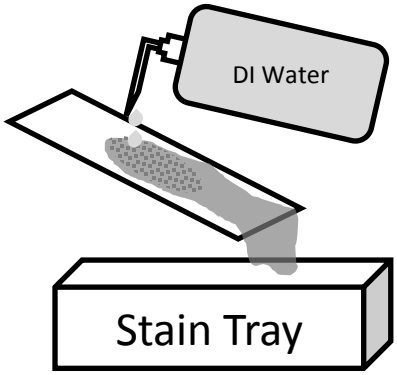
1. Prepare heat-fixed slides of each culture.
2. Place a filter paper round on the slide on top of the smear.
3. Cover the filter paper and slide with Malachite Green and at the hot plate set-ups, place the slide across the top of a steaming beaker of DI water. Steam for 5-10 minutes. While steaming, add additional stain as needed to keep the filter paper wet. DO NOT let the slide dry out.
4. Remove and discard the filter paper. DO NOT discard in the sink!
5. Gently wash with DI water (this is essentially the decolorizer step; there is no separate decolorizer).
6. Counterstain by covering the smear with Safranin for 2 minutes.
7. Gently wash with DI water, blot, and observe under oil immersion.



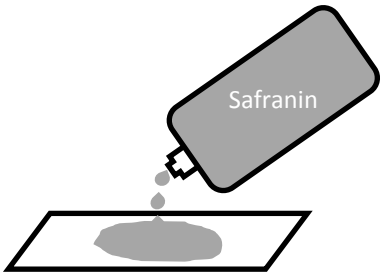
Step #1: Begin with a heat-fixed emulsion.



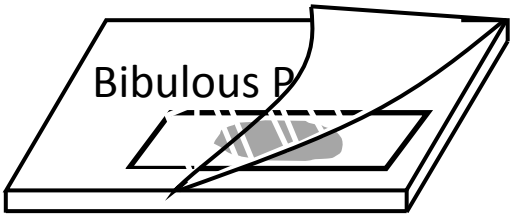
Step #2: Cover the smear with a strip of bibulous paper. Apply malachite green stain. Steam for 5 to 8 minutes. Keep the paper moist with stain. Perform this step with adequate ventilation, gloves, and eye protection.



Step #3: Grasp the slide with a slide holder. Remove the paper and dispose of it properly. Gently rinse the slide with water.



Step #4: Counterstain with safranin stain for 1 minute. Rinse with distilled water.

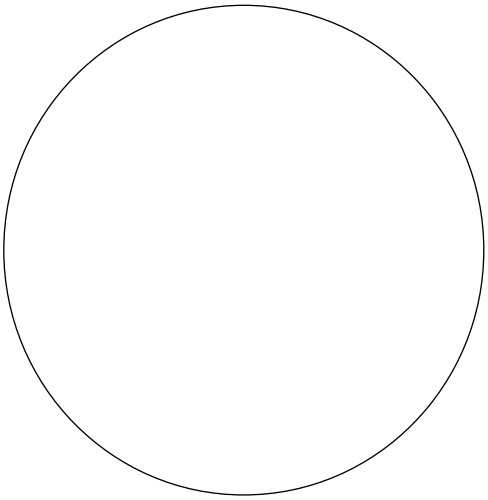


Step #5: Gently blot dry in a tablet of bibulous paper. Do not rub. When dry observe under oil immersion.

C.S. Ramey

Results:

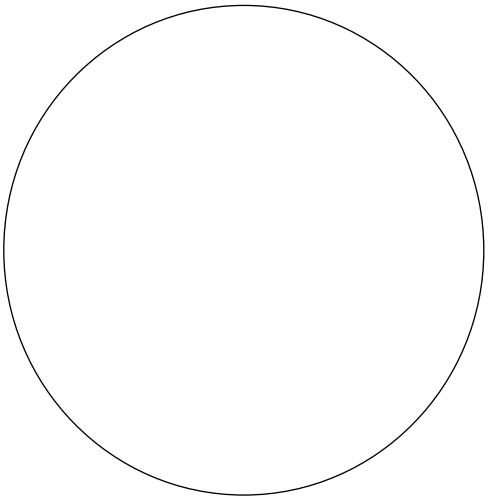
Draw and color (or indicate colors of) your Endospore stain. Label vegetative cells and endospores.



Organism_____

Size_____

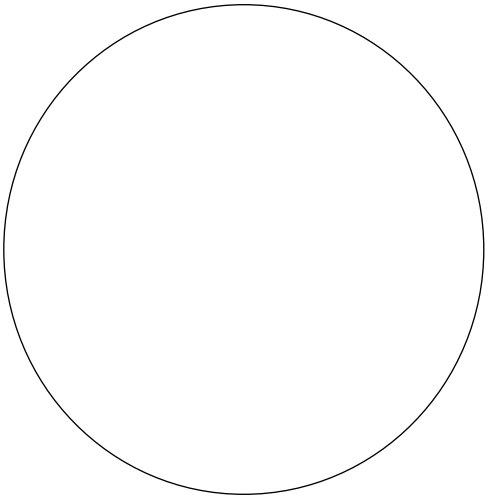
Magnification_____



Organism_____

Size_____

Magnification_____



Questions:

Organism_____

Size_____

Magnification_____

1. Does a negative endospore stain eliminate the presence of endospore forming bacteria in a specimen? Explain.

2. How might age of the culture affect the results of the endospore stain?

3. What is the purpose of heating the cells during the primary stain, Malachite Green, step?

4. Why do the spores not stain pink after the addition of Safranin?

Conclusion:

Reflect on your success with the Endospore stain. Provide a complete analysis of your stain technique and results. Did you have success in observing spores in all three cultures?

Rate your confidence from 1-5 (1=completely confident, 5=not very confident) on being able to accurately perform and assess an Endospore stain. If you had problems discuss them and your plan to improve your success next time.

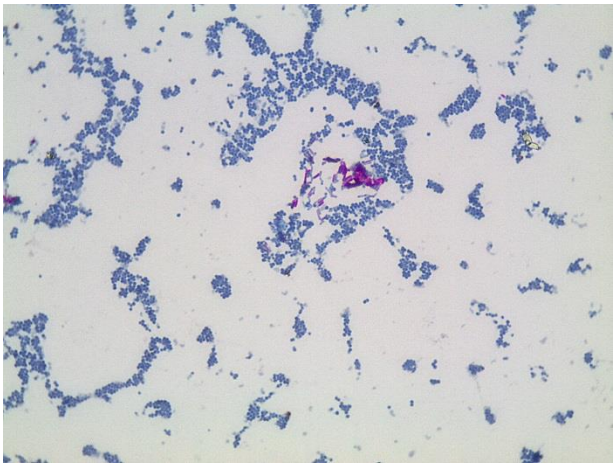
Resources:

1. Hussey, Marise A., Zayaitz, Anne. Endospore Stain Protocol, in Laboratory Protocols. ASM Microbe Library. http://www.microbelibrary.org/component/resource/laboratory-test/3112-endospore-stain-protocol Published September 29, 2007. Last updated April 2013. Accessed 1/6/16.

Acid-Fast Stain: Kinyoun Method

"Tuberculosis (TB) is one of the world's deadliest diseases:

- One third of the world's population is infected with TB.
- In 2014, 9.6 million people around the world became sick with TB disease. There were 1.5 million TB-related deaths worldwide.
- TB is a leading killer of people who are HIV infected.



Acid-Fast Stain of *Mycobacterium smegmatis*, 1000x
Kelly Carrillo Burke CC-CY-NC-SA

A total of 9,421 TB cases (a rate of 2.96 cases per 100,000 persons) were reported in the United States in 2014. Both the number of TB cases reported and the case rate decreased; this represents a 1.5% and 2.2% decline, respectively, compared to 2013. This is the smallest decline in more than a decade."[1]

"The presence of acid-fast-bacilli (AFB) on a **sputum smear** or other specimen often indicates TB disease. Acid-fast microscopy is easy and quick, but it does not confirm a diagnosis of TB because some acid-fast-bacilli are not *M. tuberculosis*. Therefore, a **culture** is done on all initial samples to confirm the diagnosis. (However, a positive culture is not always necessary to begin or continue treatment for TB.) A positive culture for *M. tuberculosis* confirms the diagnosis of TB disease. Culture examinations should be completed on all specimens, regardless of AFB smear results. Laboratories should report positive results on smears and cultures within 24 hours by telephone or fax to the primary health care provider and to the state or local TB control program, as required by law."[2]

Learning Objectives:

After this lab you should be able to:
1. Perform an Acid-Fast Stain on a mixed culture of bacteria (Acid-Fast/non-Acid-Fast).
2. Explain the principles of how the Acid-Fast Stain works.
3. Evaluate and interpret your Acid-Fast Stain results correctly.
4. Interpret and troubleshoot poor stain results.

Introduction:

Mycobacterium smegmatis is generally a non-pathogenic bacterium related *Mycobacterium tuberculosis*, one of the most important worldwide bacterial pathogens. Both organisms, as well as another genus named *Nocardia*, are considered Acid-Fast. *Mycobacterium smegmatis* is the organism you will stain today in order to learn the procedure for the Acid-Fast stain.

Hopefully you noticed while learning the Gram stain, that *Mycobacterium smegmatis* does not Gram stain well. This is because of the unique cell wall structure in the genera *Mycobacterium* and *Nocardia*. Their cell walls contain a waxy lipid called mycolic acid, which resists staining and decolorizing once it is stained. In the Acid-Fast stain a very concentrated stain solution of Carbolfuchsin (pink) is used in order to penetrate and stain the cell walls. Then, an acid based decolorizer (as opposed to the acetone based used in the Gram stain) is used to decolorize the cells. Mycobacterium and Nocardia are "acid-fast" because even the acid decolorizer will not remove the Carbolfuchsin from the cells. You can think of it as

the Carbolfuchsin "holding fast" to the cell wall. Therefore, acid-fast bacteria will be pink, non-acid-fast will decolorize (lose the pink color) and can be counterstained. You will use methylene blue for the counterstain; so non-acid-fast bacteria will be blue after the procedure. There are very few acid-fast bacteria, so this stain is a valuable diagnostic tool for finding tuberculosis and nocardial diseases in patient specimens.[3]

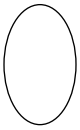
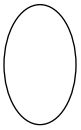
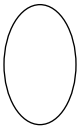
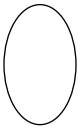
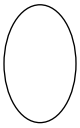
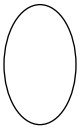
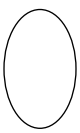
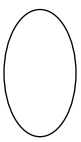
Pre-lab questions:

1. Why are some bacteria Acid-Fast, and most others are not?

2. Compare the decolorizer used in the Gram stain, with the one used in the Acid-Fast stain.

3. Why is the Acid-Fast stain and important diagnostic tool?

4. Color in the following diagram of bacteria (clear, pink, or blue) during the various stain steps:

	Non-Acid-Fast	Acid-Fast
Prior to Staining		
After Carbolfuchsin		
After decolorizer		
After Methylene Blue		

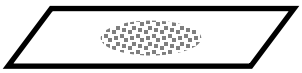
Materials:

Student Stock Cultures:
Mycobacterium smegmatis
Staphylococcus saprophyticus
Glass Slides
Stain tray and slide holder

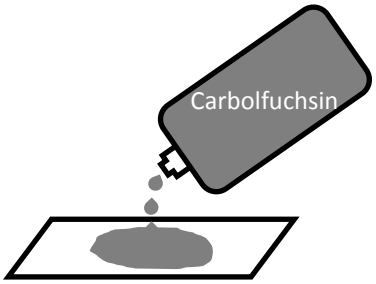
Stain kit:
Kinyoun carbolfuchsin
Acid/Alcohol decolorizer
Methylene blue
Glass bowls with Sanisol

Procedures:

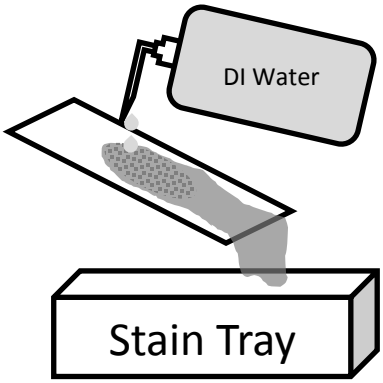
1. Prepare a heat-fixed smear prep with a mixture of *Mycobacterium smegmatis* and *Staphylococcus saprophyticus.* Aseptically place a drop of each onto the same spot on the slide and mix well. Try to break up the waxy *Mycobacterium smegmatis* so that it can spread evenly across the slide. Air dry, then heat-fix.
2. Cover the smear with Kinyoun carbolfuchsin and let stand for 5-10 minutes.
3. Gently wash with DI water.
4. Gently decolorize with Acid-Alcohol by dropping the decolorizer down the slide until the slide runs clear. Note that this decolorizer is not the same as the Acetone-Alcohol used in the Gram stain.
5. Gently wash with DI water.
6. Counterstain by covering the slide with Methylene blue for 1 min.
7. Gently wash with DI water, blot, and view under oil immersion.



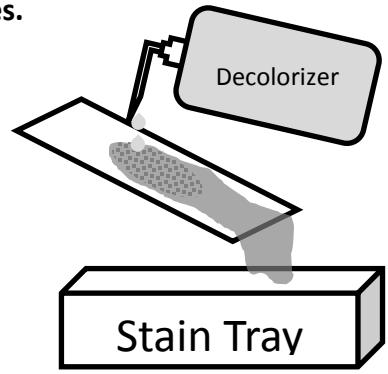
Step #1: Begin with a heat-fixed emulsion.



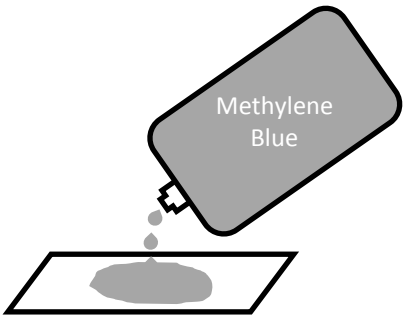
Step #2: Apply Kinyoun carbolfuchsin stain for 5 minutes. Perform this step with adequate ventilation and wear gloves.



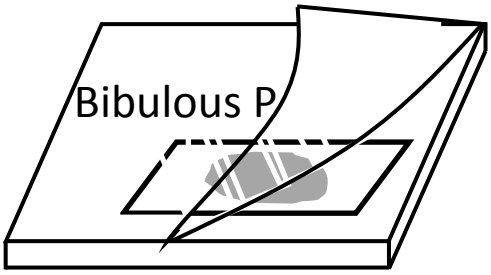
Step #3: Grasp the slide with a slide holder. Gently rinse the slide with distilled water.



Step #4: Continue holding the slide with a slide holder. Decolorize with acid-alcohol (CAUTIONI) until the run-off is clear. Gently rinse the slide with distilled water.



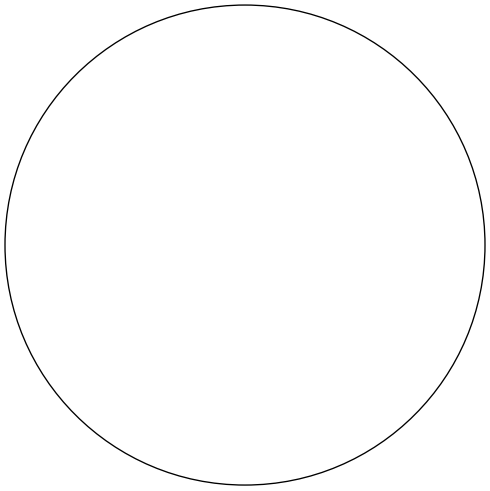
Step #5: Counterstain with methylene blue stain for 1 minute. Rinse with distilled water.



Step #6: Gently blot dry in a tablet of bibulous paper. Do not rub. When dry observe under oil immersion.

Results:

1. Draw and color (or indicate colors of) your Acid-Fast stain:



Organism/color_____ Size_____

Organism/color_____ Size_____

Magnification_____

Questions:

1. If a patient presented with a bloody cough and other symptoms of tuberculosis, what are the primary tools used for diagnosis? Hint: check out the resources section

2. Why is the Acid-Fast stain considered both a structural stain and a differential stain?

3. What is the purpose of using an acid-alcohol decolorizer? What would your results be if you used water or acetone-alcohol instead?

Conclusion:

Reflect on your success with the Acid-Fast stain. Provide a complete analysis of your stain technique and results. Did you have success in observing and differentiating *Mycobacterium smegmatis* from *Staphylococcus saprophyticus*?

Rate your confidence from 1-5 (1=completely confident, 5=not very confident) on being able to accurately perform and assess an Acid-Fast stain. If you had problems discuss them and your plan to improve your success next time.

Resources:

1. Tuberculosis, Fact Sheets. Centers for Disease Control and Prevention website.
http://www.cdc.gov/tb/publications/factsheets/testing/diagnosis.htm
December 12, 2014. Accessed 1/6/16

2. Tuberculosis, Data and Statistics. Centers for Disease Control and Prevention website.
http://www.cdc.gov/tb/statistics/default.htm
September 24, 2015. Accessed 1/6/16

3. Hussey, Marise A., Zayaitz, Anne. Acid-Fast Stain Protocols, in Laboratory Protocols. ASM Microbe Library.
http://www.microbelibrary.org/component/resource/laboratory-test/2870-acid-fast-stain-protocols
Published September 8, 2008. Last updated August 2013. Accessed 1/6/16

Physiological Tests for Characterization and Identification of Bacteria

“Rice University scientists have invented a technology that could potentially identify hundreds of bacterial pathogens simply, quickly and at low cost using a single set of random DNA probes. Rice's "universal microbial diagnostic," or UMD, uses pieces of randomly assembled DNA and mathematical techniques that were originally pioneered for signal processors inside digital phones and cameras.”

"If a laboratory today wants to test for 200 known pathogenic species, they need 200 different tests, each with its own specific DNA probe that was designed specifically to bind with DNA from a particular pathogen," said study co-author Richard Baraniuk, the lead scientist on the new study. "Our technology is fundamentally different. With a small set of DNA probes, we can test for a large number of species."

"In many U.S. hospitals, it still takes several days to definitively identify the specific bacterium that's making someone sick," said Baraniuk, Rice's Victor E. Cameron Professor of Electrical and Computer Engineering. "The lack of rapid bacterial diagnostics can promote antibiotic resistance. Having an accurate, efficient and rapid system for identifying infectious pathogens quickly and inexpensively would help, and such a system would also be a valuable tool for public health, defense, global health and environmental science."

http://phys.org/news/2016-09-id-pathogens-dna-probes.html
Research article: http://advances.sciencemag.org/content/2/9/e1600025.full

New technologies are improving both the ability to identify organisms, especially in clinical settings, and the time needed for identification. These rapid tests are essential in improving patient outcomes. As databases of information increase, more organisms will be identified more rapidly and accurately. Traditionally, before the onset of genomic databases and technological advancements in testing procedures, bacteria were identified by phenetic means. Culture characteristics, staining characteristics, and the use of biochemical tests were the gold standard for ID. These procedures, as you are discovering, are labor intensive and take several days to complete. This has been a hurdle in improving diagnosis and treatment of infectious disease, especially in the current age of antibiotic resistance. Although these traditional procedures are being used less and less, and are often automated now, they still have value in clinical, industrial, environmental, and food testing labs. New technology is expensive, may not be widely available, and will not always ID unusual strains, new organisms, etc. Laboratory personnel are still trained to utilize and evaluate traditional tests as well as new tools.

For the microbiology student, performing these exercises not only introduces you to the types of tests traditionally done to identify bacteria, and a glimpse into the clinical lab, they teach you how bacteria grow and metabolize: use of different nutrients, molecules and enzymes they produce, respiratory abilities, etc. These exercises serve to reinforce that bacteria are living organisms capable of exhibiting a diverse array of biochemical properties. That even though they are invisible in our macro world, they are alive and well and going about the business of being extremely successful organisms, regardless of whether we are aware of them or not. It is essential that students understand them in this way, and not just as abstract “germs”.

To this end, you will learn how to run the tests and evaluate the results using your known stock cultures. You will then be able to apply your knowledge and logic to the identification of unknown bacteria later in the course. As you work through the tests and fill in the charts, it's a good idea to produce your own master chart for the unknowns, and include cultural and stain characteristics.

Learning Objectives:

After this lab you should be able to:

1. Perform biochemical tests used in the identification of bacteria.
2. Evaluate the results of the biochemical tests.
3. Judge which tests are appropriate for some bacteria, but not others.
4. Formulate how you will organize and address identifying unknown bacteria based on observations of the patterns in your results.

Selective and Differential Media: MacConkey, EMB, MSA

MacConkey Agar (1)

Purpose: Selective and differential medium; identification of Enterobacteriaceae

Media: Contains bile salts to inhibit most Gram (+) bacteria except *Enterococcus* and some species of *Staphylococcus*, peptone, and lactose.

Reagents/Indicators: Contains crystal violet and bile salts, which inhibit Gram (+) bacteria, and neutral red dye, which stains microbes fermenting lactose (and thereby decreasing the pH) a pink color.

Mechanism/reactions: By utilizing the lactose available in the medium, Lac+ bacteria such as *Escherichia coli*, *Enterobacter* and *Klebsiella* will produce acid, which lowers the pH of the agar below 6.8 and results in the appearance of red/pink colonies. Non-Lactose fermenting bacteria such as *Salmonella*, *Proteus* species and *Shigella* cannot utilize lactose, and will use peptone instead. This forms ammonia, which raises the pH of the agar, and leads to the formation of white/colorless colonies.

Directions: Streak agar in a straight line and incubate for 24 – 48 hours.
Interpretation: (+) = Lactose fermentation, re/pink colonies
(Slow) = Some organisms ferment lactose slowly or weakly, and are sometimes put in their own category – these include *Serratia* and *Citrobacter*
(-) = non-lactose fermenters, white/colorless growth

Fig. 1 Lactose fermenter (left) and non-lactose fermenter on MacConkey Agar
https://commons.wikimedia.org/wiki/File:MacConkey_agar_with_LF_and_LF_colonies.jpg
By Medimicro (Own work) [Public domain], via Wikimedia Commons

EMB Agar (2)

Purpose: Selective and differential medium; identification of Enterobacteriaceae. Used primarily to distinguish coliform from non-coliform bacteria in water testing.

Media: Eosin, Methylene Blue, lactose, sucrose

Reagents/Indicators: Eosin Y and Methylene Blue

Mechanism/reactions: Selects for Gram Negative bacteria, and differentiates those enterics which ferment lactose (coliforms) from those which do not ferment lactose (non-coliforms). Indicators form a dark purple precipitate at low pH (due to fermentation products) and also inhibit gram positive bacteria. E. coli will often produce a green metallic sheen due to strong fermentation and precipitation of acid and indicator complex.

Fig. 2 Two lactose fermenters growing on EMB

Directions: Streak agar in a straight line and incubate for 24 – 48 hours.

Interpretation: (+) = Lactose fermentation, dark purple colonies with dark center.
Weak fermenters will have pink mucoid growth.
Green sheen = vigorous fermentation of lactose
(-) = non-lactose fermenters, colorless (or very faint pink) growth.

Mannitol Salt Agar (3)

(Optional during the unknowns: may be requested with justification for its use.)
Purpose: Selective and differential; identification of pathogenic Staphylococci

Media: Mannitol Salt Agar (MSA) contains mannitol, 7.5% sodium chloride, and phenol red

Reagents/Indicators: Phenol red

Mechanism/reactions: Salt concentration will inhibit most other organisms so the media is selective for staphylococci. Mannitol is fermented by *Staphylococcus aureus* and will cause a pH change in the medium (acidic) that is detected by observing phenol red changing to yellow.

Directions: Streak agar in a straight line and incubate for 24 – 48 hours.

Interpretation: (+) = Growth and yellow halo surrounding it (also record growth/no color)
(-) = No growth, no color change

Fig. 3 *Staphylococcus aureus* growing on MSA and exhibiting the typical yellow halo.

Materials:

Gloves
Student stock organisms
1 plate MAC/GNR
1 plate EMB/GNR
1 plate MSA/ GPC plus *Staphycoccus aureus*
Staphycoccus aureus

Procedures:

1. Inoculate each GNR onto MAC
2. Inoculate Each GNR onto EMB
3. Inoculate each GPC and *Staphycoccus aureus* onto MSA

Safety: *Staphylococcus aureus* is a BSL2 organism. Wear glove while handling.

Results:

Table 1. Give the results for the inoculated bacteria. Be careful to not only enter +/-, but also a short description of the colony characteristics on each media.

Bacterium	MAC	EMB	MSA

Bacterium (cont.)	MAC (cont.)	EMB (cont.)	MSA (cont.)

Questions:

1. Compare and contrast MAC and EMB.

2. Is it normal to use both MAC and EMB when identifying a bacterium? Why or why not?

3. What similarities and differences did you observe in your results with MAC and EMB?

4. What is the primary purpose of MSA?

5. What differences between the different GPCs did you notice on MSA?

6. Why is MSA optional only during the unknowns?

Conclusion:

Explain how MAC, EMB, and MSA are selective and differential media, and predict how this will help you during identification of unknowns.

Resources:

1. Mary E. Allen. 2005. Macconkey agar plates protocols. Web. 1 October 2016
http://www.asmscience.org/content/education/protocol/protocol.2855

2. Archana Lal, Naowarat Cheeptham. 2007. Eosin-methylene blue agar plates protocol. Web. 1 October 2016
http://www.asmscience.org/content/education/protocol/protocol.2869

3. Patricia Shields, Anne Y. Tsang. 2006. Mannitol salt agar plates protocols. Web. 1 October 2016
http://www.asmscience.org/content/education/protocol/protocol.3034

Chromogenic Media:

Introduction:

HardyCHROM Chromogenic Media are one brand of a type of new media developed to improve identification of certain bacteria. These media utilize chromogens (colorless molecules that can be oxidized to colored compounds) that convert to different colors when degraded by different microbial enzymes. The media were first developed primarily for the rapid detections of MRSA (Methicillin Resistant *Staphylococcus aureus*), an important drug resistant bacterium frequently occurring in hospitals and more recently in the general population.

Additional chromogenic media have been developed for a variety of important clinical isolates and pathogens especially drug resistant microbes: *E. coli*, *Candida*, *Salmonella*, *Shigella*, enterococci, etc.

HardyCHROM UTI:
This media is used specifically for the isolation and differentiation of urinary tract pathogens.

Urinary tract infections are very common, and one of the most common hospital acquired infections. Thus they are a leading cause of prescribing antibiotic treatment (1). It is important to have efficient ID of potential pathogens for timely and correct antibiotic treatment, especially in the case of antibiotic resistant organisms.

The media is streaked directly from a urine specimen, which might contain a variety of organisms. The media helps reduce the time of diagnosis and the identification of multiple organisms at once. Typically, urine specimens are grown on Blood Agar and MacConkey agar plates (or bi-plates). Although BAP/MAC plates are somewhat differential, chromogenic plates are being used increasingly in labs.

(Not available during the unknowns)

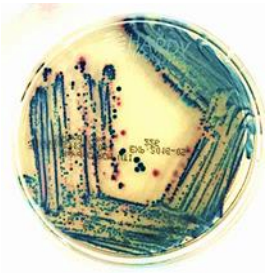


Fig. 4 HardyCHROM UTI Agar; two enteric bacteria. K.C. Burke CC BY-NC SA

Materials:

Gloves
1 HardyCHROM UTI plate/group
1 Unknown mixed broth culture

Procedures:

Each group will perform isolation streaks from two mixed bacterial cultures onto 2 separate HardyCHROM UTI plates. The cultures represent urine samples collected from patients. Students should wear gloves to mimic the correct procedure in handling patient specimens in the clinical laboratory. *Isolated colonies are necessary for the correct identification of the bacteria.*

1. Groups will streak the mixed broth onto the plate:
2. Plates are sensitive to light, so incubate the plates immediately after streaking.
3. After incubation evaluate the plates according to colony color.

Results:

Table 2. Utilize the descriptions and color charts provided in lab to evaluate the plates.

Plate #/isolate	Isolation achieved?	Color	Your Identification	Correct ID from the key
Isolate 1				
Isolate 2				

Questions:

1. Did your group achieve isolation of the 2 organisms? If not, what might be some reasons that isolation wasn't achieved?

2. Why is isolation so important?

3. Is this medium a selective medium? Why or why not?

4. According to the Hardy IFU handout and your results, would there be any additional tests that should be done on the bacteria you isolated?
https://catalog.hardydiagnostics.com/cp_prod/content/hugo/HardyCHROMUTI.pdf

5. Overall, how well did your group do at isolating and identifying your specimens?

6. "A colony count greater than or equal to 10^3 colony-forming units per mL of a uropathogen is diagnostic of acute uncomplicated cystitis.[14] However, studies have shown that more than 10^2 colony-forming units per mL in women with typical symptoms of a UTI represent a positive culture.[15] "(2)

Based on the above, is it possible to diagnose a urinary tract infection from the method we did in lab using the HardyCHROM media?

Conclusion:

In your own words, explain the mechanism behind chromogenic media (how it works), and why it is helpful in a clinical situation.

Resources:

1. Wilson, M.L, Gaido, L. (2004). Laboratory Diagnosis of Urinary Tract Infections in Adult Patients. Clin Infect Dis. 38 (8):1150-1158.doi: 10.1086/383029. Print. Web. November 2015 http://cid.oxfordjournals.org/content/38/8/1150.full

2. Colgan, R., Williams, M. (2011) Diagnosis and Treatment of Acute Uncomplicated Cystitis. Am Fam Physician. 84(7):771-6. Print. Web. November 2015 http://www.aafp.org/afp/2011/1001/p771.html

3. HardyChrom UTI:
https://catalog.hardydiagnostics.com/cp_prod/content/hugo/HardyCHROMUTI.pdf

Blood Agar Plates (BAP):

Introduction:

Blood Agar is an important clinical medium. It is both enriched and differential. The blood contains many nutrients that help support "fastidious" bacteria, those that need additional or particular types of nutritional support. Many bacteria can hemolyze blood, partially or completely, and these hemolysis patterns can help differentiate and identify certain bacterial pathogens. The blood agar we use in lab is commercially prepared and has a TSA base with 5% Sheep's blood added to it. To prepare the medium, the TSA is cooled, the blood is added aseptically, and then the plates are poured. (1)

There are two types of hemolysis. Alpha-hemolysis (α) is caused by damage (but not lysis) of the RBCs in the blood; the media is translucent with a green-ish tinge around the colonies (1). Beta-hemolysis (β) is lysis of the RBCs and the media looks completely transparent around the colonies.

Non-hemolytic (often called gamma-hemolysis, γ) bacteria exhibit neither lysis nor clearing of any kind. **(Not available during the unknowns)**

Fig. 5
Hemolysis *Streptococcus* . Spp (Left) alpha-Hemolysis (*S. Mitis*); (Middle) beta-Hemolysis (*S. Pyogenes*); (Right) gamma-Hemolysis (= Non-Hemolytic, *S. Salivarius*)
https://ja.wikipedia.org/wiki/%E8%A1%80%E6%B6%B2%E5%AF%92%E5%A4%A9%E5%9F%B9%E5%9C%B0
GFDL and CC-by-SA 2.5, 2.0, 1.0

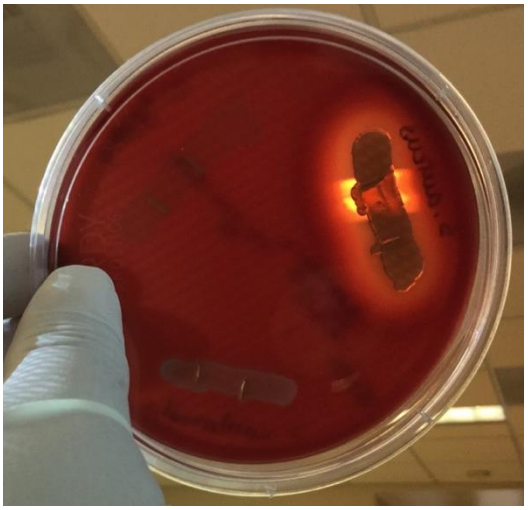


Fig. 6 BAP exhibiting hemolysis patterns, particularly beta-hemolysis in the upper right.
K.C. Burke CC BY-NC SA

Materials:

Gloves
1 Blood Agar Plate (BAP)/group
Lactococcus lactis
Staphylococcus aureus One slant per table is provided at the front table)
Staphylococcus epidermidis

Procedures:

1. Label the plates, and "split" into 3 quadrants, labeling each quadrant with the name of one of the 3 organisms.
2. Inoculate the 3 organisms onto a BAP and stab the inoculation with the loop along the streak line.

Alpha (α) Hemolysis (*Lactococcus lactis*)
Beta (β) Hemolysis (*Staphylococcus aureus*)
Gamma (γ) Hemolysis (*Staphylococcus epidermidis*)

Safety: *Staphylococcus aureus* is a BSL2 organism. Wear glove while handling.

Results:

Table 3. Record your observations.

Bacterium	Hemolysis pattern	Description

Conclusion:

In your own words, explain the mechanism behind Blood Agar (how it works), and why you think it is a good growth medium for fastidious bacteria.

Resources:

1. Rebecca Buxton. 2005. Blood agar plates and hemolysis protocols. Web. 1 October 2016 http://www.asmscience.org/content/education/protocol/protocol.2885

Fermentation and Utilization Media: Durham Sugar Tubes, MRVP, Oxidase, Catalase, Citrate

Introduction:

Bacteria can be differentiated based on their ability to respire (aerobically, anaerobically, or facultative), or their ability to produce ATP via fermentation. Respiration produces ATP via oxidative phosphorylation, which occurs along an Electron Transport Chain (ETC). In aerobic respiration, O_2 is the final electron acceptor. In anaerobic respiration, molecules other than O_2 are the final electron acceptors. Fermentation also happens after glycolysis, however, ATP is produced by substrate level phosphorylation without an ETC or utilizing O_2. Much less ATP is made, but it is sufficient for many bacteria. Acid is also produced during fermentation. The acid produced depends on the sugar that was consumed. Fermentation also may produce gas, CO_2. Think of wine vs. champagne!

These tests help identify bacteria based on the sugars they utilize, the acids, the produce, their ability to utilize O_2, etc.

Phenol Red Broths (aka Durham Tube Sugar Fermentations): Dextrose, Lactose, Sucrose

Purpose: To distinguish carbohydrate fermenters from non-fermenters, to detect and distinguish utilization of specific carbohydrates by the products formed.

Media used: 0.5% to 1% carbohydrate broth- Dextrose (glucose), Lactose, or Sucrose, Peptone, with Phenol Red and an inverted Durham tube for detection of gas.

Reagents and/or indicators: Phenol Red

Mechanism/reaction: Carbohydrate fermentation results in acid and sometimes gas production causing a pH change and possibly gas being trapped in the Durham tube.

Directions: Inoculate tubes and incubate at 35ºC for 24 – 48 hours.

Interpretation: Observe for color change and gas production.

- (+)= color change from re to yellow, pH < 7.0
- (-)= no color change, pH = or > 7.0
  (Note: Color may change to a darker red than an uninoculated tube. This darker color indicates alkaline metabolic products due to the utilization of the peptone instead of the sugar.)
- Gas production (+)= bubble trapped in the inverted Durham tube
- No gas production (-)= no bubble trapped in the inverted Durham tube

Record results as:

1. AG = acid with gas production
2. A = acid, no gas
3. (-) = negative for acid and gas

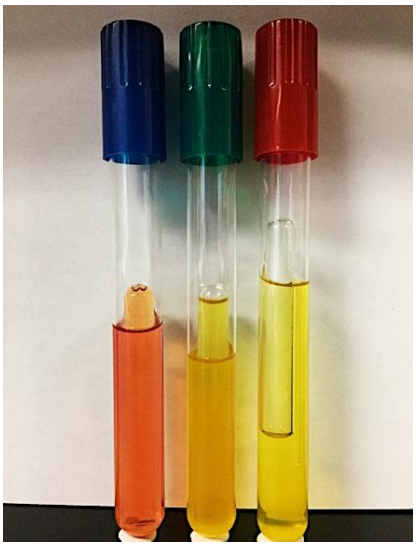


Fig 7. Sugar tubes: non-fermentive (L), Acid production (center), Acid and Gas production (R)
J. Gallai CC BY-NC SA

Methyl Red Test: Mixed Acid Fermentation

Purpose: To determine mixed acid fermentation (lactic, acetic, formic, etc). Part of the IMViC tests--(**I**ndole, **M**ethyl Red, **V**oges-Proskauer, **C**itrate)

Media: MRVP broth--buffered peptone glucose broth used for both MR and VP tests.

Reagents/indicators: Methyl Red- red in pH under 4.4, yellow in pH over 6.2, and orange in between

Mechanism/reactions: If the organism uses the mixed acid fermentation pathway and produces large amounts of organic acids from glucose, the acids will overcome buffers in the medium and the culture will be acidic.

Directions: Broth is inoculated and incubated for 48 hours – 5 days. After incubation, add 5 drops of Methyl Red indicator, do not shake the tube, read the results immediately.

Interpretation:

- (+) = bright red color immediately upon the addition of methyl red (pH < 4.4)
- (-) = yellow color (pH > 6.2)
- Weak (+) = orange color

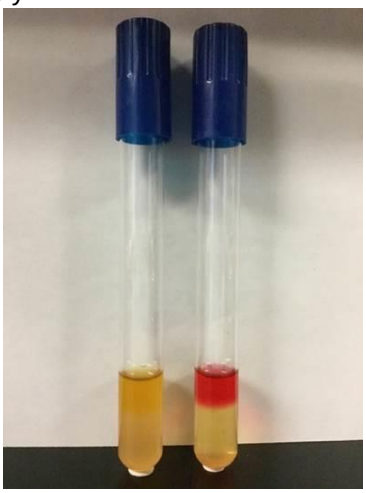


Fig.8 Methyl Red test; negative (L), positive (R)
J. Gallai CC BY-NC SA

Voges Proskauer Test: Butanediol Fermentation

Purpose: To detect the production of acetoin (acetylmethyl carbinol) or 2,3 butainediol (acetoin is the precursor) from glucose broth. Part of the IMViC tests.

Media: MRVP broth-- buffered glucose peptone broth used for both MR and VP test.

Reagent/indicators: Barritt's reagents A- Napthol and B- Potassium Hydroxide (KOH)

Mechanism/reactions: Glucose + diacetyl + KOH + O_2 + arginine → pink color
(Acetoin is oxidized to diacetyl in the presence of KOH)

Directions: Inoculate broth and incubate 48 hours. After incubation add 20 drops of Barritt's Reagent A (napthol) and 20 drops Barritt's Reagent B (KOH). Shake well at frequent intervals and allow reaction to develop up to 1 – 2 hours if necessary.

Interpretation: (+) = red layer at top in 10 minutes. (earliest detection), progressing downward.

() = no red color, disregard any copper or brownish-purple color.

Fig.9 Vogues Proskauer test; positive (L), negative (R) *http://www.asmscience.org/content/education/protocol/protocol.3204*

Catalase Test

Purpose: Production of catalase.

Media: TSA

Reagents/indicators: 3% Hydrogen Peroxide (H_2O_2)

Mechanism/reactions: Catalase converts hydrogen peroxide, a by-product of oxidative respiration, to oxygen and water. Anaerobes and aerotolerant anaerobes lack this enzyme.

Directions: Apply several drops of 3% hydrogen peroxide to growth from a TSA plate.

Interpretation: Vigorous bubbling due to the release of oxygen via catalase.

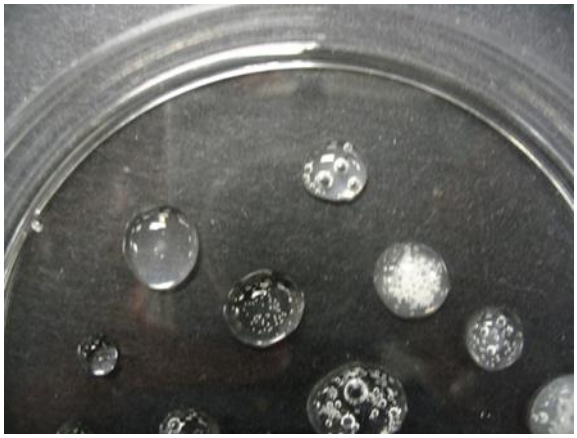


Fig 10. Catalase test
K.C. Burke CC BY-NC SA

Oxidase Test:

Purpose: The oxidase test identifies organisms that produce the enzyme cytochrome oxidase

Media: Use growth from a TSA plate or slant

Reagents/indicators: Oxidase dry slides

Mechanism/reactions: In organisms that use oxygen as the terminal electron acceptor in the electron transport chain, cytochrome oxidase transfers electrons to the oxygen. In the test, the reagent in the dry slide acts as the electron acceptor and changes from yellow to purple when it is oxidized.

Directions: Using a sterile wooden stick (do not use an inoculating wire) pick a small amount of bacteria from a TSA plate or slant and touch an area on one section of the dry slide. Look for the color change to purple within about 30 sec. DRY SLIDES CAN BE USED FOR MANY TESTS (4 / SQUARE, 4 SQUARES PER SLIDE. USE UP EACH SLIDE BEFORE OPENING A NEW SLIDE PACKET).

Interpretation:

(+) = Color change to purple within 30 sec.
(-) = No color change, or a change after more than 30 sec.

Fig 11. Oxidase test
K.C. Burke CC BY-NC SA

Citrate Test:

Purpose: To determine an organism's ability to use citrate as the sole source of carbon. Part of the IMViC tests.

Media: Simmons Citrate Agar- contains sodium citrate as sole carbon source, mineral salts, and pH indicator Bromothymol blue

Reagents/indicators: Bromothymol Blue is a pH indicator. Yellow at less than pH 6.0, green between pH 6.0-7.6, Prussian blue at pH greater than 7.6.

Mechanism/reactions: Utilization of citrate leaves a sodium residue, increasing pH of the medium

Directions: Streak slant, cap loosely (this is an aerobic process), incubate 24 – 48 hours.

Interpretation:

- (+) = medium changes color from green to Prussian blue
- (-) = no change, medium remains green
-

***Caution, Simmons Citrate can sometimes give a false positive result. If the media is blue, then check for growth on the slant to confirm a positive.

Fig.12 Citrate test. Positive (L), negative (R) K.C. Burke CC BY-NC SA

Materials:

Student stock organisms
1 tube Dextrose/GNR
1 tube Lactose/GNR
1 tube Sucrose/GNR
2 tubes MRVP broth/GNR (label one tube MR, the other tube VP)
Hydrogen Peroxide, empty petri dishes, sterile wooden sticks
Oxidase Dry Slides
1 tube Simmons Citrate/GNR

Procedures:

1. Inoculate each GNR into each of the sugar tubes.
2. Inoculate each of the GNRs into an “MR” tube.
3. Inoculate each of the GNRs into a “VP” tube.
4. Perform the catalase test on all organisms.
5. Perform the oxidase text on the GNRs.
6. Inoculate each of the GNRs onto the slants of the Simmon’s Citrate tubes.

Results:

Table 4. Give the results for the inoculated bacteria.

Bacterium	Sugars (A/G) D L S	MR/VP	Catalase	Oxidase	Citrate

Bacterium	Sugars (A/G) D L S	MR/VP	Catalase	Oxidase	Citrate

Conclusion:

Examine your results and state which test or set of tests, if any, would be best to help identify (it would be a good idea to do this for all of your stock cultures!) the following-

E. coli

Pseudomonas

staphylococci

Lactococcus lactis

Hydrolytic and Miscellaneous Media: Starch, Skim Milk, Gelatin, Indole, Urea, Kligler's/TSI

Introduction:

Many bacteria can produce enzymes that they secrete into the environment. These exoenzymes break down nutrients that are too large to enter the cell. The smaller hydrolyzed products can enter the cell and be metabolized. In this series of tests you will detect the production of the exoenzymes by observing the hydrolysis of the nutrient in question, or the product of hydrolysis itself.

The final test is a combo pack. Kligler's or Triple Sugar Iron (two very similar tests) can detect sugar utilization and gas production via fermentation, and the ability to produce hydrogen sulfide (H_2S). The production of H_2S is a unique ability of some bacteria and can be a very identifying factor.

Indole Test--Tryptophan Hydrolysis:

Purpose: To detect production of tryptophanase. Part of the IMViC tests.

Media: Tryptone Agar plates

Reagents/indicators: Indole dry slides

Mechanism/reactions: Tryptophanase causes the hydrolysis of Tryptophan → indole + pyruvic acid

Directions: Using a sterile wooden stick (do not use an inoculating wire) pick a small amount of bacteria from a TA plate and touch an area on one section of the dry slide. Look for the color change to pink/red within about 30 sec. DRY SLIDES CAN BE USED FOR MANY TESTS (4 / SQUARE, 4 SQUARES PER SLIDE. USE UP EACH SLIDE BEFORE OPENING A NEW SLIDE PACKET).

Interpretation:

- (+) = Color change to red within 30 sec.
- (-) = No color change to red

Fig. 13 Indole Dry slide, positive (lower squares) J. Gallai CC BY-NC SA

Starch Hydrolysis:

Purpose: To detect production of amylase.

Media: Starch Agar plates (1% starch)

Reagents/indicators: Gram's Iodine

Mechanism/reactions: When iodine comes in contact with starch it turns blue-black.

Directions: Streak starch agar in a straight line. After incubation add Gram's iodine, drop wise, sparingly, just to cover growth and surrounding area on medium.

Interpretation:

- (+) = Colorless zone around colonies where starch has been hydrolyzed by amylases.
- (-) = No zone, medium is blue-black immediately adjacent to growth.

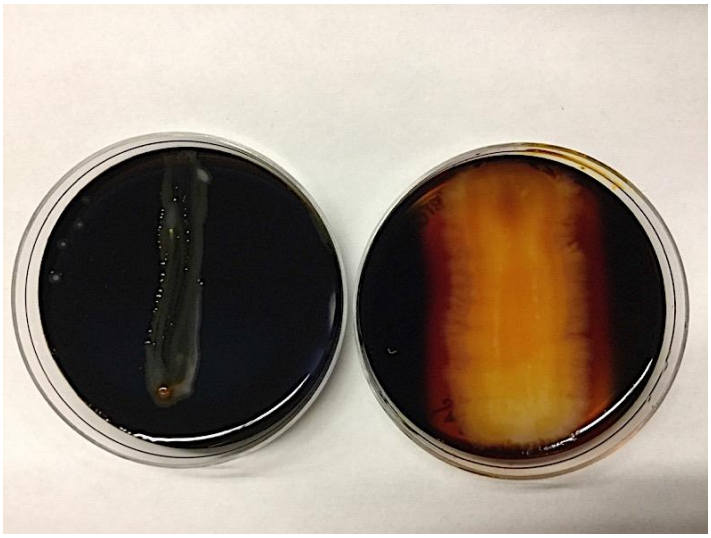


Fig. 14 Starch test. Left: negative for amylase after the addition of Gram's Iodine, the black shows the starch-iodine complex. Right: after the addition of Gram's Iodine, showing the clear zone around the colonies where starch was hydrolyzed. J. Gallai CC BY-NC SA

Urease Test:

Purpose: To detect production of urease

Media: Urea broth

Reagents/indicators: Phenol Red

Mechanism/reactions: Urease hydrolyzes urea to ammonia and carbon dioxide. Ammonia increases the pH of the culture causing the phenol red to go from yellow to bright pink.

Fig.15 Urease test. Positive (L), negative (R) K.C. Burke CC BY-NC SA

Directions: Inoculate urea broth and incubate for 24 – 48 hours.
Interpretation:

- (+) = Red or bright pink color (pH > 8.4)
- (-) = Yellow color (pH < 6)

Casein Hydrolysis (Skim milk):

Purpose: To detect the production of casease.

Media: Skim Milk Agar

Mechanism/reactions: Casease proteolyses casein into peptides and amino acids.

Directions: Streak agar in a straight line and incubate for 24 – 48 hours.

Interpretation:

- (+) = Clear zone around growth indicating casein hydrolysis via casease
- (-) = No clear zone.

Fig.16 Skim milk test. Positive (L), negative (R) J. Gallai CC BY-NC SA

Gelatin Liquefaction:

Purpose: To determine the production of gelatinase.

Media: Nutrient Gelatin Deep (12- 15% gelatin)

Mechanism/reactions: Gelatinase causes the breakdown and liquefaction of gelatin→polypeptides→amino acids.

Directions: Deep stab inoculation, ⅔ of the way down the center of the tube. After incubation, refrigerate for 1 hour before reading.

Interpretation:

- (+) = Liquefaction (after refrigeration)
- (-) = Gels when refrigerated, no liquefaction.

This test does not detect weak positives and concentration of gelatin used may inhibit growth of some organisms.

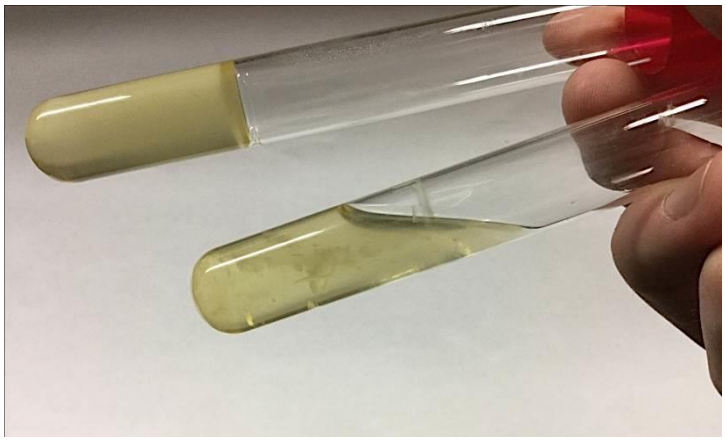


Fig. 17 Gelatin liquefaction (after refrigeration)
J. Gallai CC BY-NC SA

Triple Sugar Iron Agar (TSI):

(Note that either TSI or KIA will be used and, the difference is in the sugars available in the test)

Purpose: The differentiation of Enterobacteriaceae by their ability to ferment dextrose (glucose), lactose, and sucrose, and produce gas and/or hydrogen sulfide.

Media: TSI contains ferrous sulfate, phenol red, dextrose (glucose), lactose, and sucrose.

Mechanism/reactions:
Fermentation: Phenol red turns yellow in an acid environment.
Dextrose is fermented; the butt of the medium turns yellow and the slant remains red.
Lactose or **sucrose** is fermented; the butt and slant both turn yellow.
Gas: Gas from fermentation may appear as breaks or cracks in the medium.
Hydrogen Sulfide: A few bacteria are capable of reducing the SO_4= to H_2S (hydrogen sulfide). The iron combines with the H_2S to form FeS (ferrous sulfide) a black compound. This will turn the butt black. Thus, a black butt indicates H_2S production.

Directions: Dual inoculation with a needle: streak surface of agar slant, then stab, incubate for 24 – 48 hours.

Interpretation: Slant/Butt, Gas, H_2S

- ALK/A: (+) Dextrose = Alkaline slant (red) over acid butt (yellow)
- A/A: (+) Dextrose, Lactose and/or Sucrose= Acid slant over acid butt
- ALK/ALK: No change, alkaline slant over alkaline butt
- G: Gas production
- H_2S (+): = Hydrogen Sulfide production = black (produced in acid environment)

Kligler Iron Agar (KIA):

Purpose: The differentiation of Enterobacteriaceae by their ability to ferment dextrose (glucose), lactose, and produce gas and/or hydrogen sulfide.

Media: KIA contains ferrous sulfate, phenol red, dextrose (glucose), lactose.

Mechanism/reactions:
Fermentation: Phenol red turns yellow in an acid environment.
Dextrose is fermented; the butt of the medium turns yellow and the slant remains red.
Lactose is fermented; the butt and slant both turn yellow.
Gas: Gas from fermentation may appear as breaks or cracks in the medium.
Hydrogen Sulfide: A few bacteria are capable of reducing the SO_4= to H_2S (hydrogen sulfide). The iron combines with the H_2S to form FeS (ferrous sulfide) a black compound. This will turn the butt black. Thus, a black butt indicates H_2S production.

Directions: Dual inoculation with a needle: Stab into the center of the deep, then streak surface of agar slant, incubate for 24 – 48 hours.

Interpretation: Slant/Butt, Gas, H_2S

- ALK/A: (+) Dextrose = Alkaline slant (red) over acid butt (yellow)
- A/A: (+) Dextrose and Lactose = Acid slant over acid butt
- ALK/ALK: No change, alkaline slant over alkaline butt
- G: Gas production
- H_2S (+): = Hydrogen Sulfide production = black (produced in acid environment)

Materials:

Student stock organisms
1 plate Starch Agar/GP
1 plate Skim Milk Agar/GP
1 tube Gelatin/all organisms
1 plate Tryptophan Agar/2 GNRs (Inoculate 2 GNRs on each plate)
1 tube Urea broth/All except GNRs
1 tube of TSI or Kligler's/GNR

Procedures:

1. Inoculate each GP onto a Starch Agar plate
2. Inoculate each GP onto a Skim Milk Agar plate
3. Perform a stab inoculation with a needle of each of your stocks into a Gelatin tube
4. Inoculate 2 GNRs each onto a Tryptophan Agar plate, inoculate all GNRs total
5. Inoculate all your stocks, except the GNRs, into a Urea broth tube
6. Inoculate each of the GNRs into TSI or Kligler's (needle inoculate, stab then streak slant)

.

Fig. 18 *Kligler's. Negative (L), Acid/Acid, Alk/Acid/Gas (C), Acid/Acid/H_2S, Alk/Acid/ H_2S (R)*
K.C. Burke CC BY-NC SA

Results:

Table 5. Give the results for the inoculated bacteria

Bacterium	Starch	Skim milk	Gelatin	Indole	Urea	TSI/Kligler's S/B G H_2S

Conclusion:

Examine your results and state which test or set of tests, if any, would be best to help identify (it would be a good idea to do this for all of your stock cultures!) the following-

E. coli

Mycobacterium smegmatis

B. cereus

Lactococcus lactis

Unknown #1

What is yellow, wrinkled, round,...?

Learning Objectives:

After this lab you should be able to:
1. Use pure culture techniques, such as the Isolation Streak, selective media, etc., to subculture an unknown bacterial isolate from a broth culture.
2. Identify the bacteria in the culture using the appropriate techniques, media, etc.
3. Complete a lab report about your unknown investigation.

Introduction:

Identification of unknown bacterial isolates is an important part of clinical diagnostics, research, quality control, etc. There are many characteristics of individual bacteria that aid in their identification. These can be as simple as observing unique cultural characteristics like colony pigmentation and metabolic characteristics like sugar fermentation. Today, clinical isolates can be identified by utilizing sophisticated metabolic and genetic tests. The state of the art is MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight), a mass spectrometry method. This method places a bacterial isolate into a matrix material to stabilize it and then a laser is focused on the sample causing ionization of proteins in the sample. The ions are analyzed and a "mass spectrum" of the proteins is produced. The bacterial "fingerprint" spectra can be compared with large databases of bacteria and identification of bacteria can be done in minutes.

However, most clinical labs utilize a combination of high-tech methods and traditional cultural methods to identify bacteria. These low-tech methods are important in situations where high-tech equipment (costing hundreds of thousands of dollars) is not available or when an isolate doesn't match anything in the database, or exhibits unexpected growth patterns or test results.

During the next few labs you will perform the appropriate tests on an unknown isolate in order to identify it.

Pre-lab questions:

1. Describe several examples/situations of when it might be important to isolate and identify a bacterium.

2. Why are cultural characteristics, physiological tests, staining results still important in a clinical diagnostic lab?

Materials:

Day 1:

Unknown cultures A and B
2 TSA plates
2 TSA slants
2 TSB tubes
Stains
Subsequent days: Reagents and stains are available at all times. Media is available by request only.

TSA plates and slants
TSB
Stains
Physiological media:
- PR Dextrose Broth
- PR Lactose Broth
- PR Sucrose Broth
- MR-VP Broth
- Simmon's Citrate Slant
- TSI Agar Deep Slant
- Nutrient Gelatin Deep
- Urea Broth
- Starch Agar Plate
- Skim Milk Agar Plate
- EMB Agar Plate
- MacConkey Agar Plate
- Tryptone Agar Plate
- Mueller-Hinton Plate (Novobiocin 30 disk)

Test Reagents:
- Catalase Reagent-3% Hydrogen Peroxide
- Oxidase Dry slides
- Indole Dry Slides
- Methyl Red
- Barritt's Reagents A & B

Procedures:

Each person will subculture their unknown samples, perform stains, run tests, etc., according to the instructions given on their bacteria. It will be up to the individual to determine which tests are appropriate and necessary to use.

Individual results should be recorded below and a final write-up will be required. Your instructor will advise you as to the requirements of the write-up.

Day 1:
You will receive a broth culture and should record the number of the unknown. You will subculture the unknown on a plate, slant, and broth and incubate them. Be sure to label each correctly including the number of the unknown.

Next, you should perform a gram stain and negative stain to determine gram reaction, morphology, arrangement, and size.

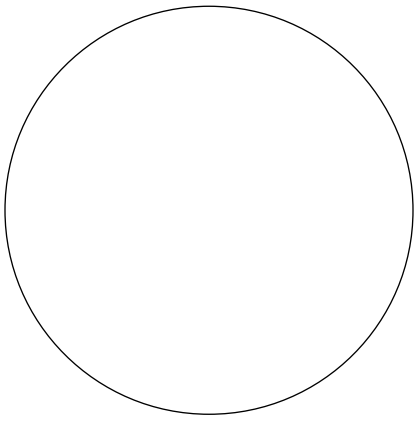
Your Unknowns:
_____A

Gram reaction _____

Cell Morphology _____

Cell Arrangement _____

Cell Size _____



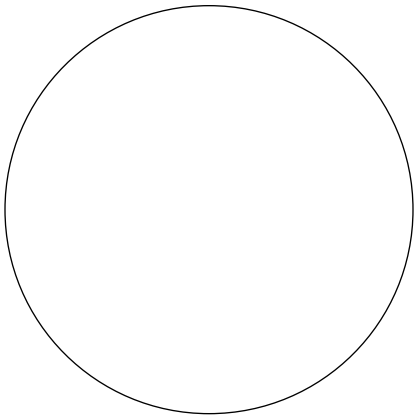
_____B

Gram reaction _____

Cell Morphology _____

Cell Arrangement _____

Cell Size _____



Day 2:
At this point you should have determined the gram reaction, cell morphology, and cell arrangement of your unknown. After plating the culture you will be able to observe and record its culture characteristics. Refer back to the lab on culture characteristics in order to use the correct terms to describe its growth characteristics.

_____A
Culture characteristics:

Plate- **Slant-**

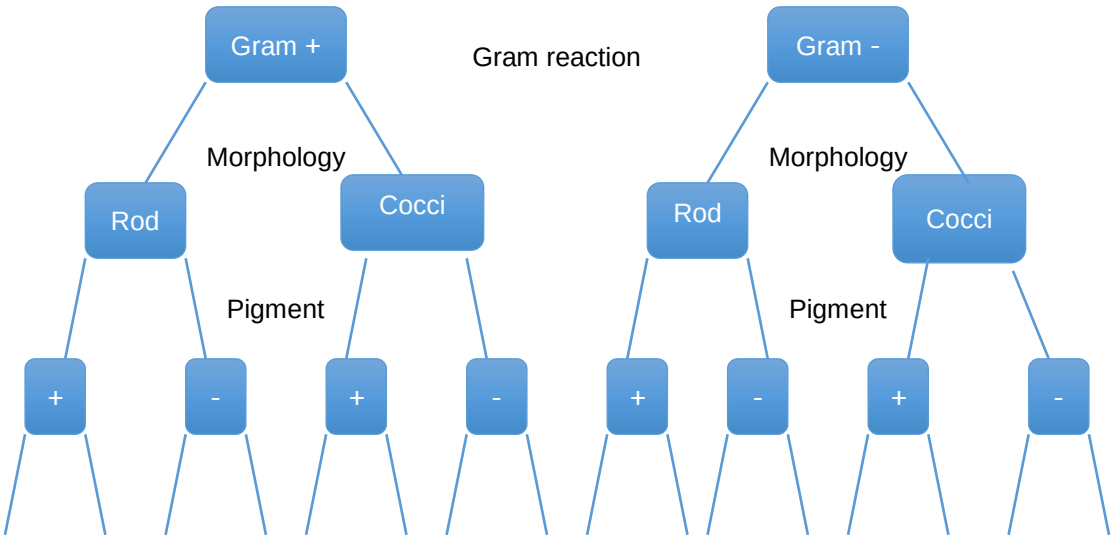
Broth-

_____B
Culture characteristics:

Plate- **Slant-**

Broth-

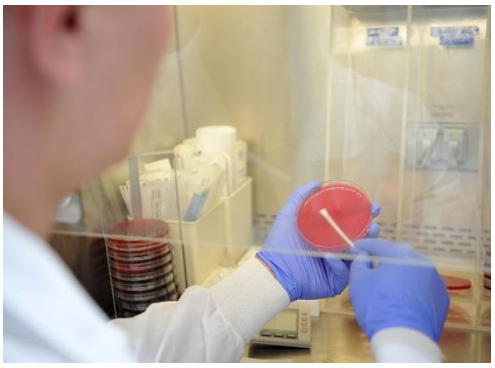
Day 2 and beyond:
Decide which tests you need to help ID your unknown. Identification of unknown bacteria can be aided by the use of a dichotomous key, or flowchart, like the one below. The example below illustrates how you might begin to design a flow chart. There is no one set way to construct it!



Results and Conclusion:

Complete your lab report as directed.

Unknown #2: Mixed Culture



Staff Sgt. Grant Wendland, 51st Medical Support Squadron medical laboratory technician, streaks out a streptococcal pharyngitis, or strep throat, culture plate as part of a rapid strep test in the 51st Medical Group at Osan Air Base, Republic of Korea, April 17, 2014. The 51st MDG's laboratory is staffed by a 10-person team that supports healthcare providers in their treatment of diseases. (U.S. Air Force photo/Airman 1st Class Ashley J. Thum). Public Domain.

Learning Objectives:

After this lab you should be able to:
1. Separate a mixed culture using pure culture techniques, such as the Isolation Streak method, selective media, etc.
2. Identify the bacteria in your mixed culture using the appropriate techniques, media, etc.
3. Complete a lab report about your unknown investigation.

Introduction:

Bacteria rarely arrive in pure culture, unless ordering them from a supply house. Most natural samples have a variety of bacteria in them. In order to identify bacteria using physiological tests, they need to be separated and cultured individually. In this round of unknowns, you will be given a mixed culture of bacteria that will contain 2-3 bacteria. Using pure culture methods, you will separate and subculture your isolates and then work to identify them. You will utilize all the observable characteristics of the isolates as was done in Unknown #1-stain characteristics, cell morphology, arrangement, size, culture characteristics, and physiological test results.

Pre-lab questions:

1. What are methods you have used in lab to separate or select for certain organisms?

2. Describe your experiences with contaminated cultures in lab. Why will it be important to maintain a high level of aseptic technique during Unknown #2?

3. List the important first steps and procedures you will take with your mixed culture. Try to list them in the order you will do them.

Materials:

Unknown mixed culture
2 TSA plates
Stains

Subsequent days: Reagents and stains are available at all times. Media is available by request only.

TSA plates and slants
TSB
Stains
Physiological media:
- PR Dextrose Broth
- PR Lactose Broth
- PR Sucrose Broth
- MR-VP Broth
- Simmon's Citrate Slant
- TSI Agar Deep Slant
- Nutrient Gelatin Deep
- Urea Broth
- Starch Agar Plate
- Skim Milk Agar Plate
- EMB Agar Plate
- MacConkey Agar Plate
- Tryptone Agar Plate
- Mueller-Hinton Plate (Novobiocin 30 disk)

Test Reagents:
- Catalase Reagent-3% Hydrogen Peroxide
- Oxidase Dry slides
- Indole Dry Slides
- Methyl Red
- Barritt's Reagents A & B

Day 1:

Procedures:

Day 1:
1. Receive mixed culture, label it, and record #. DO NOT discard this original culture.
2. Streak culture onto two TSA plates, incubate.
3. Gram Stain mixed culture.

Subsequent Days (activities will be individual in nature, and not everyone will do the same thing on Day 2, Day 3, etc.):
1. Subculture ISOLATED colonies only onto a TSA plate, TSA slant, and into a TSB tube, incubate. After incubation observe and record culture characteristics of your plate, slant, and broth. Request media to re-streak cultures if isolation of both bacteria is not achieved.
2. Re-Gram stain isolated colonies.
3. Request media and run appropriate tests on ISOLATED colonies as needed.
4. Record all results.

Results and Conclusion:

Complete your lab report as directed.

Bacterial Examination of Food: Standard Plate Counts

The presence of microbes in food may or may not be a disease or spoilage issue. Bacteria could be present that will not cause human disease, or could be present as an intrinsic part of the food—think yogurt! However, high counts of bacteria in food could be problematic, in both the potential for disease and also for food spoilage. If high counts of bacteria are present in food in a manufacturing/production setting, additional tests could be called for. This is especially true with foods where bacteria are not expected to be present and in foods that have been through a process like pasteurization. High plate counts could also be a sign of a breach in the manufacturing process.

Learning Objectives:

After this lab activity you should be able to:
1. Perform a serial dilution.
2. Determine the population count of bacteria in a food sample.
3. Reflect on the presence/absence of bacteria in food.

NIH, Public Domain.1988

Introduction:

Most foods are solid at least in some way, and so food must be re-suspended in water in order to perform a plate count. In addition, because most foods will not contain extremely high numbers of bacteria the dilution scheme is set-up accordingly. Finally, because coliforms are indicators of possible contamination by intestinal pathogens, food samples are routinely tested for them.

Your group will bring in a sample of a fresh food that should be safe to eat, yet might contain bacteria. A Standard Plate Count (SPC) will be performed to determine the number of viable bacteria in the original sample.

Pre-lab questions:

1. What foods might contain bacteria, yet, is safe to eat?

2. What kind of results might you predict for foods like potato chips, cookies, or canned soup?

3. What is “food spoilage”?

Materials:

One food sample/group, at least 20g
Sterile blender container
1 ml pipettes
1-180 ml sterile water blank
1-99 ml sterile water blank
Sterile spatula and weigh boats
3 Eugon Agar pours
3 sterile empty petri dishes
1 EMB plate

Procedures:

1. Using the best aseptic technique possible, weigh out 20g of food and transfer to the sterile blender container.
2. Add the 180 ml of sterile water to the container and blend the food for 5 minutes.
3. Use the 99ml water blank for diluting, mix thoroughly, and then plate out the sample to the following dilutions: 1:100, 1:1000, 1:10,000.
4. Pour the Eugon pours into the plates, gently mix the plates using a slow figure-eight motion on the lab bench. Set aside the plates to harden.
5. From the food in the blender container, use a sterile loop to streak an EMB plate to look for coliforms.
6. Incubate plates at 35C for 24h.
7. After incubation, evaluate plates.
    a. Count the colonies on the appropriate plate and report total #CFUs/ml.
    b. Look for presence of coliforms on the EMB plate.

Results:

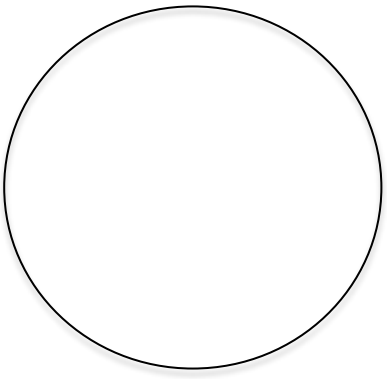
1. Standard Plate Count

Food that you tested:_____

Plate Dilution	1:100	1:1,000	1:10,000
Colonies Counted			

Calculate the SPC, the number of bacteria/g of food for your group:

2 EMB plate observations:



EMB agar

3. Class Results: Place all class results in the table below.

Group	Food tested	SPC	EMB +/- for coliforms
1			
2			
3			
4			
5			
6			

Questions:

1. Were your group's results what you expected? Why or why not?

2. Which foods had the highest SPCs? Is there a relationship between the types of foods tested and the SPCs?

3. Were there any mistakes made during the procedure? How might these have affected your results? What would you do differently next time?

4. Does a high plate count necessarily mean that a food should not be eaten? Why or why not?

Conclusion:

Reflect on the class's results. What did you learn about food and bacterial counts?

Bacterial Examination of Water: Multiple Tube Test, Standard Plate Count, and Membrane Filter Technique

"The most important microbiological drinking water tests are for bacteria. Water is tested throughout the systems weekly for Total Coliform bacteria. The MCL for total coliforms is 5% of all monthly tests showing positives for larger systems. The presence of Escherichia coli (E. coli) indicates fecal contamination of waters. No E. coli was detected in any drinking waters in the SCV last year."
The Santa Clarita Valley 2015 Water Quality Report

Learning Objectives:

After this lab you will be able to
1. Define the term "coliform".
2. Perform the Multiple Tube Test and explain the principles and each step of the test.
3. Calculate the total number of bacteria in the food sample utilizing the SPC method.
3. Utilize the membrane filter technique to assess and possibly quantify the presence of coliforms in a water sample.
4. Describe the principles of the Membrane Filter Technique.

Introduction:

Many intestinal pathogens can be waterborne and transmitted by drinking contaminated water. It's important to be able to test drinking water sources for contamination by pathogens, but it's not very practical or expedient to look for the many types of pathogens that could be found (and in small numbers and often hard to grow in culture). Methods have been developed to detect organisms that are normally found in the human gut but are not normally found in soil or water. The presence of these organisms in water indicates that there may be fecal contamination of the water and therefore, intestinal pathogens might also be present. These organisms are called "indicator" organisms and the group of choice is the coliform group—Gram-negative, facultative anaerobic, non-endospore forming rods that ferment lactose to produce acid and gas in 48h at 35C. Typical coliforms that we have observed in lab are *Enterobacter aerogenes* and *E. coli*. The following tests are conducted to detect the presence of coliforms, particularly *E. coli*, in water samples.

Pre-lab Questions:

1. Define the term "coliform".

2. Why aren't pathogens, like *Vibrio cholerae*, tested for directly?

3. Why is the Presumptive test lactose broth and the confirmed test EMB?

Materials:

Water sample, at least 150ml, collected by student

Presumptive Testing-Multiple Tube Test (Most Probable Number-MPN):
- 3 Double Strength Lactose Broths (DSLB)
- 6 Single Strength Lactose Broths (SSLB)
- 1-10ml sterile pipette
- 1-1ml sterile pipette
- (MPN Table needed for analysis)

Confirmed:
- 1 EMB plate

Completed:
- None, this will not be performed

Standard Plate Count:
- 1 TGEA Pour
- 1 Sterile Petri plate
- 1-1ml Sterile pipette

Membrane Filter Method:
- 1 Sterile Nalgene Membrane Filter Unit (0.45um)
- 1 mEndo agar plate
- 1 Sterile forceps
- 1 Sterile scalpel

Procedures:

Multiple Tube Test (MPN)

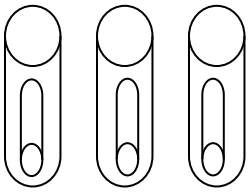
Presumptive Test:

1. Set up three DSLB and six SSLB tubes as shown by your instructor. Label each tube with the amount of water that is to be dispensed into it: 10ml, 1.0ml, and 0.1ml.

(10ml in each DSLBs, 1.0ml in 3 SSLBs, 0.1ml in remaining 3 SSLBs as directed below)

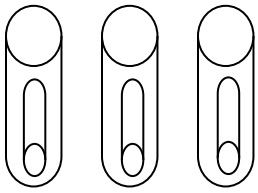
2. Shake/swirl water sample 25 times if possible.

3. Inoculate Durham tubes as follows



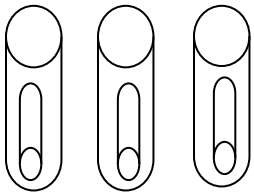
DSLB

10.0ml water in each



SSLB

1.0ml water in each



SSLB

0.1 ml water in each

A. With a 10 ml pipette, transfer 10 ml of water to each of the DSLB tubes.

B. With a 1.0 ml pipette, transfer 0.1 ml of water to each of the last set of SSLB tubes, and 1.0ml to each of the middle three SSLB tubes (this way only one pipette is used and prevents carry over from the lower volume to the higher volume).

5. Incubate the tubes at 35C for 24h.

6. Day Two: Examine the tubes and record the number of tubes in each set that have gas present.

7. Determine the Most Probable Number of coliforms in your sample by referring to MPN Determination Table provided.

Confirmed test:
If a water sample is positive for gas then it is presumed that the sample contains coliforms. The confirmed test would then be performed via inoculation of a plate of EMB agar from a gas positive tube. In this lab you will instead conduct the confirmed test prior to getting results from the presumptive test.

1. Inoculate an EMB plate with a loop of your original sample of water.

2. Incubate at 35C for 24h.

3. Day two: Observe plate for coliforms; purple colonies with dark centers. *E. coli* may exhibit a green sheen. You can compare this to your Membrane Filtration test.

Completed test:
Coliform colonies from EMB would be inoculated again into Lactose Broth with a Durham tube and checked for gas, and inoculated on NA or TSA and checked via Gram stain for GNRs. If these tests are positive it shows that coliforms (not another gas producer) are present and indicates that the water sample is contaminated. Due to time constraints you will not be performing the Completed Test.

Standard Plate Count

A standard plate count can be done to determine total numbers of bacteria in a sample, but is not specific for coliforms. Depending on the water source a dilution series might be appropriate. However, since we don't suspect your water samples to have high numbers of bacteria you will plate directly from your water sample.

1. Transfer 1ml of your sample to the empty petri dish and add the TGEA pour. Rotate the plate on the table in a slow figure-eight motion, then set aside to harden. Incubate at 35C for 24h.

2. Day two: Count total colonies, determine #CFU/ml in your sample.

Membrane Filter Technique

The Membrane Filter Technique utilizes an apparatus containing a filter membrane with a 0.45um pore size that will trap microorganisms larger than that on the surface of the membrane as the water sample passes through the membrane. The membrane is then placed onto a plate of m-Endo agar, which is similar to EMB agar. It has lactose to indicate lactose fermenters and basic fuchsin, which causes the colonies to appear red and/or the green sheen typical of coliforms. The media also contains ingredients that inhibit Gram-positive bacteria. Nutrients in the media diffuse into the membrane filter to support growth of organisms on the surface. If coliforms are present, the number of coliforms/mL in the original sample can be calculated.

1. Attach the Membrane Filter apparatus to the vacuum line.
2. Remove the lid and pour the remaining water sample (optimally 100ml) into the filter and turn on the vacuum line.

3. Once the water has been filtered turn off the vacuum line. Remove the filter by scoring around the edge of the filter with a sterile scalpel. Gently lift the filter with sterile forceps and place it onto the mEndo agar plate, right side-up.
4. Invert the plate and incubate at 35C for 24h.
5. Day two: Evaluate for the presence of coliforms. If coliforms are present and countable, quantify the amount (CFU/ml) in your original sample.

Results:

Water sample source: _____

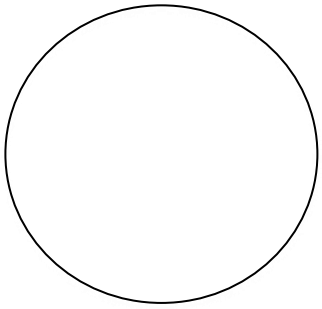
1. Presumptive test:

Inoculum	LB with 10ml	LB with 1.0 ml	LB with 0.1ml
# of tubes with gas			

Can you presume that there are coliforms present in your sample? _____

MPN (use the MPN chart): _____

2. Confirmed test: Draw and describe your observations on the EMB plate.



EMB Agar

If you had a positive presumptive test, did your EMB plate confirm coliforms? _____

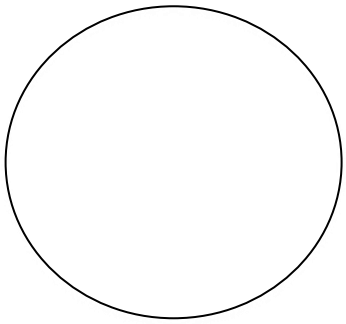
3. SPC:

A. # of colonies on plate:_____

B. Calculate the number of bacteria in the original sample:

4. Membrane Filter Technique

A. Draw and describe what you observe on your membrane filter/m-Endo agar plate:



m-Endo Agar

B. Number of coliforms: _____

C. Number of coliforms in your original sample/mL: _____

Show your calculation:

5. Class Results

Group	Water Source	MPN	EMB +/-	SPC	Mb Filter +/-
1					
2					
3					
4					
5					
6					

Questions:

1. Why is *E. coli* used as the test organism in water testing?

2. Why isn't it necessary to include a pH indicator in the lactose broth tubes?

3. What is the MPN chart based on?

4. We omitted the completed test. However, based on your results, what would you have expected to see in the completed test and gram stain?

5. Why would water samples for the Membrane Filter Technique need to be fairly clear and not turbid?

6. What tests that you did today were qualitative? Which were quantitative?

7. Can media be sterilized by filtration? Explain your answer.

Conclusions:

Reflect on these tests, your data. Discuss why coliforms are the bacteria chosen for water testing and discuss what the class's results indicate about the water samples tested.

References/Resources:

Santa Clarita Valley 2015 Water Quality Report
http://clwa.org/docs/wp-content/uploads/2015/06/2015-CCR-Water-Report-.pdf

Immunology:
ELISA-Simulation
StaphTEX-Agglutination Reaction

"In April 2013, the U.S. Preventive Services Task Force (USPSTF) recommended that clinicians screen adolescents and adults ages 15 to 65 years for HIV infection. Younger adolescents and older adults who are at increased risk should also be screened. They also recommend screening all pregnant women for HIV, including those who present in labor whose HIV status is unknown."[1]

Introduction:

Immunological diagnostic methods have become an essential, integral part of diagnosing disease. One can either look for the pathogen (antigen), or the patient's immune response to the pathogen (patient serum antibodies). These tests have become very specific and sophisticated technologies. Today there are "5th Generation" assays which are rapid, highly accurate molecular tests that test for and differentiate signals from both Ag and Ab.

The tests in today's lab are tried and true tests that, though older, are still used in clinical and research labs. Often ELISA and Agglutination tests are used as screening tests; if tests are positive then further testing is done.

Learning Objectives:

After this lab you should be able to:
1. Explain the immunological basis of the ELISA and Slide Agglutination tests.
2. Explain the procedures of the tests.
3. Interpret the results of a simple ELISA and the StaphTEX test.
4. Compare and Contrast the immunological mechanism of these tests.

A. ELISA: See handout for all instructions

B. StaphTEX-Agglutination Reaction

Introduction:

Rapid tests are often used in a doctor's office or clinic to identify bacteria. A very common one is a latex agglutination test for the causative agent of Strep Throat. A similar test, StaphTEX, can be done for *Staphylococcus aureus* a potentially serious pathogen. StaphTEX is also a latex agglutination test which looks for antigen (Ag)—the bacteria itself—in a patient sample. The test utilizes microscopic latex beads in a solution (blue in this case). Each latex bead is covered in antibodies (Ab) to the pathogen, and often other proteins. The StaphTEX beads are coated with both IgG Ab to *S. aureus* and the protein Fibrinogen. *S. aureus* produces Protein A which will attach to the IgG, and often also coagulase which affects fibrinogen. The latex beads have two ways then, of being affected by the *S. aureus.*

If *S. aureus* is present in the patient isolate an agglutination reaction occurs with the latex beads and will be visible as the precipitation of blue clumps. A negative test will not have clumping.

Materials:

Gloves
Hardy StaphTEX Blue Kit:
- Reagents
- Wooden Sticks
- Latex Agglutination Cards

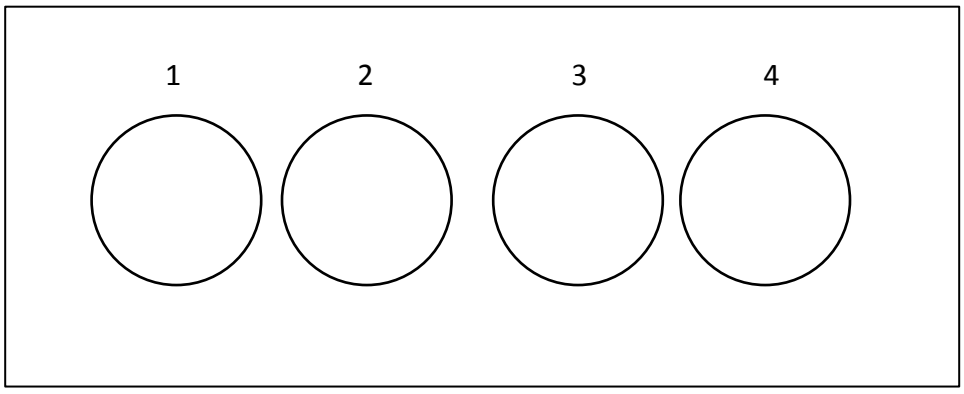
Disposable inoculating loops
Even and Odd numbered tubes of unknown bacterial cultures
StaphTEX product information sheet

Procedures:

1. Shake well, and then place a drop of the LATEX REAGENT in each of the first 4 reaction circles on the card.
2. Add 1 small drop of (+) control to the first circle. Mix into the Latex Reagent to the edges of the circle with the wooden stick.
3. Add 1 drop of the (-) control reagent to the second circle. Mix with the wooden stick.
4. Add one loop of growth (2 small colonies) from an EVEN numbered unknown culture to the third circle. Mix with the wooden stick.
5. Add one loop of growth (2 small colonies) from an ODD numbered unknown culture to the fourth circle. Mix with the wooden stick.
6. Gently rock the card back and forth for 20 seconds and read the reaction immediately. A positive reaction will have visible clumps in the mixture—it looks like "blue pepper." A negative test will be a uniform suspension.
7. Compare the unknowns to the controls to determine if an unknown is *S. aureus.*

Results:

1. Draw your reaction here, label each circle with the reagents/cultures mixed in each:



2.Record your results:

Unknown tube #	Agglutination Reaction (+ or -)	Positive ID of *S. aureus* ? (+ or -)	ID according to the instructor key: (name of organism)

Questions:

1. Explain how the StaphTEX test works.

2. How is this test different than looking for antibodies in the serum of a patient?

3. After reading the StaphTEX product information handout, what are some limitations of this test, and what might go wrong and result in an incorrect test?

Conclusion--ELISA and slide agglutination:

Compare and contrast the immunological mechanisms between the ELISA and Slide Agglutination tests

Resources:

1. "HIV Testing." cdc.gov. CDC, 30 June 2015. Web. 5 Nov. 2015.
http://www.cdc.gov/hiv/testing/
2. "StapTEX Blue Kit." Hardy Diagnostics. n.d. Web. 5 Nov. 2015.
https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/StaphTexBlueKit.htm

Microbescopes and Observation of Natural Samples

Microbes are ubiquitous.
A course in microbiology should convince you of this. However, most of the work done in an introductory microbiology course is in the laboratory, often with a slant toward the clinical world. Yet we know that microbes are found in all environments. So, let's go outside. Let's find some microbes around campus, grab some samples from natural sources outside the classroom and take a look.

Learning Objectives:

After this lab you should be able to:
1. Collect, observe, record, and identify microbes from natural samples.

Introduction:

View the Microbescope website to orient yourself and learn the basic steps of how to use the scopes. See handout.
http://www.microbescope.com/
Microbescopes were invented and funded through a kick-starter campaign. This site has photos and videos of the scope and some samples.
https://www.kickstarter.com/projects/1446839815/the-microbescope/video_share

Pre-lab questions:

After viewing the two sites above answer the following questions.

1. How much sample should one put on the lens (estimate "size")?

2. What do you need to do if you have a dry sample?

3. What is the total magnification achievable?

4. How does one view the specimen?

5. How does one capture images or video?

6. How can one increase depth of field and resolution?

7. How should you clean the lens and scope?

Materials:

Microbescope and phone stand
Smart phone or iPad
Transfer pipette
Spatula
Sample cups
DI Water
Alcohol wipes
Diffuser
Natural samples (students will collect these)

Procedures:

1. You will collect specimens in the sample cups. Look for:
-Soil
-Water
-Algae
-Lichen
-Other?
2. If the sample is dry you will need to add a small amount of water to the sample cup.
3. Reconvene with the class to view and image your samples.
    a. Turn on the scope.
    b. Place a very small amount of liquid on the lens.
    c. Take images/video.
    d. While observing and after imaging, attempt to ID the microbes that you see. Imaging tip: If you want a more magnified image or video, increase the magnification (zoom in) on your phone prior to taking the image or video.
4. Use the Protist handout to try and ID any Protists you may find. You may only be able to ID the Domain/Kingdom, but you may be able to ID it further.
5. Fill out the following chart.
6. Clean up the scope and return all parts neatly together. Use the wipes to GENTLY clean the lens.
7. Post image/video on CANVAS. Send images to partners without iPhones.

Results:

Sample type and location	Describe observation	Diagram	Organism Characteristics	Possible ID/Taxonomy	Image/video?

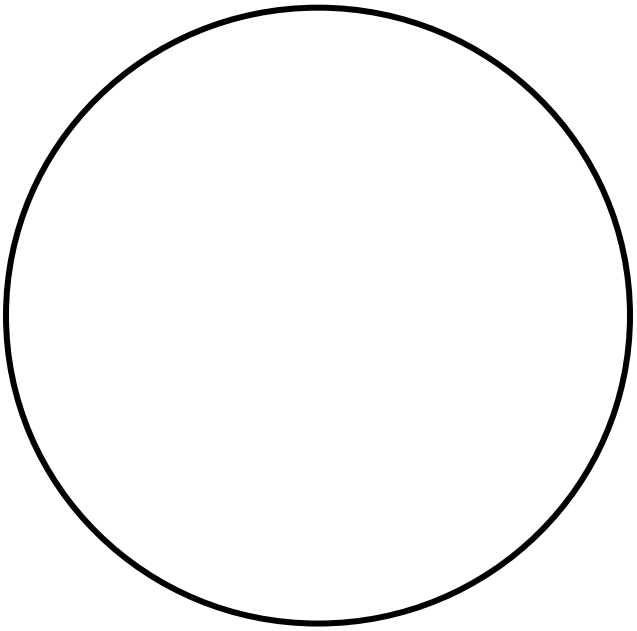
Sample type and location	Describe Observation	Diagram	Organism Characteristics	Possible ID/Taxonomy	Image/video?

Questions:

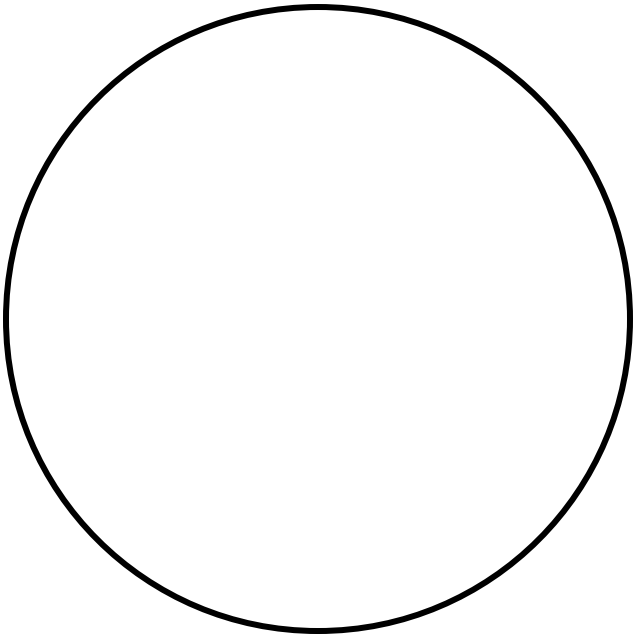
1. Did you have difficulty using the scope and/or finding any organisms? Explain.

2. Which did you observe--Bacteria, Protists, Fungal, Animal microbes (rotifers, etc.)? Explain.

3. Draw and describe two microbes that you observed and give their major characteristics, size estimate. (Base this on your experiences in lab and measurements. You may have to go back to you lab manual to look at your drawing/measurements), and a possible ID.



Description and ID:



Description and ID:

4. If you have an iPhone, were you able to record images or video?

Conclusion:

Reflect on what you observed. Discuss how this activity relates to ideas, topics, etc., that you have studied in this course.

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2017