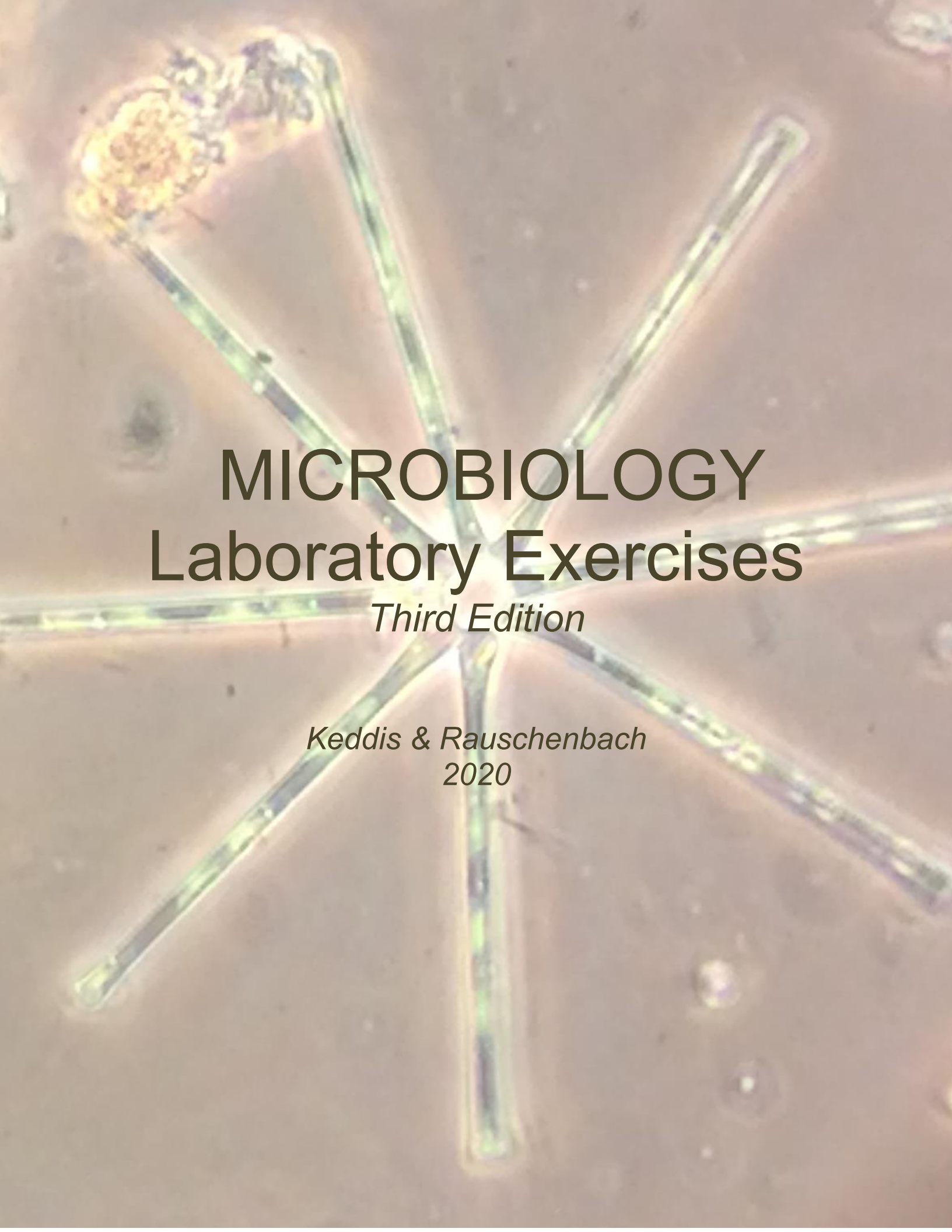

MICROBIOLOGY
Laboratory Exercises

Third Edition

Keddis & Rauschenbach

2020

Photo Credits (in order of contribution):
Diane Davis, Ines Rauschenbach & Ramaydalis Keddis

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Many thanks to those in the Department of Biochemistry and Microbiology, Rutgers University, who have through the years inspired our enthusiasm for the science and teaching of microbiology, with special thanks to Diane Davis, Douglas Eveleigh and Max Häggblom.

Safety:
The experiments included in this manual have been deemed safe by the authors when all necessary safety precautions are met. The authors recommend maintaining biosafety level 2 in the laboratory setting and using risk level 1 organisms for all exercises.

Microbiology Laboratory Exercises

Third Edition
2020

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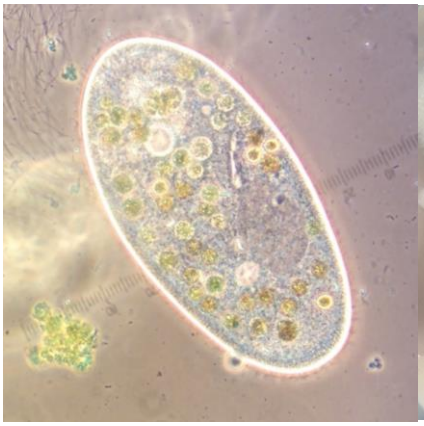
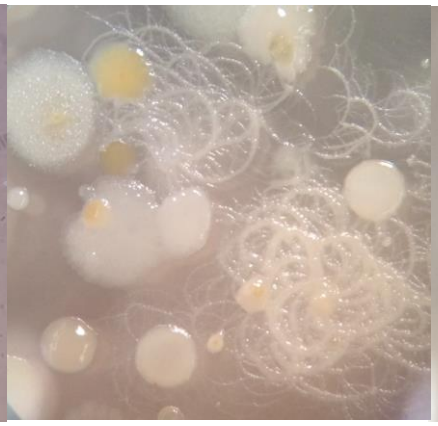

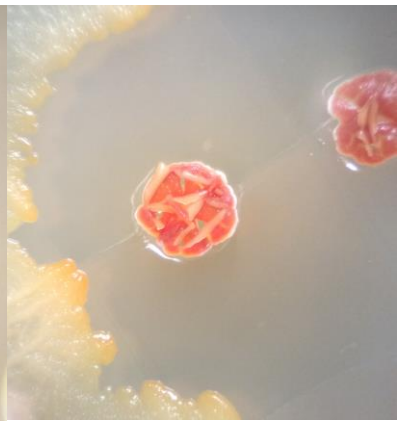

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LABORATORY SCHEDULE

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2	Follow Up: Culturing and handling microorganisms Isolation of a Pure Culture Counting bacterial populations: Plate counts
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4	Microscopy – Introduction Control Of Microorganisms – Lab Work
5	Microscopy – Phase Contrast (Yeast and Bacteria) Control of Microorganisms (Poster Work)
6	Microscopy – Phase Contrast (Unknowns) Control of Microorganisms (Poster Work) Selection of Fungi or Bacteria
7	Follow Up: Selection of Fungi or Bacteria Microbial Staining Control of Microorganisms (Poster Work)
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Best Laboratory Practices

The organisms used in General Microbiology are classified as **Risk Level 1** organisms by the ABSA (**American Biological Safety Association**) as they are not known to cause disease in healthy humans. You will learn the techniques of safe microbiology using risk level 1 organisms; however, we will maintain bio safety 2 standards when working with all organisms. The following list of best practices is printed here to remind you of the safety discussion presented during the first laboratory meeting.

- Do not bring food or drinks into the laboratory.
- No sandals – toes must be covered.
- No shorts or short skirts. Long pants or skirts must be worn at all times.
- Cell phones and other non-lab electronic devices are not permitted.
- Lab coats and safety glasses are required.
- Long hair must be tied back.
- Always use aseptic technique when working with organisms in culture.
- Wash your hands before leaving the laboratory.
- Place coats, books, and other personal possessions in assigned space.
- Cleanliness: Before beginning your laboratory work, wipe down the benchtop with a microbiocide (e.g. Konflict)
- Instructions for disposal will be provided during your lab period. Never throw anything down the drain. The following will help when you are unsure.

General Instructions for disposal:

- Microscope slides and cover slips go in the red “sharps” containers.
- Pour excess stains into labeled hazardous waste containers.
- Broken glass should be disposed off in the broken glass container (Figure I-1).

Figure I-1. Receptacle for broken glass.

Working in the Laboratory

Working in a microbiology lab is likely different from most lab work you have experienced. Some tools and equipment commonly found in microbiology laboratories are shown in Figures I-2 & I-3. The biggest difference you will notice is the use of aseptic technique when working with cultured organisms. Since most of the supplies we will be using have been sterilized either by autoclaving or gamma irradiation, sterility must be maintained. This requires a new set of lab practices. Besides the new lab practices and techniques there are many new terms to learn. Many of the terms (see Appendix B) in boldface in this introduction and throughout the manual are among those that are extremely important for you to be familiar with (understand completely and be able to describe) and may be new to you.

Most of the exercises and experiments that are performed in General Microbiology will extend over two or more weeks. This requires careful record keeping. It is a necessity since we are studying the characteristics of microorganisms and learning set of tools and techniques used to accomplish this. Generally, we will inoculate the culture media and the organisms will need to be incubated for 24 – 48 hours and then stored in the refrigerator until you return to lab the following week. This is not ideal! If you were working in the research lab you would be checking your results immediately after incubation.

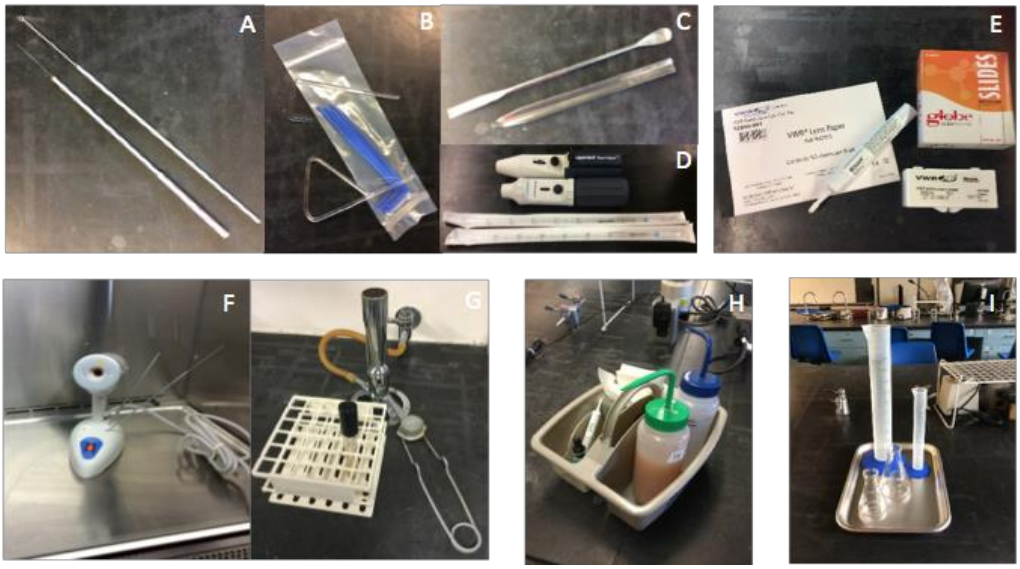


Figure I-2 Common Tools Used in the Microbiology Laboratory.

A Needle and loop
B Spreaders
C Spatulas
D Pipettes and sterile tips
E Lens paper, immersion oil, and slides
F Incinerator
G Bunsen burner, striker, and tube rack
H Caddy with disinfectant, water bottle, paper towels, lens cleaner, and oil
I Graduated cylinders and Erlenmeyer flasks

Figure I-3 Common Equipment Used in the Microbiology Laboratory.

A Biosafety Cabinet with Incinerator
B Water Bath
C Electrophoresis gel box and power supply
D Centrifuge
E Shaker incubator
F Incubator
G Light microscope

(1) PREPARATION OF A CULTURE MEDIUM

The study of microorganisms in the laboratory generally requires growing organisms on a nutrient medium. Culture media must meet the nutritional requirements of the organism you wish to culture. Minimal nutritional considerations include the macronutrients; carbon, nitrogen, and phosphorus, and various other minerals, such as iron and magnesium, in lesser amounts. In addition, most microbiological media incorporate buffers, weak acids or bases that resist changes in pH. Of course, all living organisms require water.

Many different types and formulations of media are used for growing bacteria and fungi in pure culture. There are also media specific for the selection and differentiation of a vast variety organisms. For our routine laboratory work, a complex medium (exact composition is not known) prepared from plant, fungal and/or animal extracts is appropriate to meet the nutritional needs of most of the organisms we study. Many different types of media are available commercially as pre-formulated dry powders to be reconstituted in the laboratory. Appendix C at the end of this manual describes all media used in General Microbiology.

A broth medium is one in which the ingredients are simply dissolved in water; a solid medium is usually prepared with agar, a seaweed polysaccharide that acts as a gelling agent. Agar is a superior gelling agent in microbiological media because of its unique physical properties. Once gelled, agar remains a solid up to 90°C (gelatin melts at 35°C). Solid agar does not melt until it is heated to 100°C; liquid agar does not solidify until it is cooled to about 42°C allowing liquid agar to be poured into Petri plates at a temperature relatively easy to handle (Figure 2.1). For most microorganisms, agar is an ingredient which they cannot use as a nutrient.

The media prepared in this exercise will be sterilized to kill microorganisms introduced during preparation and used in subsequent experiments during the course. Growth media are routinely sterilized in the autoclave by heating at 121°C under pressure (15 psi) for 15 minutes (Fig.1.1). The autoclave is also necessary, as discussed in the safety training, to kill cultures after use.

Figure 1.1 Left: Autoclave; Right: Bench top Sterilizer

MATERIALS

Dehydrated trypticase soy broth (BBL)
Dehydrated agar
Balance and weighing paper
Pipette and tips
Autoclave
Graduated cylinder (100ml)
Flask (250 ml)
Test tubes & caps

PROCEDURE

1. Work by bench-side. Each group will prepare 100 ml of the medium. This will be enough for making ~14 slants (7 ml per slant).
2. Measure 100 ml distilled water. Weigh out the dehydrated medium according to directions on the label but recalculated for the volume you are preparing. Pour the weighed media into the 250 mL flask. In addition to the medium, add 1.5% w/v dehydrated agar. Pour the water in the cylinder into the flask, Try to wash down the neck of the flask where powder may be sticking. Swirl the flask to suspend the ingredients; the TSB will dissolve rather quickly; but the agar will not dissolve until the medium is boiled in the microwave.
3. Heat the medium in a microwave oven - boil to dissolve the agar; use occasional light agitation. Watch it carefully. Take great care as the agar can boil over. After the agar has completely dissolved, cool the medium in a water bath to about 60°C.
4. To prepare agar slants, dispense about 7 ml of the melted cooled agar into clean tubes, using a Brinkman or Eppendorf pipette (See Appendix D). Cap the filled tubes with plastic caps.
5. Autoclave the medium 121°C, 15 psi for 15 min. (Autoclaving and slanting will be done for you after class.)

(2) CULTURING AND HANDLING MICROORGANISMS

Basic laboratory techniques in microbiology must be mastered not only by the microbiologists, but also by molecular biologists, geneticists, biochemists and many other scientists, as these techniques are used as tools in many disciplines. These techniques include the transfer of microbial cells from one medium to another without introducing extraneous species from the environment or contaminating the in a variety being studied (aseptic technique); of formats. The preparation of culture media that will support the growth of the microorganisms being studied; and the isolation of pure cultures from mixed populations and their maintenance in the laboratory (Figure 2.1 and Table 2.1).

Figure 2.1 Assortment of Media

Table 2.1 Overview of media formats.

Media type	Description
Liquid Medium	
Broth	Any liquid medium dispensed in a tube or flask, used for growth of a variety of organisms
Solid Media	
Deep	Provides little surface for growth, must be stabbed for inoculation; used for testing oxygen limitation on bacterial growth
Slant	Maintenance and storage of pure cultures
Plate	Provides a large surface area for growing bacteria but dries out fairly rapidly; commonly used for the isolation of individual colonies

In this exercise you will accomplish aseptic culture transfer. Aseptic technique requires working in a different way; for most students, General Microbiology Lab is the first time they have worked aseptically in the lab. The cardinal rule for working aseptically is – **Don't open anything until the instant you need it & and don't leave it open longer than absolutely necessary.** You will use a sterile inoculating needle to transfer cells from a pure bacterial culture to the surface of an agar slant, establishing a fresh culture of cells on a new culture medium.

You should be able to accomplish this simple task without introducing, into either the old or the new culture, contaminating species of bacteria or fungi. Aseptic technique also protects the work space and environment around the workspace from contamination by the organism being transferred.

MATERIALS

Various slant cultures

1 TSA slant per student, Bunsen burner/Striker, Test tube rack, Inoculating needle

PROCEDURE

There are numerous methods to accomplish the transfer of microorganisms. The following method is generally easiest for beginning students. Before you get started, be sure to watch the microlab clip on Canvas about culture transfer. Whenever you make an aseptic transfer in lab you will use a Bunsen burner. The flame of the Bunsen burner is used to sterilize equipment and also provides convection currents that keep the air near your work free of spores and bacteria. You should always work near your own burner flame for this reason as well as the safety concerns of reaching from a distance to flame your needle.

1. Sterilize the inoculating needle in the flame of a Bunsen burner. Hold the needle nearly vertically and heat the entire length of the wire portion of the needle until it glows red. Allow 10 seconds (count to 10 slowly or quickly to 20) for the wire to cool before proceeding. Do not wave the needle around in the air to cool.
2. Hold the culture to be transferred in the left hand (for a right-handed person) and remove the cap using the last two fingers of the right hand, the same hand in which you are holding the sterile needle. Never put the cap down on the bench. Note: We do not flame the open tube either before or after the culture sample is removed. This flaming is historic, a reflection of times when cotton plugs were used (Figure 2.2). There is still debate on this topic (1)

Figure 2.2 Cotton-Plugged Tubes

3. Insert the needle and touch the medium to ensure that it is cool, then with the needle, remove a **small** amount of the surface growth from the slant. Do not gouge the agar. Replace the cap on the culture tube and put it back into the test tube rack. Pick up the fresh agar slant and follow the same procedure to aseptically remove the cap from the tube.
4. Evenly streak the surface of the new slant from the bottom upward, with a back and forth motion. Replace the cap on the tube and flame the inoculating needle to kill any residual inoculum before putting it down.
5. Label this tube appropriately and make sure to record the name of the culture transferred in your notebook. Incubate the culture @ 35°C. The culture will be removed from the incubator after ~24 hrs. and refrigerated until the next laboratory period.

WEEK TWO

1. Examine the growth on your slant macroscopically. Describe any growth in your notebook. Do you assess your culture transfer to be successful?

(3) ISOLATION OF A PURE CULTURE

The microorganisms found in any environmental niche are a diverse group consisting of several to many species. Before studying the characteristics of any one of the species in the laboratory, it is often desirable to isolate that species as a *pure culture*. A pure culture contains only the progeny of a single microbial cell, in contrast to a *mixed culture* which may contain several different species and strains. The *quadrant streak plate* (or isolation streak plate or QSP) technique is a convenient method for obtaining an isolated colony that can be used to generate a pure culture from a mixed culture. In this method, an inoculating loop (for a liquid culture) or needle is used to streak cells from a mixed population across an agar surface. Large numbers of cells are deposited along the streak lines initially; as the number on the loop thins out the cells deposited become isolated (separated) from each other. Each *isolated colony* that subsequently grows is assumed to have developed from a single, isolated cell. The isolated colony on the plate is not a pure culture as it has not grown in the absence of other cells types. Figure 4.1 shows successful QSPs; Figure 3.2 shows unsuccessful QSP attempts. Why do you think they were successful/unsuccessful?

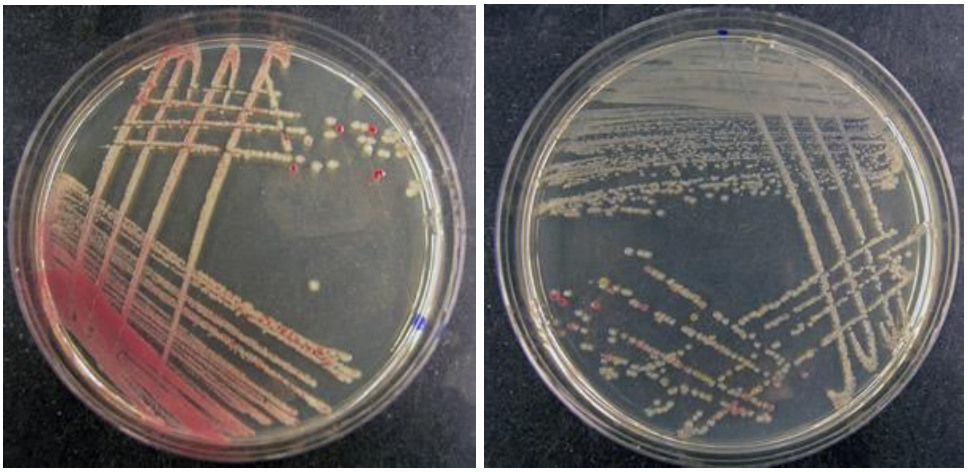


Figure 3.1 Expected examples of successful QSPs.

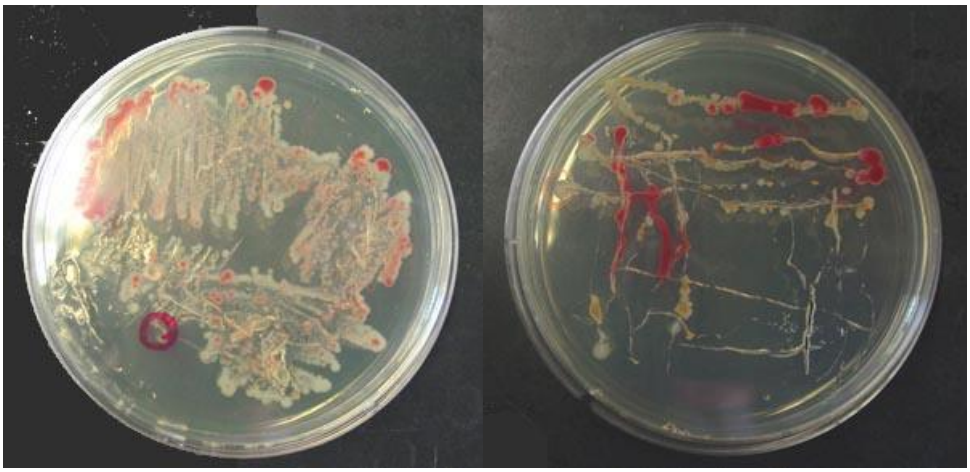


Figure 3.2 Unsuccessful QSPs.

To obtain a pure culture, cells are picked from an isolated colony with an inoculating needle and transferred to a fresh culture medium. Cells that grow on the new medium are a subculture of the cells from the original isolated colony. Subcultures are used for subsequent study of the isolated organism. Contaminating organisms from the original culture remain on the streak plate.

In this exercise you will use the quadrant streak plate technique to isolate a pure culture from a mixed culture containing four different bacterial species. The culture that you isolate and establish as a pure culture will be used in later exercises to demonstrate methods for identifying unknown microorganisms.

MATERIALS

Week 1
A mixed broth culture containing three to four unknown organisms
One plate of TSA, Bunsen burner/Striker, Test tube rack, Inoculating loop

Week 2
1 TSA slant per student, Bunsen burner/Striker, Test tube rack, Inoculating needle

PROCEDURE – WEEK ONE

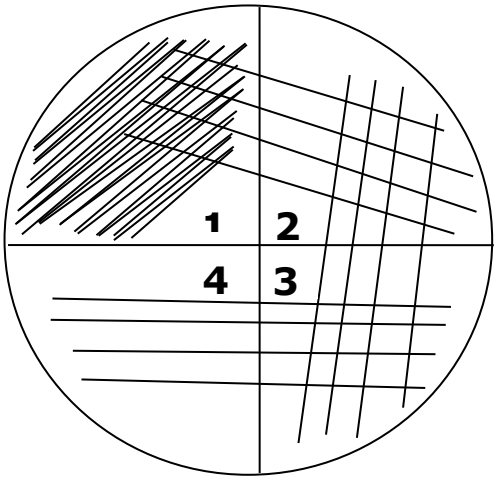


Figure 3.3 The quadrant streak plate method.

1. Use your sharpie and divide your Petri dish into four quadrants. Number each quadrant 1 – 4. (Figure 3.3) Label your Petri dish. Please be sure to also review the micro lab clip on Canvas about the proper QSP procedure.
2. Sterilize the inoculating loop in the flame of the Bunsen burner and cool. Either quickly count to 20 or slowly to 10. Remove a loopful of the mixed culture to the agar surface and inoculate this area with a continuous back and forth motion as if you were trying to rub off all of the cells clinging to the loop.
3. Flame the loop to sterilize and cool. Touch the loop to a clean spot on the agar plate to assure that it is cool. Streak through the area in the inoculated sector and into the next sector four times. Use straight lines making sure to lift the needle before each subsequent streak line.
4. Repeat as in step 3 until the four sectors on the plate have been inoculated. The idea is to streak so that each succeeding sector contains fewer cells than the preceding one, resulting in at least one sector with cells spaced far enough apart to allow isolated colonies to develop.
5. Label the agar side of the plate; incubate the cultures, agar side up, @ 35°C. The plates will be removed from the incubator after 24 hrs and refrigerated until the next laboratory period.

WEEK TWO

1. Examine the pattern of growth to confirm that the procedure was followed correctly, and that isolated colonies were obtained in one of the sectors. If you failed to get isolated colonies, streak another plate.
2. Transfer cells from an isolated colony to the agar slant provided. Incubate @ 35°C for 24 hrs and refrigerate until the next laboratory period. Save your slant in the refrigerator since it will be used as your unknown organism for identification in later exercises. Save the original plate (in the refrigerator) until the next class period.

(4) COUNTING BACTERIAL POPULATIONS

One of the most common procedures for determining the number of bacteria in water samples is the pour plate method. In the pour plate (plate count) method, serial dilutions of a sample are made and portions of the dilution are "poured" onto a suitable agar growth medium. For the streak or spread plate method, the cells are diluted and then spread onto the medium. For both, the cultures are incubated under conditions that permit colonies to develop. It is assumed that each colony develops from a single cell. By counting the number of colonies and taking into account the dilution factor, the number of bacteria in the original sample can be determined.

Because the number of bacteria in a sample is normally large (10^5 - 10^{11} cells/ml), it is almost always necessary to prepare a series of dilutions, and plate samples from several of the dilutions. In this way, plates that contain a reasonable number of colonies can be obtained. By statistical analysis, this reasonable number is established to be in the range of **30 - 300 colonies** per plate. In practice, ten-fold dilutions are made, usually from 10^1 to 10^8. The number of dilutions necessary to achieve a countable sample can be reduced depending upon what is known about the sample. For example, water that is not visibly turbid will carry less than 10^7 bacteria per ml so the highest dilution necessary in such a sample would be ~10^5. Any approximation of population density can help to reduce the number of dilutions necessary. Estimating the cell concentration as a function of optical density is a common starting point for plate counts.

In the pour and spread plate methods only viable cells are assessed. Bacterial cells in the diluted sample are mixed with liquefied agar so cells are distributed evenly throughout the medium before the agar solidifies.

MATERIALS

E. coli sample
Sterile test tubes
1 bottle sterile buffered water, about 150 ml
1 bottle of TSA per 2 students (@ 50°C)
Pipette, micropipettes, and sterile tips
3 sterile Petri dishes

METHODS

1. As a lab, you will be given the $O.D._{660}$ of an *E. coli* culture. Your instructor will show you how to approximate the number of cells based on the turbidity of your culture. Use this approximation for working out a dilution scheme. Work with your neighbor and think out an optimal dilution scheme. Ideally, one of your plates will have about 30-300 cells/plate; one plate will have 10 fold more cells and one 10 fold less**.** Plating these three dilutions should assure a countable plate, remember, the O.D. is only an approximation that helps determine a starting point. **Please be mindful of the amount of materials (test tubes, pipettes etc.) that you will be using. You want to keep it to a minimum.** As a class we will discuss whether your dilutions schemes are appropriate.
2. Prepare your dilutions. Aseptically dispense buffered water into the test tubes. All work must be done aseptically. Make sure to vortex before making the next dilution.

3. Label the bottom of the Petri dishes with your name and the dilution to be counted. Dispense the appropriate volume (1.0 ml) of the dilutions you are counting into the bottom of sterile Petri dishes. Aseptically, dispense agar (melted and held at 50°C in the incubator) into the Petri dish as follows. Anytime you are working aseptically, you need to work near a Bunsen burner. Never share a Bunsen burner with someone else, as this will not allow you to work close enough to the burner to take advantage of the convection currents it generates.
3. Before you start, make sure to watch the micro lab clip on Canvas to see the demonstration of the technique. The procedure is as follows: lift the cover of a Petri dish and **aseptically** pour the liquefied agar into the bottom section of the dish. Replace the lid as soon as the agar has been poured. Repeat the procedure with the second medium. Allow about 5 to 8 minutes for the agar to solidify.
4. Gently mix the agar with the dilution sample by swirling gently in a "Figure 8" pattern as demonstrated by your instructor, and let the agar harden. Incubate the cultures by placing the plates, agar side up, in the bins provided. The cultures will be incubated at room temperature for three days and refrigerated until the next lab period.

WEEK TWO

1. To determine the number of cells in the original culture, select the plate that has between 30 and 300 colonies; multiply the number of colonies by the dilution factor for that plate. Plates with more than 300 colonies are recorded as Too Numerous To Count (TNTC).

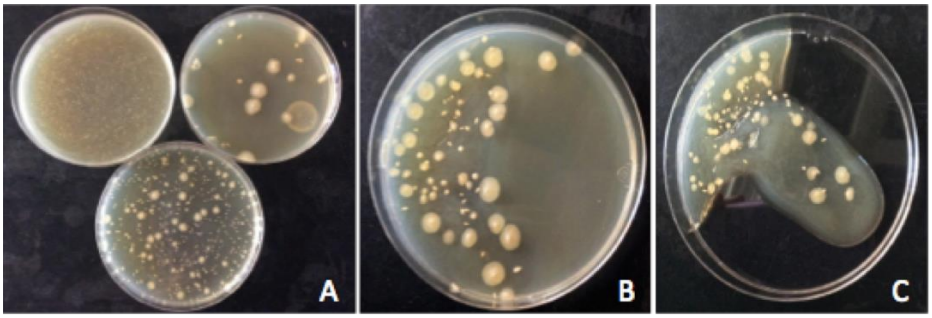


Figure 4.1 Dilution Plates. A: Successful dilution series; B: Unsuccessful, the culture was not mixed properly before the agar solidified; C: Agar was not distributed properly in the Petri dish before it solidified.

(5) CONTROLLING MICROBIAL GROWTH

We will be working in the groups for this exercise. Each group will select one of the following microbial control agents: antiseptics, disinfectants, antibiotics, hand washing or UV light. As a group, formulate an original testable hypothesis, using the methods in the manual as a guide to design your experiment. Groups will perform the experiment (make sure you include controls) deciding on incubation temperature and time, examine results and draw conclusions as a group. The collected information will then be compiled in a poster and presented at the end of the semester.

CHEMICAL METHODS TO CONTROL MICROBIAL GROWTH

5-1 DISINFECTANTS AND ANTISEPTICS

Chemicals that kill microorganisms or prevent the growth of microorganisms are called antimicrobial agents. They are widely used in various industries including, for example, health care settings and food service. Antimicrobial agents are found in a broad range of consumer products from mouthwash to cereal boxes and beyond. Concentration and contact time are the critical factors determining the effectiveness of an antimicrobial agent against a given microorganism. Microorganisms vary in their sensitivity to antimicrobial agents. The activity of many antimicrobial agents is aimed at blocking active metabolism and preventing the organism from generating the macromolecular constituents needed for reproduction. Because resting stages such as spores are metabolically dormant, they are not affected by such antimicrobial agents. Similarly, viruses are more resistant than other microorganisms to antimicrobial agents because they are also metabolically dormant outside host cells.

Antimicrobial agents are used in a wide variety of applications and are classified according to their application and spectrum of action. **Germicides** are chemical agents that kill microorganisms. Such chemicals may exhibit selective toxicity. Depending on the target organisms they are classified as viricides (viruses), bactericides (bacteria), algicides (algae), or fungicides (fungi). Whereas germicides kill growing microorganisms, “static” agents inhibit the growth of microorganisms but do not kill them i.e. when a bacteriostatic agent is removed, bacterial growth resumes.

Disinfectants are used on surfaces and equipment but not biological tissue. They can be either the germicidal or germistatic agents. Household cleaning agents often contain disinfectants to control the growth of microorganisms. Ammonia and bleach (hypochlorite) are widely used disinfectants. In general, the agents that oxidize biological macromolecules such as hypochlorite are effective disinfectants. Antiseptics are similar to disinfectants but may be applied safely to biological tissues. These substances are used for topical (surface) applications (e.g. on the skin) but are not necessarily safe for consumption. Alcohol (70%) is effective in reducing the numbers of microorganisms on the skin surface and is probably the most widely used antiseptic. It may be used on the skin in the area of a wound as well as for the disinfection of various contaminated objects. Alcohol may denature proteins, extract membrane lipids, and/or act as a dehydrating agent, contributing to its effectiveness as an antiseptic. Even viruses are inactivated by alcohol. Soap

and water reduce the number of microorganisms on the skin but are not as effective as antiseptic agents. Iodine is another effective antiseptic agent, killing all types of bacteria including spores. It is frequently applied to minor wounds to kill microorganisms preventing infection. Various dyes used in selective media, such as crystal violet, are similarly used as antiseptic agents. Such dyes are normally effective bactericidal agents at concentrations of less than 1:10,000. Dilute solutions of heavy metals are also used in antiseptic formulations. Mercuric chloride, copper sulfate, and silver nitrate are examples of heavy metal containing compounds that are used to kill microorganisms. Silver nitrate is applied to the eyes of newborn human infants to kill possible microbial contaminants and is particularly important in precluding the transmission of gonococcal (gonorrhea) infections from an infected mother to the infant's eyes. Both cationic and anionic detergents are also used as antiseptics. Detergents containing quaternary ammonium salts are effective germicidal agents. In this exercise, you will examine the effectiveness of various products that are marketed as antiseptics and disinfectants.

Figure 5.1 Examples of common antiseptics and disinfectants available. Left and center: Antiseptics Right: Disinfectants

MATERIALS

Broth cultures of your choice
Petri plates of nutrient agar (NA) or other general growth medium
Forceps
Cotton swabs
70% alcohol
Glass spreading rod
Sterile filter-paper disks
Ruler in millimeters
Various antiseptics and disinfectants

PROCEDURES

1. Uniformly "swab inoculate" the **entire** surface of the Petri plates with **one** of the broth cultures. This may be accomplished by swabbing to cover one-half of the plate area, rotating the plate 90°, swabbing over the initial inoculum with the same swab and covering one-half of the plate area, rotate the plate 90°, following the same procedure, and rotate the plate one more time repeating procedure. You will be swabbing **4** different times.

2. Label the **agar side** of the Petri plates with the name of the microorganism being tested. Indicate which substance is being evaluated for each disk placed on the agar surface.
3. Using sterile forceps, dip a sterile filter disk into one of the solutions provided and place the saturated disk onto one of the inoculated agar plates. (If there is an excess of solution on the filter paper disk, drain it on a paper towel prior to placing on the agar.)
4. Repeat this procedure for all solutions being tested.
5. Incubate the cultures for 24-48 hr.

WEEK TWO

1. Examine the plates for clear areas (zones of inhibition) in the lawn of confluent bacterial growth surrounding the disks. Measure and record the sizes of the zones of inhibition.

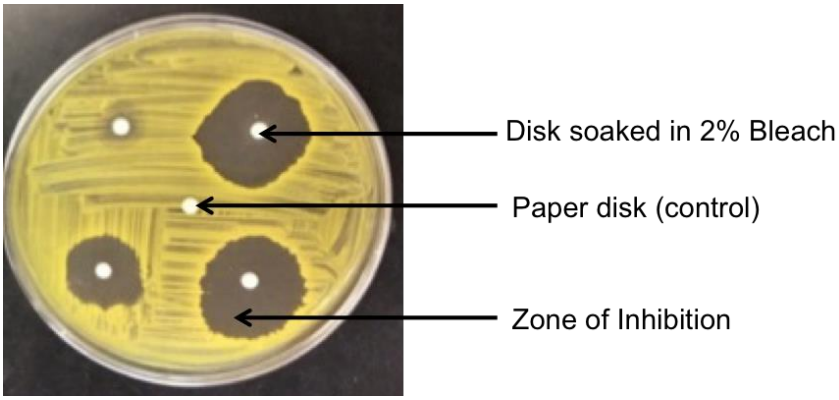


Figure 5.2 Example plate showing zones of inhibition.

5-2 ANTIMICROBIAL AGENTS USED *IN VIVO*: SUSCEPTIBILITY TESTING

Resistance of microbes to the effects of antimicrobial agents is a serious problem in the world today. In 2014, the World Health Organization Global Surveillance Report (2) stated the problem is “so serious that it threatens the achievements of modern medicine. A post-antibiotic era – in which common infections and minor injuries can kill – far from being an apocalyptic fantasy, is instead a very real possibility for the 21st Century.” Monitoring the susceptibility/resistance of microbes has become critically important.

The Kirby-Bauer method (3) is the classical method for determining the susceptibility of an organism to an antimicrobial agent. An agar plate is inoculated with the culture to be evaluated. The antimicrobial agent is allowed to diffuse into the agar medium, usually from a filter paper disc. The entire surface of a Petri dish is swab inoculated with the test organism to create a “lawn” of growth. A filter paper disk impregnated with an antimicrobial agent is applied to the surface of the inoculated agar plate. The antibiotic diffuses radially from the round filter paper into the agar: the concentration decreases as a function of the square of the distance from the disk. At some distance from each disk, the antimicrobial agent is diluted to the point that it no longer inhibits microbial growth. The effectiveness of a particular antimicrobial evidenced by the presence of growth inhibition-zones (Fig 5.3). These

zones of inhibition appear as clear areas surrounding the disks. The diameter of the zones is measured, and the relative efficacy of the antimicrobial agent is determined by comparing measured zone sizes to standards. The relative effectiveness of different antibiotics provides the basis for the sensitivity spectrum of the organism. The disk diffusion method represents a simple procedure for screening substances to determine if they have significant antimicrobial activity.

The relative sensitivity of the organism is one factor considered in selecting antimicrobial agents for treatment. A number of experimental variables including viscosity of the culture medium, solubility of the antimicrobial agent etc. influence the size of the clearing zone. Many antimicrobial compounds produce adverse effects that must be considered when prescribing chemotherapeutic agents.

In this exercise, you will test the susceptibility of bacteria to a number of antimicrobial agents. Although we tend to think of antibacterial agents as antibiotics, this is not the proper terminology. In 1947, Selman Waksman (4) defined antibiotics as "chemical substances that are produced by microorganisms and that have the capacity, in dilute solution, to selectively inhibit the growth of and even to destroy other microorganisms." Antibiotics are then, by definition, natural products affecting any other microbe. The term "antimicrobial agent" broadly refers to any compound, natural or synthetic, that inhibits the growth of a microbe (bacterial, fungal, viral, protozoal etc.) Please use these terms correctly.

MATERIALS

Cultures of your choice
Filter disks containing a number of different antimicrobial agents/various dosages
Ethanol
Mueller Hinton Agar Plates, 150 mm in diameter
Ruler graduated in millimeters
Tweezers

PROCEDURE – WEEK 1

1. Uniformly "swab inoculate" the **entire** surface of the Petri plates with **one** of the broth cultures. This may be accomplished by swabbing to cover one-half of the plate area, rotating the plate 90°, swabbing over the initial inoculum with the same swab and covering one-half of the plate area, rotate the plate 90°, following the same procedure, and rotate the plate one more time repeating procedure. You will be swabbing 4 different times.
2. Aseptically place antimicrobial disks on the culture plates. Using sterile forceps place the antimicrobial disks on the surface of the agar plate inoculated with the test organism; space them in equal sectors such that no more than 8 disks are placed on a plate.
3. Incubate at 30°C for 24h.

WEEK TWO

1. Measure the zones of inhibition for each antimicrobial agent for each of the cultures. Record your results.
2. Compare your results with standards (Thermo Fisher for product insert from the Clinical Laboratory Standards Institute document M100-S23) provided for each antimicrobial agent and determine the sensitivity, resistance, or intermediary relationship of each organism to each antimicrobial agent.

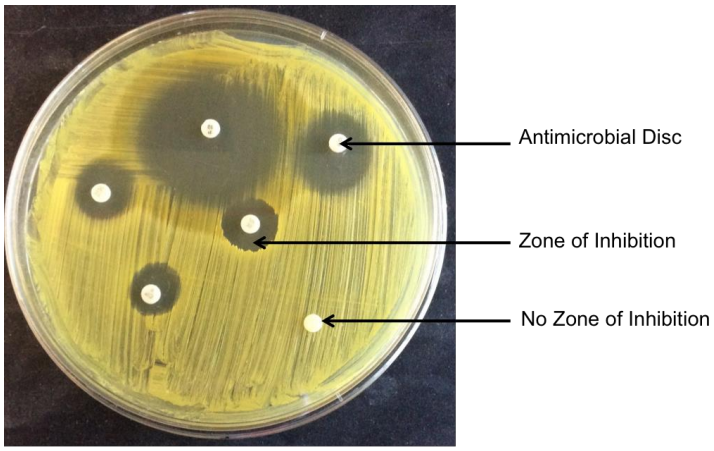


Figure 5.3 Mueller Hinton Agar Plate Showing Antimicrobial Zones of Inhibition.

5-3 HANDWASHING

Prior to the discovery of microorganisms, little attention was paid to handwashing and other routine methods of cleanliness that are commonplace today. In the mid-nineteenth century, the relationship between handwashing by obstetrical workers and the development of puerperal fever after delivery was recognized. When the obstetrical staff orderlies washed their hands in hypochlorite, there was a tenfold decrease in the rate of puerperal fever (other doctors working in a different ward ignored this result!). Today we routinely wash our hands and surgeons thoroughly scrub before an operation. Washing removes transient microorganisms that accumulate on the skin surface and also removes excess populations of the normal microbiota that are indigenous to the skin.

In this exercise, you will determine the effectiveness of handwashing in removing microorganisms from the skin. We will be using RODAC (Replicate Organism Detection and Counting) plates. These plates are specifically suited to testing surfaces as they have a positive agar meniscus as they are slightly over-filled.

MATERIALS
Soap
Water
Sterile swabs
Trypticase soy agar plates (RODAC (Replicate Organism Detection and Counting) plates)

PROCEDURES – WEEK ONE

1. Each student in the group will be assigned a different handwashing product.
2. Each student will then place his or her **unwashed** hand on a Rodac plate (i.e. inoculate the plate with the palm of your hand).
3. Hands will then be washed with ONE of the soaps and antimicrobial products provided and rinse your hands with tap water (you decide the time frame).
4. The washed hand (use the one that previously placed on the plate) will now be applied to a second plate.
5. As a control, swab a plate with tap water, using a cotton swab.
6. Incubate the cultures at 35˚C for 48 hr.

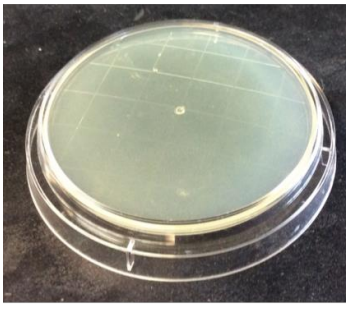


Figure 5.4 Rodac Plate

WEEK TWO

7. After incubation, observe the agar plates and record the density of bacterial growth according to your experimental design. If individual colonies develop, count the number of colonies and record the number.

PHYSICAL METHODS TO CONTROL MICROBIAL GROWTH

5-4 ULTRAVIOLET LIGHT

Ultraviolet radiation has a lethal effect on microorganisms. Its penetrating power, however, is less than gamma radiation (as UV is longer wavelength electromagnetic radiation than gamma rays). In spite of its limited penetrating power, UV radiation is useful and is used primarily for sterilizing air entering hospital operating rooms, and air and surfaces such as table tops in transfer rooms. The greatest germicidal activity of ultraviolet radiation is at the wavelength of 260 nm, the absorption maximum of deoxyribonucleic acid (DNA). The principal mechanism by which UV light exerts its lethal effect is by damaging the DNA molecule. UV radiation causes the crosslinking of adjacent thymine bases to yield thymine-thymine dimmers, which inhibits replication of DNA. Microorganisms vary in their susceptibility to the lethal effects of UV radiation. Some bacteria have very efficient repair mechanisms that are responsible for specifically repairing the DNA damaged by UV light. Furthermore, UV radiation is relatively ineffective against bacteria such as *Bacillus* and *Clostridium*, which can form endospores (resting cells) in which DNA is not synthesized. An alternative defense is of pigments that protect organisms from the effects of radiation.

MATERIALS

Trypticase soy agar plates (TSA)
3 x 5 index cards
UV lamps
Broth cultures of your choice

PROCEDURES

1. Turn on the UV lamp and allow it to warm up for about 10 minutes. **UV radiation can cause severe damage to the eyes,** at all times wear protective glasses when you are working with UV lamps.
2. Uniformly swab the entire surface of a TSA plate with one organism. This may be accomplished by first streaking with a cotton swab in one direction and then rotating the plate and streaking at 90^{o} to the initial streaking. Repeat this procedure with another organism (or two, depending on your experimental design) on another Petri dish.
3. Place under the UV light. **Remove the cover of the Petri plate.** Place an index card over the "0-time" section as shown. Push the index card so as to cover the next section of the plate at the timed intervals. Continue this procedure, by pushing the index card over the Petri dish to result in exposure times of 0s,10s, 20s, 30s, 1m, 2m.
4. Incubate according to your experimental procedure.

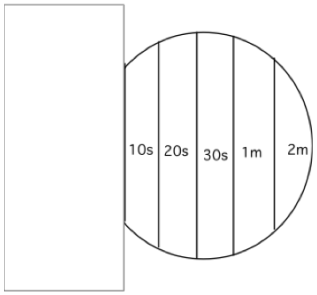


Figure 5.5 Marked TSA Plate

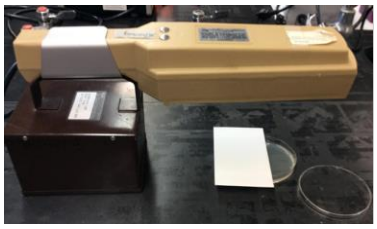


Figure 5.6 UV Light

WEEK TWO

5. Record your observations.

(6) SELECTION OF FUNGI FROM AIR

In this exercise, you will exploit the ubiquity of microorganisms in the air, to demonstrate the principle of selection. Microbiologists use selective media to favor (or select for) the growth of one group of organisms over other organisms that might be present. Selection is an important concept that we will revisit many times during the semester. **The selective media that we will be using is Mycophil**. This medium is selective by pH, it has a pH of ~5. We will also use **TSA (tryptic soy agar), a general-purpose, non-selective medium** that will allow growth of many different organisms. The pH TSA is ~7.

You will expose the agar surface to the air (for approximately 5 min) in an area outside the building. Some of the organisms in the sampling area will settle on the surface of the agar during the sampling time. Most of the organisms floating in the air outside will have come from the soil. The cultures are then incubated at room temperature until the colonies become visible. If the colonies are spaced apart from one another, we assume that each colony consists of the progeny of a single cell – or, more correctly, a single "colony forming unit" (CFU) since colonies may develop from a cell, a spore or even a bit of fungal hyphae. Hyphae (singular – hypha) are the threadlike filaments that form the fungal mycelium.

The microorganisms that grow on the plates under the conditions used in this exercise will be either bacteria or fungi. The basis for selection in our experiment is pH. Generally, fungi will grow at a pH slightly on the acidic side of neutral whereas bacteria generally favor a neutral pH. The population of organisms in the air is quite variable depending on place, time, and weather (we see a big difference between fall and spring semesters). However, as part of our experimental methods, we assume that the same population of organisms fall on each plate at any given time, place, or weather condition. You will use aseptic technique to pour the prepared media into Petri plates. Aseptic technique requires working in a different way in the lab than you may be used to. The cardinal rule for working aseptically is – don't open anything until the instant you need it and don't leave it open longer than absolutely necessary.

The first thing you will do is to pour growth medium containing agar, in addition to nutrients, into Petri dishes. Agar is a gelling agent; it provides solid support for bacterial growth when a solid medium is desired. Agar is a polysaccharide (the basic carbohydrate building block is galactose) derived from seaweed. Although it is used primarily for microbiological media (certainly this is true in the U.S.), agar is used as a food gelling agent in many cultures – it is "vegetarian JELLO" (gelatin, the solidifying agent in JELLO is an animal product). Primarily two properties of agar make it a more suitable gelling agent for most microbiological purposes than gelatin.

1. Most microorganisms can't utilize the polysaccharide in agar as a carbon source. What do you think would happen to the solid medium if the organism growing on it could use the agar as a food source?
2. The physical properties of the agar polymer make it very handy to work with. As you will see when you make TSA in lab this week agar is purchased as a dry powder. This powder will not go into solution until the medium is heated to boiling (100°C). The medium will remain a liquid until it cools to ~42°C. This

allows you to pour it into which ever format you desire (Petri plates, test tubes etc.). Once the agar solidifies it remains a solid until it is heated to 100°C. This means you can incubate cultures at elevated temperatures and the culture medium will remain solid. This wouldn't work with gelatin.

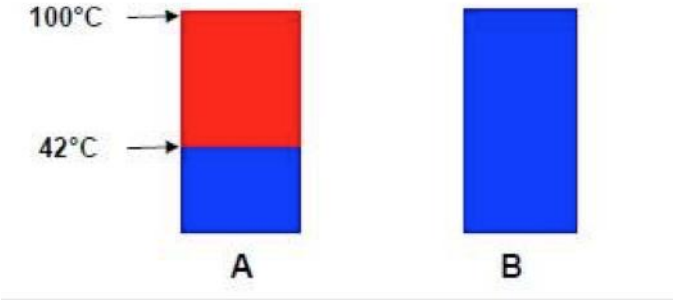


Figure 6.1 Physical Characteristics of Agar

A. If agar starts out at 100°C it is a liquid; once cooled to 42°C it becomes solid. Agar remains solid until heated to 100°C.
B. If agar starts out at say room temperature (~25°C) it will be a solid and remain solid until heated to 100°C.

MATERIALS

Tryptic soy agar (TSA) agar medium (pH ~7.0); Mycophil (pH ~5); 2 sterile Petri dishes

PROCEDURE

1. The agar media have been melted and placed in the incubator (55°C).
2. Aseptically pour each medium in an appropriately labeled, sterile petri dish.
3. Select a sampling area (outside the building). The area underneath a bush will generally yield a nice variety of microorganisms. Remove the lids from both Petri dishes to expose the surface of the agar medium for about 5 minutes. Both dishes should be exposed in the **same** area for the **same** length of time. **It is important to carefully note the time of exposure and where you exposed your plates. Choose a spot with a location that is easy to describe accurately.** After exposure, replace the lids. Incubate the cultures at room temperature in the bins provided. Place Petri plates agar side up. This prevents condensation on the lid from dripping onto the agar surface that causes dispersal and coalescence of the cultures.

WEEK TWO

1. Examine the plates for the number and type of colonies. Describe some interesting colonies by using the colony morphology guide. Typical results are shown in Figure 6.2. You will need to include this digital image of your plates in your formal lab report.

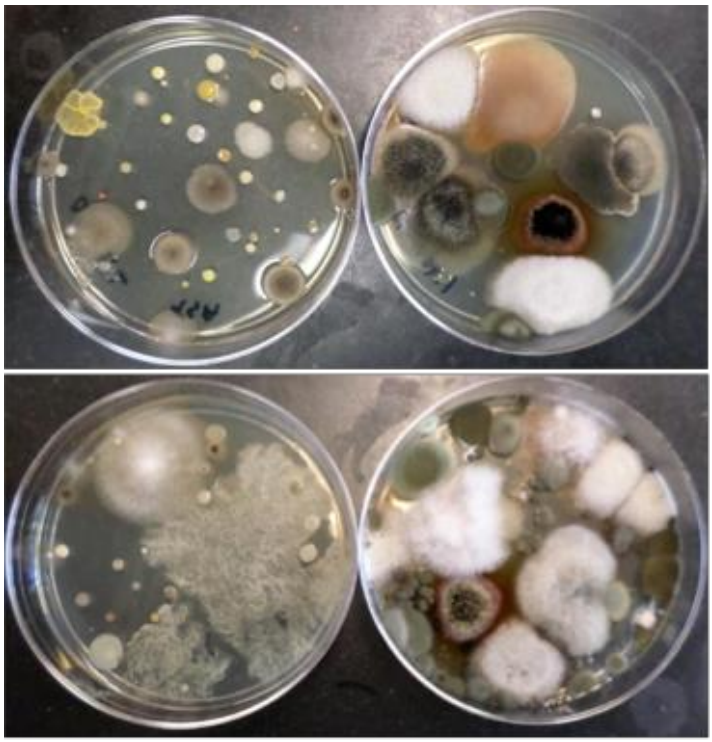


Figure 6.2 Typical Experimental Results. Left: TSA (pH7); Right: Mycophil (pH5)

2. We will use differences in colony appearance to distinguish between fungal and bacterial colonies. Can you distinguish fungal colonies from bacterial colonies in the images above (Figure 6.2)? To tabulate the number of colonies of each (fungal and bacterial), we will start with the **assumption** that the colonies that are round, shiny and smooth are bacterial and the filamentous (fuzzy) colonies are fungal. As you make your observations you will need to test these assumptions, as not all colonies will easily be assigned. Record "TNTC" if your colonies are "too numerous to count" (Figure 6.3).

Figure 6.3 Two Plates that are TNTC. Can you still distinguish TSA and Mycophil?

You may encounter other results that make counting difficult. For example, ants or other insects may get into your plate when you expose them (Figure 2.4). You may observe secondary colonies formed as the insects moved around the plate. Is antibiotic production visible on any of the other plates shown here?

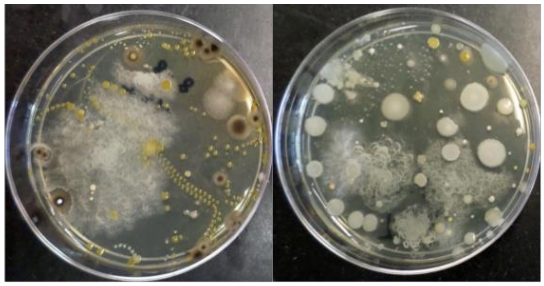


Figure 6.4 Examples of Common Observations. Left: Ant Tracks; Right: Antibiotics Production.

Record your colony counts in table form as shown below:

NUMBER OF COLONY FORMING UNITS

	Fungal	**Bacterial**	**% FUNGAL (FUNGAL/FUNGAL + BACTERIAL)**
pH 5 (MYCOPHIL)			
pH 7 (TSA)			

3. To test the assumptions of colony appearance, select colonies difficult to classify as either fungal or bacterial. Take a look at those colonies using one of the stereomicroscopes set up at the end of the benches. If you don't know which colonies to test, your TA will help you select colonies for observation. Record your observations in your notebook. Remember to note the magnification used and to indicate the relative size of the colonies.

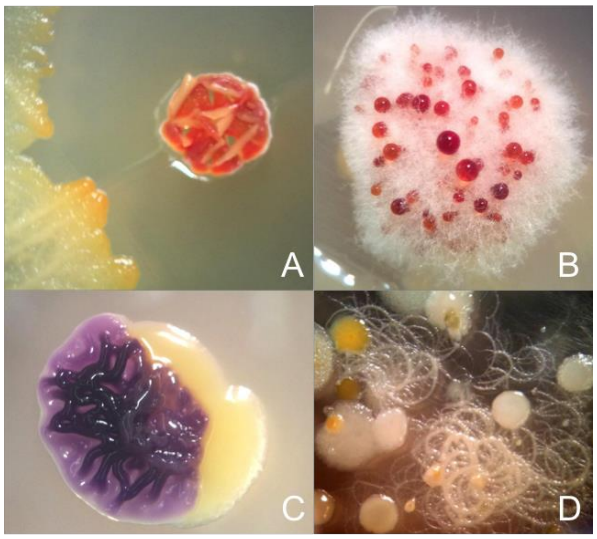


Figure 6.5 Colony Morphologies

A. Bacterial colony displaying antimicrobial activity
B. Fungal colony
C. Bacterial colonies
D. Bacterial colonies

How would you describe each colony morphology?

(7) MICROSCOPY

Because microbes, by definition, are not visible with the naked eye, microscopes are important tools for their study. There are several important types of microscopy for a variety of applications. Table 7.1 lists several common types of microscopes. All are light microscopes except electron microscopes. The wavelength of light used influences resolution, the shorter the wavelength the higher the resolution. Electron microscopes use electrons (electromagnetic radiation with much short wavelength than visible light) and so they have much greater resolving power.

Table 7.1 Microscopy types and common applications

Types of microscopy	Application
Brightfield	Stained or naturally pigmented samples
Phase Contrast	Enhance the contrast in colorless samples
Darkfield	Motility determination
Fluorescent	Fluorescent tag added for visualization/differentiation
Confocal	Three-dimensional morphology
Differential Interference (DIC)	Enhance the contrast in colorless samples
Electron	Visualization of internal components (transmission) and three dimensional morphology

The light microscope is one of the most important instruments available for the study of microorganisms. Invented in the seventeenth century by Robert Hooke, the compound microscope (more than one lens makes a microscope “compound”) brought to light the existence of the microbial world. Hooke observed fungi (*Mucor*). It was several years later that Antonie van Leeuwenhoek observed bacteria. He called them “animalcules” as he did not know what he was seeing. It was two centuries later that the connection was made between these “animalcules” and first, fermentation (Louis Pasteur) and later, disease (Robert Koch). Modern light microscopes are superb instruments that can provide considerable information about a specimen to a trained observer.

Light microscopes with phase contrast optics allow much better imaging of specimens with little contrast versus their aqueous environment. Phase contrast optics exploit differences in refractive index between specimen and background to generate contrast. Figure 7.1 below compares the same field of view in brightfield and phase contrast. Photosynthetic cells are green and so are visible in both brightfield and phase contrast. Phase contrast optics allow visualization of even those structure lacking contrast.

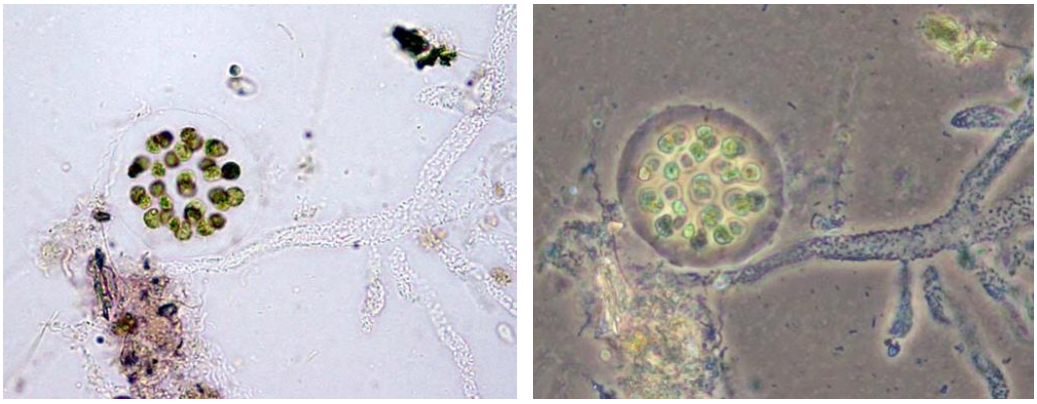


Figure 7.1 Sample under light microscopy (left) and phase contrast (right)

During the course of the semester you will use microscopy several times to view prokaryotes. You will also practice with yeast (fungal eukaryotes) and may have the opportunity to look at a hay infusion. A hay infusion is made by placing hay or other dried grass into a sample of water (tap or pond if available) and allowing the mixture to sit in the sun for a week or two. Hay infusions are teeming with prokaryotic and eukaryotic life. The following micrographs were produced from imaging hay infusions. Can you tell which were viewed with phase contrast optics and which with brightfield?

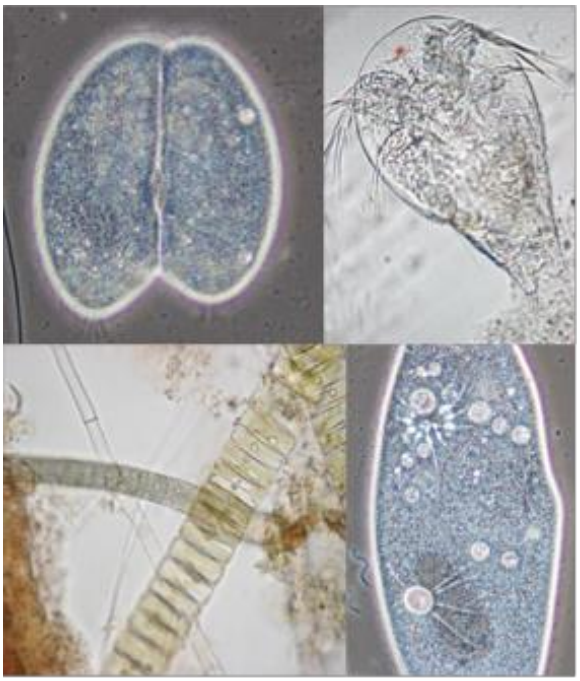
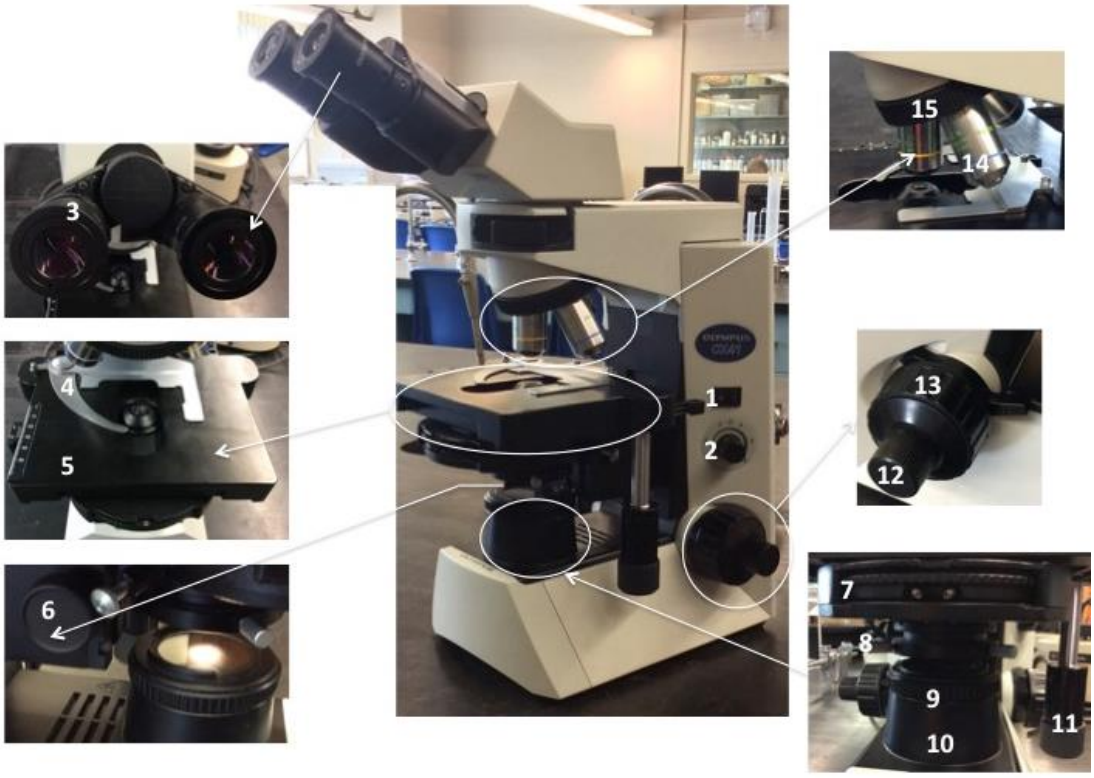


Figure 7.2 Hay Infusion. Brightfield and phase contrast observations of eukaryotic organisms.

The Microscope - Overview



1	On/Off Switch	9	Field diaphragm adjustment
2	Light intensity adjustment	10	Light source
3	Ocular (Eye piece)	11	Stage position adjustment
4	Stage clip	12	Fine adjustment knob
5	Stage	13	Coarse adjustment knob
6	Condenser height adjustment	14	Objective lens
7	Condenser position selection wheel	15	Revolving nosepiece
8	Brightfield iris adjustment		

Figure 7.3 Olympus phase contrast microscopes similar to those in our lab.

Preparing a Wet mount from a Broth Culture

MATERIALS
Microscope, Microscope slides and cover slips
Specimen (may include all or some of the following): Yeast (*Saccharomyces cereviseae,* a eukaryote*)* suspension, *Bacillus subtilis* (bacterial rods); *Enterococcus durans* (bacterial cocci).

PROCEDURE
1. Flame your loop, cool it and insert it into your bacterial culture (use aseptic technique). Then place the drop of the bacterial suspension on a microscope slide and cover with a coverslip. Although you will be observing both bacterial species don't prepare both slides at the same time. The second slide will dry out before you make your observations.
2. Use phase contrast microscopy. Follow the instructions provided on the back cover of the manual. Record observations in your notebook as drawings and any necessary descriptive text. Indicate length and width of the organisms.

Preparing a Wet mount from a Bacterial Slant

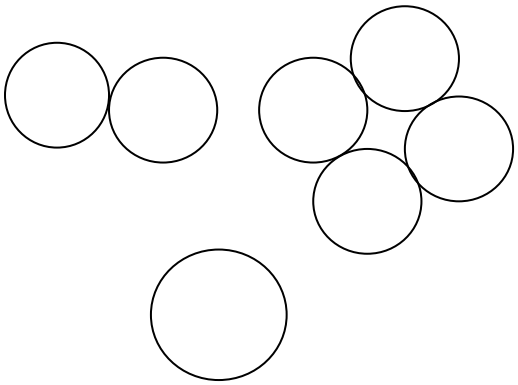
MATERIALS
Phase contrast microscope
Microscope slides and cover slips
Your unknown culture and other cultures available

PROCEDURE
1. Using a sterile loop, place two loopful of H_2O on a clean slide each about 1 cm from the center of the slide. DO NOT squirt the water onto the slide, remove the top from the water bottle and dip the loop in.
2. Using a sterile **NEEDLE**, touch the culture on the slant and mix the culture from the needle into the first drop of water.
3. Flame your needle, cool, and mix a small amount of the water from the first drop into the second. Place a coverslip on each drop.
4. Now you have two dilutions to observe - one that likely has too many organisms present to get a good view but will be easy to find, and a second drop that will provide a better view of individual organisms.
5. Use phase contrast microscopy. Follow the instructions provided on the back cover of the manual. Record observations in your notebook as drawings and any necessary descriptive text. Indicate length and width of the organisms.

Recording your Observations

Each drawing should include the following (Figure 7.4):

Name of Organism
Magnification
Type of microscopy used
A size or scale marker (This provides the reader with a ruler to measure anything in your diagram)
A few descriptive words as appropriate



Micrococcus giganticus
1000x TM
PC

Cells are cocci (spherical) and appear in clusters of two or four. Some single cells observed.

50µm

Figure 7.4 Representative Picture and Appropriate Labels/Description for *Micrococcus giganticus.*

Figure 7.5 Phase contrast

SPECIMEN SIZE DETERMINATION

Magnification	**µm/small division (eyepiece scale)**
100x (10 x 10)	10
400x (10 x 40)	2.5
1000x (10 x 100 oil)	1.0

(8) MORPHOLOGY AND STAINING OF BACTERIA

The Gram Stain:

Christian Gram, a Dane working at the Berlin City Hospital, developed a staining technique to differentiate *Schizomycetes* from mammalian tissue cells microscopically. Fortuitously it was later found to differentiate two basic types of bacterial cell walls. These two basic groups of bacteria became known as Gram-negative organisms and Gram-positive organisms (based on the ability to stain with in the Gram procedure).

In his initial single stain he did not clearly see all of the bacteria in his tissue preparation (i.e. bacteria now known as a Gram-negative did not stain) and was thus disappointed. However, the subsequent distinction of Gram positive and Gram-negative proved most useful. Furthermore, the Gram-positive characteristic is unique to some bacteria, yeasts, and a few molds. Most other living cells are Gram-negative or Gram variable. A few are Gram-nonreactive, e.g. *Mycobacterium*.

The major component of the cell wall of Gram-positive bacteria is peptidoglycan, a polysaccharide with peptide cross-linkages. After staining, the crystal violet-iodine complex cannot pass through the peptidoglycan during decolorization, as the solvent causes the thick peptidoglycan to contract and thus retain the stain/complex. In the Gram-negative bacterial cell wall, peptidoglycan is a minor component and there is also a proteolipid present neither of which prevents the loss of the stain complex. If the peptidoglycan is removed from a gram-positive cell (for example, by the action of lysozyme), the cells will not retain the stain complex and will appear Gram-negative. The Gram stain provides easy and fast and the information that is quite valuable (e.g. a general correlation of the sensitivity of bacteria to antimicrobial agents).

General Protocol

1. Primary stain - crystal violet, stains all cells purple.
2. Mordant - used to enhance a complex formation between the dye and the bacterial cell. An iodine solution serves as the mordant.
3. Decolorizing agent — a solvent, acetone, alcohol, or a mix. This removes the primary stain from the Gram-negative cell and “dehydrates” the Gram-positive cell wall.
4. Counterstain - used to recolor cells that have lost the primary stain after alcohol treatment; it contrasts with the primary stain, e.g. red safranin vs. the primary crystal violet.

Gram-positive organisms do not lose the primary stain when treated with the decolorizing agent and appear **purple** after the staining procedure. The **Gram-negative** organisms lose the primary stain (decolorization), and after counterstaining with **safranin** they **appear pink**. If Gram-positive cells are not treated with the iodine mordant after primary staining, they too will lose their primary stain at the decolorizing step and appear as Gram-negative cells. Why is decolorization a critical step in the procedure?

Examples of Gram(+) and Gram(-) organisms:

Gram-Negatives	**Gram-Positives**
Enterobacter aerogenes	*Bacillus subtilis*
Escherichia coli	*Staphylococcus epidermidis*
Pseudomonas fluorescens	*Micrococcus luteus*
Citrobacter freundii	*Enterococcus durans*

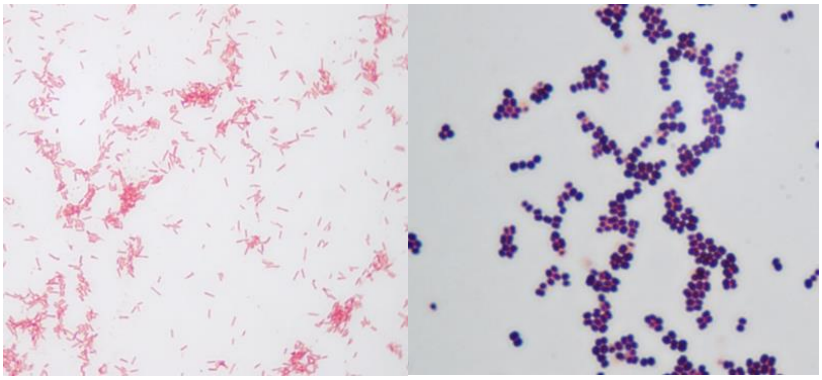


Figure 8.1 Gram reactions. Left: Gram-negative; Right: Gram-positive

MATERIALS

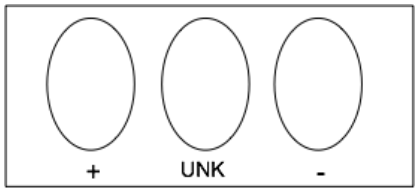
Glass Slide
Gram (+) and Gram (-) Controls
Gram Stain Reagents
- Crystal violet
- Gram iodine
- Safranin
- Decolorizer: Acetone-Ethanol (50:50 - Red squirt bottle)

Staining Kit (Staining tray, slide holder, clothespin to hold slides)
Paper towels

Figure 8.2 Staining Kit

STAINING PROCEDURE

1. Preparation of smears. We will always include positive **and** negative controls on **all** of our Gram stains. This is the best way to evaluate our technique and learn the Gram reaction of the organism of interest. **Use your loop** to add one loopful of water to each oval drawn on the slide with wax pencil as shown below. **Use your needle** to mix a **SMALL** sample of culture into the appropriate water drop and spread it over the whole area of the oval. This will facilitate drying.



2. Dry the smears in the air and then gently heat fix by passing through a flame. Your instructor will demonstrate heat fixing.

3. Crystal violet staining: Place the slide on a staining rack and flood each smear with crystal violet. **Stain for 1 minute**.
4. Wash the crystal violet from the slide with water. Drain any excess water onto a paper towel by tapping slide gently on the towel.
5. Flood the smears with iodine and retain iodine on slide for 1 minute.
6. Wash the iodine from the slide with tap water and drain off any excess water. **BLOT DRY** with paper towels.
7. Decolorize: Place the slide on a staining rack and flood each smear with decolorizer until there is no purple color in the draining liquid.
8. Briefly wash the smears with tap water. Drain any excess water.
9. Counterstain the bacteria with safranin for at least 2 min.
10. Wash with tap water. Blot dry.
11. Examine the slides under bright-field with no cover slip. Focus the slide beginning at 100x total magnification (10x objective). You will need the oil-immersion objective (1000X) to judge the quality of your Gram stain.

Negative Stain:

Negative staining is an excellent way to determine an organism's cellular morphology and to view capsules. Since the cells themselves are not stained, their morphology is not distorted in any way. The nigrosin provides a dark background against which the shapes of the unstained cells are clearly visible. This method provides a high degree of contrast not available in most other staining procedures.

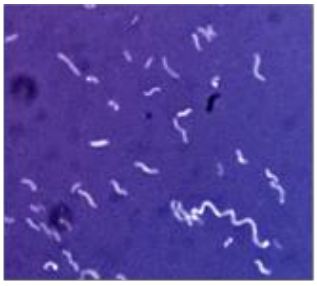


Figure 8.3 Negative Stain

Staining Procedure:

1. Place a single drop of nigrosin on a clean microscope slide, adjacent to the edge.
2. Using a flamed loop and sterile technique, remove some organism from your tube or plate and mix it into the drop of nigrosin. Be sure there are no large clumps of organism, but try to avoid spreading the drop.
3. Place the end of another clean microscope slide at an angle to the end of the slide containing the organism and spread the drop out into a film. This is done by contacting the drop of nigrosin with the clean microscope slide and using the capillary action of the dye/microscope slide to spread the nigrosin across the smear.
3. Allow the film to air dry.
4. Observe the slide under the microscope, using proper microscope technique.

FLAGELLAR STAIN

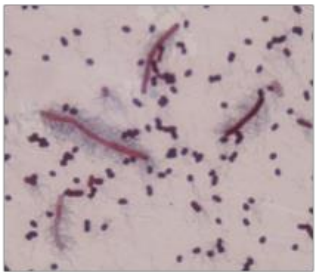


Figure 8.4 Flagellar Stain

Flagella are responsible for bacterial motility. They are long, thin appendages which are free at one end and attached to the cell at the other end. They are comprised of helical protein filaments, uniform in length and diameter. The rotation of the flagella propel the cell through liquid medium. Motile bacteria are categorized by the placement and quantity of flagella present. Bacteria with one flagellum (at either end) are polar, or **monotrichous**. A tuft of several polar flagella is called **lophotrichous,** while bacteria with flagella distributed over the entire surface are termed **peritrichous**. *E. coli*, which is peritrichous, possesses few flagella while *Proteus vulgaris* may contain several hundred flagella. Motility can be visualized using wet mount observations. However, flagella generally must be stained to be seen with the light microscope.

SPORE STAIN

During the stationary growth of bacteria, when the nutrients have been depleted and conditions for growth are poor, some bacterial species can form endospores. Endospores (spores formed within the cell) represent a dormant form which is capable of surviving for prolonged periods of time. When suitable environmental conditions recur, spores germinate and reestablish vegetative growth. The discovery of bacterial endospores had great impact on microbiology. Knowledge of heat resistant forms is essential for adequate sterilization of culture media and food, and was also a key factor in disproving the theory of spontaneous generation. These unique structures are formed by *Bacillus*, *Clostridium*, and *Sporosarcina* species.The bacterial endospore is unique in its ability to withstand heat, resist drying, radiation, acids and chemical disinfectants. These structures are impermeable to standard dyes and require special staining protocols for their identification (Fig. 8.5).

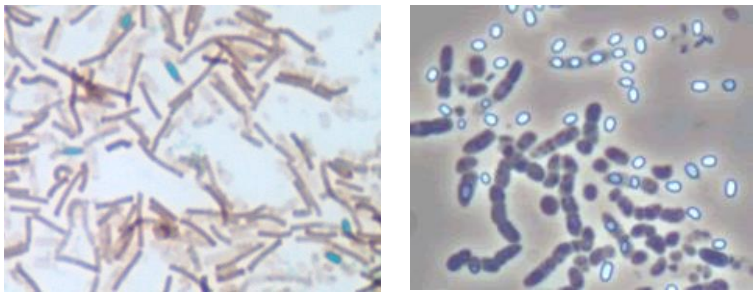
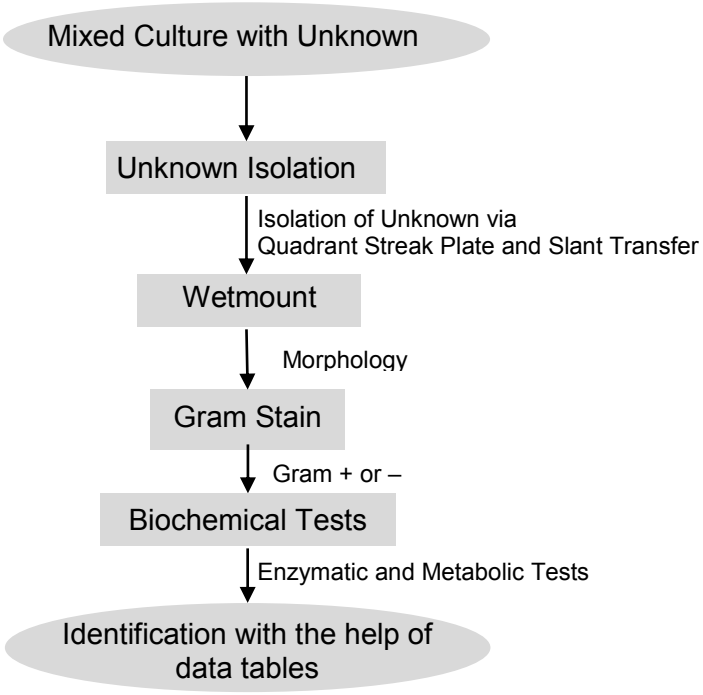


Figure 8.5 Visible spores. Left: spores stained with Schaeffer-Fulton staining procedure; Right: spores observed with phase contrast microscope.

(9) MICROBIAL METABOLISM AND UNKNOWN IDENTIFICATION

So far, you isolated your unknown from a mixed culture, observed a wet mount, and identified its Gram reaction. In this exercise, you will now identify your unknown organism based on metabolic capabilities. The schematic reviews what you have done so far and what will be done to finish the identification of your unknown:



The last step to positively identify your organism is to assess its metabolic capabilities. The metabolic potential of a microorganism is specified by that organism's genetic information. The complement of enzymes a given organism possesses determines the organism's metabolic capabilities. Nearly all enzymes are proteins, and since proteins are encoded for by DNA the organism's genetic make-up determines what enzymes are present. The observed differences in the metabolic capabilities of organisms are a reflection of genetic differences. Observation or measurement of these enzyme reactions or metabolic processes requires a means of visualization.

As scientists we spend much of our time trying to "see" things that are essentially invisible. Very few metabolic reactions are directly observable, i.e. have a product that can be differentiated visibly from the reactants without some input on the observer's part. The simplest strategies for visualizing metabolic pathways or individual enzyme reactions involve manipulations that bring about a color change. If your experiment requires detection of very small amounts of material an approach exploiting light emission or radioactive isotopes may be necessary.

The metabolic tests you will use in lab this week all involve one or more of the very common methods for visualization listed below.

1. **Inherently visible reaction product**
   Can be directly observed without any addition of indicators or chemicals
   Example: Catalase test = reaction product is bubbles

2. **Inclusion of an appropriate pH indicator**
   Observation of a color change in the medium; observation of either an acidic or alkaline byproduct
   Example: Phenol Red Lactose or Glucose broths, citrate slant, urease test

3. **Addition of a chemical compound after the organism has been grown**
   A direct observation of inclusion of a pH indicator is not possible to visualize a reaction, thus a chemical compound will be added to the reaction that will modify the product or intermediate to form a colored compound.
   Example: Oxidase test or Kovac's reagent is added to detect the presence of indole

You will work independently to identify your unknown. You will first test your unknown for catalase. You will then inoculate your unknown onto the growth test media and analyze the results next week. You will then compare your results to a table of known organisms to identify your unknown.

9-1: Enzyme Catalyzed Reactions

Enzymes are biological catalysts that lower the energy of activation for biochemical reactions and permit essential metabolic steps to proceed rapidly even at the relatively low temperatures at which living systems exist. Enzymes, like all catalysts, are not consumed in the reaction and so can convert (or turn over) many substrate molecules to product. The rate of an enzyme-catalyzed reaction is dependent on temperature, the concentration of enzyme, the concentration of substrate, and the affinity of the enzyme for the substrate.

Microorganisms possess many different enzymes. The activities of these enzymes determine the very nature of the organism; a microbe's physiological properties are determined largely by its enzymatic capacities (the phenotype reflects the genotype). A microorganism's habitat and its interactions with other organisms - its ecological relationships - are also a direct reflection of the organism's enzyme complement.

Most microbial identification systems are largely based on determining specific enzymatic potentialities that reflect the organism's taxonomic status. In this exercise, we shall assay for the presence of one enzyme (catalase) frequently used in the classification of microorganisms and evaluate growth of your unknown organisms on media.

CATALASE (Gram-positive organisms only)

Catalase catalyzes the breakdown of hydrogen peroxide (H_2O_2) to water and gaseous oxygen.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Hydrogen peroxide is a toxic by-product of aerobic respiratory metabolism. Most aerobic microorganisms use catalase to remove hydrogen peroxide. The presence of catalase is easily demonstrated by adding a drop of hydrogen peroxide to a loopful of bacteria spread on a microscope slide. Bubbles of oxygen are released and easily visible.

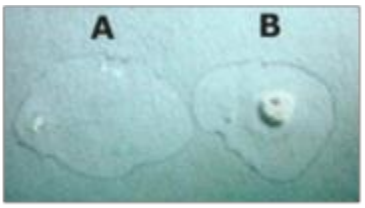


Figure 9.1 Catalase Results. A: Negative; B: Positive

MATERIALS

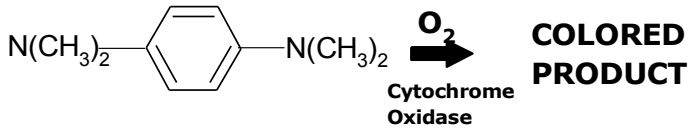
Control cultures and your unknown isolate
Hydrogen peroxide (3%)
Inoculating needle

PROCEDURES

1. Aseptically pick up some bacterial cells from slant growth with an inoculating needle or applicator stick.
2. Smear the growth onto a clean slide. You can put three organisms on each slide.
3. Pipette a drop of hydrogen peroxide onto cells.
4. Observe for bubbles and record your results. The production of bubbles indicates the presence of catalase. Your instructor will advise as to which cultures should be tested.

OXIDASE (Gram-negative organisms only)

During respiration, electrons are transferred through a series of oxidation-reduction reactions to a terminal electron acceptor, such as oxygen. This membrane-bound electron transport chain establishes an electrochemical gradient across a membrane that drives the formation of ATP. The terminal link in electron transport chains where oxygen is the terminal electron acceptor is a lipoprotein complex known as cytochrome oxidase. There is wide variation in the specificity and enzymatic activity among cytochrome oxidases found in different microorganisms that use oxygen. The oxidase test is used to learn if an organism produces certain cytochrome c oxidases. This test is commonly used to differentiate between the families of Pseudomonadaceae (oxidase positive) and Enterobacteriaceae (oxidase negative).



MATERIALS

Sterile cotton swabs
1% tetramethyl-ρ-phenylenediamine dihydrochloride (freshly prepared solution).

NOTE: These reagents are potentially toxic!

PROCEDURES

1. Using aseptic technique, gently swab slant to get a **small** amount of the culture on the swab.
2. Use the dropper bottle to put two or three drops of 1% tetramethyl-ρ-phenylenediamine dihydrochloride onto the culture on the swab.
3. Observe for immediate color change. The development of purple color within 10 seconds indicates a positive test. This reagent will turn purple due to oxidation by the air. False positive tests can occur if results are read after 20-30 seconds. Record your observations. Your instructor will advise as to which cultures should be tested.

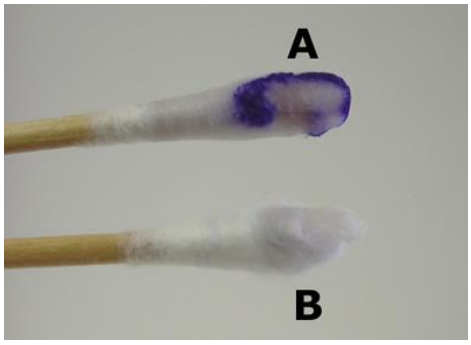


Figure 9.2 Oxidase Test.
A: Positive, B: Negative

9-2: METABOLIC CAPABILITIES

The metabolic capabilities of an organism depend, of course, on the genes present. Which genes are expressed depend on what substrates are available; in other words, what it is fed.

A wide range of biochemical and serological procedures are used in clinical microbiology laboratories for the identification of microbial isolates. Accuracy, reliability, and speed are important factors that govern the selection of clinical identification protocols. The specific procedure to be employed for the identification of pathogenic isolates depends on presumptive identification of the organism at the genus or family level. Presumptive identification is based on the observation of colonial morphology and other growth characteristics on the primary isolation medium as well as microscopic observation of stained specimens. The results are integrated with a series of biochemical tests.

A number of commercial systems are available for identification of unknowns. They are widely used in clinical microbiology laboratories because many pathogens are indistinguishable except for characteristics determined by detailed biochemical and/or serological testing. Species identification is determined by comparison of the pattern of test results generated to an established database. As nucleic acid sequencing becomes faster, cheaper and more easily automated; identification via sequencing is gaining prominence.

You will inoculate your unknown into each of the following growth media as described. Pay close attention, there are a few differences in the media used to identify Gram-negatives and Gram-positives.

WEEK 1

GRAM-NEGATIVES ONLY:

Citrate Utilization: Growth Using a Single Carbon Source ~ Blue Cap

It is often useful to determine whether a particular microorganism can meet all of its energy and carbon requirements by the metabolism of a particular compound. In this experiment, we will examine the abilities of microorganisms to utilize citrate as a sole source of carbon.

We will use Simmons citrate agar: sodium citrate as the carbon source, ammonium salts as the nitrogen source, and bromthymol blue as a pH indicator. Growth of an organism on this medium results in a rise in the pH, causing the pH indicator to turn from green to royal blue. Bromthymol blue is yellow below pH 6.0 and blue above pH 7.6.

PROCEDURE

1. Using a needle, aseptically inoculate an agar slant of Simmons citrate medium with your unknown culture. Streak gently several times across the agar surface.
2. Incubate the cultures at 35°C for 24-48 hrs. After incubation the cultures will be stored in the refrigerator until the next class.

MR-VP The Mixed Acid Fermentation Pathway (Methyl Red Test) & The Butanediol Fermentation Pathway (Voges-Proskauer Test) ~ Black Capped Liquid Medium

The Mixed Acid Fermentation Pathway (Methyl Red Test)

The mixed acid fermentation pathway produces a variety of end products, including acetic, formic, lactic, and succinic acids, plus ethanol, molecular hydrogen, and carbon dioxide. The mixed acid fermentation is carried out by many bacteria, including *Escherichia coli.* The proportions of the products vary depending on the bacterial species. However assuming that this is the primary fermentation pathway, sufficient acidic products accumulate to reduce the pH drastically. The accumulation of lactic, acetic, succinic and formic acids drops the pH below 5.0 even when the broth is slightly buffered with peptone and potassium phosphate. Methyl red is red below pH 4.8 and yellow above pH 6.0.

The Butanediol Fermentation Pathway (Voges-Proskauer (VP) Test)

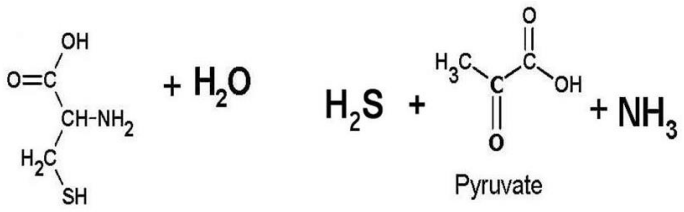
Enterobacter cloacae are positive for the butanediol pathway, *Escherichia coli* is not. Butanediol, an end product of this fermentation pathway, is rather unreactive. The diagnostic test for this pathway is based on the detection of an intermediary metabolite, acetoin, as this compound is far more reactive. Acetoin is also known as acetyl methyl carbinol. This compound is easily detected by the Voges-Proskauer test.

PROCEDURE

1. Aseptically inoculate MR-VP broth with appropriate cultures. Use your needle to introduce a small amount of growth from the slant into the liquid medium.
2. Incubate the inoculated tubes at 35°C for at least 48 hr. After this time they will be refrigerated until the next class.

SIM (Sulfide, Indole, Motility) ~ Green-Capped Agar Deep

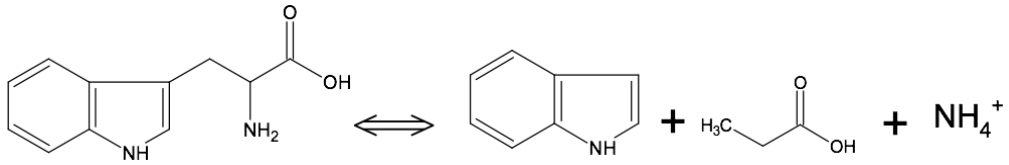
SIM Medium aids in distinguishing microorganisms based on three important characteristics: Hydrogen sulfide (H_2S) production, indole formation and motility. H_2S production is visualized via the reaction shown below. H_2S gas formed by the microorganism from cysteine reacts with the iron salt in the medium to produce FeS.



H_2S + Ferrous ammonium sulfate ⟶ FeS

(iron salts) (black precipitate)

The ability to hydrolyze tryptophan to indole is characteristic of certain enteric bacteria that possess the enzyme tryptophanase. Tryptophanase catalyzes the hydrolysis of tryptophan with the production of indole, pyruvic acid and ammonia.



Indole is visualized by the addition of Kovac's reagent after sufficient incubation (48 hrs). The casein peptone in this medium is rich in tryptophan.

MATERIALS
Kovac's reagent (*p* dimethylamino benzaldehyde - 5% in isoamyl alcohol:HCl).
SIM medium.

PROCEDURE
1. Aseptically inoculate SIM medium using needle to make straight stab inoculation.
2. Incubate the cultures at 35°C for 24-48 hr. They will then be stored in the refrigerator until the next laboratory class.

Nitrate Reduction ~ Dark Blue Capped Broth
This test is used to determine if an organism reduces nitrate (NO_3) to nitrite (NO_2) and also test if the organism has the ability of nitrification on both nitrate and nitrite to produce molecular nitrogen. The source of nitrate in the broth comes from both nutrients and potassium nitrate. The ability to perform this process is by presence of the enzymes nitrate reductase and nitrite reductase. If the organism reduces nitrate into nitrite **only**, the reaction will yield a red color. If in turn, the organism reduces nitrite into nitrogen gas a bubble will appear in the Durham tube. In this exercise, you will be testing the ability of your unknown organism to reduce nitrate. The inverted Durham tube in the media is used as a trap for nitrogen gas.

Materials:
Unknown organism
One tube of nitrate broth with Durham tube
Sulfanilic acid
α-naphtylamine
Inoculating needle
Incubator (35□C)

PROCEDURE:
1. Aseptically inoculate the broth with the appropriate bacterial culture. Use your needle to introduce a small amount of growth from the slant into the liquid medium.
2. Incubate the inoculated tubes at 35°C for at least 48 hr. After this time they will be refrigerated until the next class.

Eosin-Methylene Blue ~ Petri Dish

This medium is slightly selective for Gram-negative organisms. It is used to differentiate between organisms that ferment lactose and those that do not ferment this sugar. The eosin and methylene blue slightly inhibit Gram-positive organisms; these dyes also allow us to differentiate between lactose-fermenters and non-fermenters. Both *Escherichia coli* and *Citrobacter freundii* will grow as blue-black colonies with a green metallic sheen due to the rapid fermentation of lactose. *Enterobacter* (lactose-fermenter) colonies will appear blue-black; *Pseudomonas* and *Serratia* (non-lactose fermenters) colonies will appear colorless and mucoid.

Materials:

Unknown organism
One Eosin Methylene Blue plate
Inoculating loop
Incubator (35□C)

Procedure

Follow the procedure as described in the quadrant streak plate method exercise.

GRAM-POSITIVES ONLY:

Bile Esculin Slant

Bile esculin is a selective and differential medium. It is used to select for bile tolerant bacteria and will differentiate between bacteria that can or cannot convert esculin to esculitin.

PROCEDURE

1. Using a needle, aseptically inoculate a bile esculin agar slant with your unknown culture. Streak gently several times across the surface of the agar
2. Incubate the cultures at 35°C for 24-48 hr. After incubation the cultures will be stored in the refrigerator until the next class.

Mannitol Salt Slant

Mannitol Salt is both a selective and differential media. It is **selective** in that it selects for halotolerant organisms and **differential** in that it lets us tell apart organisms that can ferment mannitol and those who cannot.

PROCEDURE

1. Using a needle, aseptically transfer your unknown organism into the provided slant.
2. Incubate the inoculated slant at 35°C for at least 48 hours. After this time they will be refrigerated until the next class.

6.5% Salt Broth Test

Sodium chloride was added to tryptic salt broth to an overall concentration of 6.5%. This broth is used to grow halotolerant organisms such as *Staphylococci* and *Enterococci*. *Streptococci* will not survive in this environment.

PROCEDURE

1. Aseptically inoculate 6.5% salt broth with your unknown, Gram-positive organism. Use your needle to introduce a small amount of growth from the slant into the liquid medium.
2. Incubate the inoculated tubes at 35°C for at least 48 hr. After this time they will be refrigerated until the next class.

Mueller Hinton Agar and Novobiocin

Mueller Hinton Agar is an all-purpose medium that is used for antimicrobial susceptibility testing. You will be adding a Novobiocin disc. Novobiocin is an antimicrobial agent that targets the DNA gyrase of bacteria. It will help you to differentiate various species of *Staphylococci*.

PROCEDURE

1. Uniformly swab the entire surface of the Mueller Hinton plate with your unknown organism. This may be accomplished by first streaking with a cotton swab in one direction and then rotating the plate and streaking at 90° to the initial streaking. Make sure to work accurately so that the entire surface will be evenly covered with the organism.

BOTH GRAM-POSITIVE AND GRAM-NEGATIVE UNKNOWNS

Carbohydrate Utilization – Acid Production (and Gas Production of Glucose Fermenters) ~ Glucose: Red Cap, Lactose: Yellow Cap

Microorganisms may produce acidic end products (e.g., lactic acid) and/or hydrogen gas or neither acid nor gas from a particular carbohydrate substrate. The products depend on the ability of the microorganism to utilize the particular substrate and the fermentation pathway(s) that the organism is competent to carry out. Recognition of the production of acid and gas can be accomplished by using a medium that contains a **pH indicator** and a way of capturing the gas produced.

You will be inoculating tubes of phenol red broth that contain either **glucose, lactose.** The tube containing glucose also contains an inverted Durham tube to determine if gas is produced. Phenol red is red above pH 8.0 and yellow below pH 6.6. The Durham tube will collect any insoluble gas produced. View the API reactions online: the nature of the gas produced is discussed there.

PROCEDURE

1. Aseptically inoculate the broth with your unknown culture. Use your needle to introduce a small amount of growth from the slant into the liquid medium.
2. Incubate the cultures at 35°C for 24-48 hr. At the end of the incubation period they will be stored in the refrigerator until the next laboratory session.

Oxygen Tolerance ~ Yellow-Capped Deep

Oxygen can exist in a number of energetic states; some are more toxic than others. Singlet oxygen ($^1O_2^*$) is highly reactive and toxic to living organisms. Peroxidase enzymes in saliva and phagocytes (blood cells involved in one defense mechanism of the human body against invading microorganisms) generate singlet oxygen, accounting in part for the antibacterial activity of saliva and the ability of phagocytes to kill invading microorganisms. The reduction of molecular oxygen to water, which occurs when oxygen serves as a terminal electron acceptor, involves the formation of another toxic form of oxygen; the superoxide anion (O_2^-), also called super oxide radical. The superoxide anion is converted to hydrogen peroxide and water by the action of the enzyme superoxide dismutase. Hydrogen peroxide is toxic as well (it is frequently used commercially as an antiseptic); therefore, any organism living in an oxygen environment, whether it uses oxygen as a terminal electron acceptor or not, must produce enzyme(s) to get rid of peroxide; generally catalase(as we read and tested for in the catalase test) or peroxidase are responsible for the destruction of hydrogen peroxide.

- Both **obligate aerobes** and **facultative aerobes** usually produce both superoxide dismutase and catalase. These enzymes permit such microorganisms to use oxygen and continue growing without accumulating toxic forms of oxygen that would kill them. In contrast, obligate oxylabile anaerobes generally lack both of these enzymes. The inability of these organisms to enzymatically remove toxic forms of oxygen probably accounts for the fact that they are obligately anaerobic and sensitive to oxygen. The oxyduric anaerobes generally produce super oxide dismutase but not catalase, which is evidence that they can survive in the presence of oxygen.

Under controlled laboratory conditions, it is possible to adjust oxygen concentrations to maximize the growth rate of a particular microorganism. Because oxygen diffuses relatively slowly into liquids, the concentration of oxygen frequently limits the growth rate of aerobes and facultative aerobes in liquid culture. Liquid cultures can be agitated at high speed on a shaker or by an impeller within the culture vessel, or oxygen can be sparged into the culture vessel through forced aeration to maintain optimal oxygen concentration. Interruption of the supply of oxygen to an actively growing culture for even a brief period of time can lead to anaerobic conditions. In some cases a rapid die-off of the microorganisms can occur, in extreme cases some microbial populations can lose viability if a rotary shaker is turned off for only a few minutes, such as may occur when changing flasks.

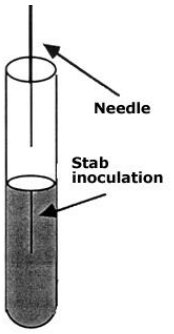
In this exercise, you will study the relationship of several types of microorganisms to oxygen by growing them under conditions of different oxygen concentrations.

MATERIALS

One tube (per student) of thioglycolate medium containing 0.075% agar,
Inoculating needle
Incubator (35°C)

METHODS

1. Label your tube with your section and seat number.
2. Use a needle to stab inoculate (aseptically) straight down through the center of the medium to the bottom of the tube.
3. Incubate at 35°C for 48 hours. The cultures will be stored in the refrigerator until the next laboratory session.



WEEK 2 – GRAM NEGATIVE UNKNOWNS

Citrate Utilization ~ Blue Cap

Observe the color of the medium. A royal blue color is a positive test for citrate utilization; a green color is a negative test. Record your results.

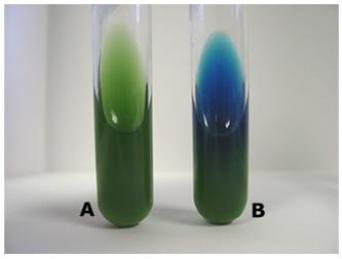


Figure 9.3 Citrate Results.
A: Negative; B: Positive

Methyl Red (MR) and Voges Proskauer (VP Test ~ Black Cap)

MATERIALS

MR-VP medium (Methyl Red: Voges - Proskauer).
40% KOH, VP Reagent 1
α-naphthol, VP Reagent 2 (6% α-Naphthol in ethanol).
Methyl red indicator in dropper bottle
Micropipette and sterile tips

NOTE: These reagents are caustic — be careful.

VP TEST

1. **After incubation, pipette 1 ml of culture to a clean test tube.** Add ~10-15 drops VP Reagent 1 (40% KOH) and ~10-15 drops of VP Reagent 2 (α-Naphthol). Vortex to mix.
2. Observe the tubes for the gradual formation of a red/burgundy color – positive test for the presence of acetoin. A brown color is negative. Some cultures react within 1 minute, but others may take 1-2 hours. Record your results.

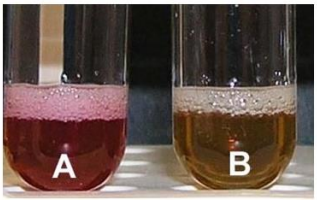


Figure 9.4 Voges-Proskauer test.
A: Positive result;
B: Negative result.

METHYL RED TEST

1. **The remaining broth culture will be used to perform the Methyl Red test.** Once you are sure you have done the Voges-Proskauer test correctly proceed with the next step.
2. Add 3 or 4 drops of methyl red solution directly to the remaining broth culture grown in the MR-VP medium. If the indicator remains red, the test is positive for mixed acid fermentation. A yellow color is negative. Record your results.

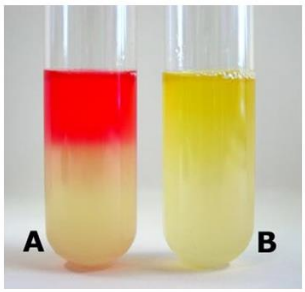


Figure 9.5 Methyl red test.
A: Positive result;
B: Negative Result.

SIM (Sulfide, Indole, Motility) ~ GREEN-CAPPED AGAR DEEP

1. Determine the results for the H_2S production and motility reactions **before** adding the Kovac's reagent. H_2S is indicated by the presence of a black precipitate. Growth radiating from the central stab line indicates motility of the organism.
2. Carefully layer ~10 drops of Kovac's reagent directly on the top of the medium in the culture tube. The presence of indole will be detected by the immediate formation of a magenta layer (positive results may range from pinkish to deep magenta) at the top of the medium.

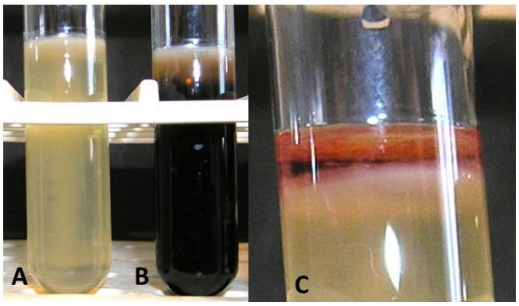


Figure 9.6
SIM (Sulfur, Indole, Motility) test.
A: negative sulfide result;
B: Positive sulfide result;
C: Positive indole result.

Nitrate Reduction- Dark Blue Cap

1. Observe the Durham tube inside your culture. If there are bubbles in the Durham tube, this shows the presence of both nitrate reductase and nitrite reductase (nitrates have been converted to nitrites, and then to nitrogen gas). No bubbles in the Durham tube will require the addition of reagents.
2. Add 5 drops of both solution A (Sulfanilic acid) and solution B (Alpha-naphtylamine). A red color indicates a positive result for nitrate reduction. If it remains colorless and no bubble is present in the Durham tube it may still be positive. As a way to verify your result, Zn powder will be provided as a way to confirm that the organism completely reduces nitrate to nitrogen gas and that your test either is positive or negative. After the Zn addition to the sample, formation of bubbles means it's positive for complete nitrate reduction whereas no reaction ensures it's a negative result.

Eosin Methylene Blue – Petri Plate

Escherichia coli and *Citrobacter freundii* will grow as blue-black colonies with a green metallic sheen due to the rapid fermentation of lactose. *Enterobacter* (lactose-fermenter) colonies will appear blue-black; *Pseudomonas* and *Serratia* (non-lactose fermenters) colonies will appear colorless and mucoid.

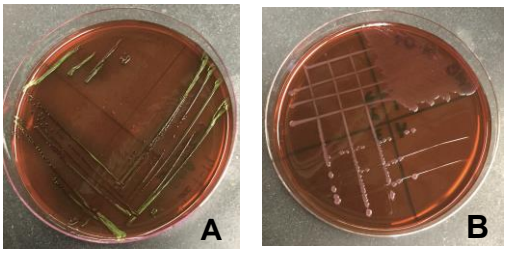


Figure 9.7
A: Lactose fermenter
B: Non-lactose fermenters

WEEK 2 – GRAM POSITIVE UNKNOWNS

Mannitol Salt – Slant
Observe the growth and the color of your medium. No growth and no change in color will indicate an overall negative result. Growth and yellowish agar indicates a positive result for mannitol fermentation and halotolerance. Growth and pink agar indicates a negative result for mannitol fermentation but positive for halotolerance.

6.5% Salt Broth Test
Observe the medium. A turbid medium indicates growth; clear medium indicates no growth.

Bile Esculin Slant
Observe the color of the medium. A black color indicates the organism is bile tolerant and can convert esculin to esculitin; a clear, amber color indicates a negative test result.

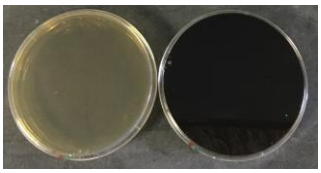


Figure 9.8 Bile esculin plates. Left: Negative result; Right: positive result.

Mueller Hinton Agar and Novobiocin
Observe the plate for growth and a zone of inhibition. No zone will indicate that the organism is resistant to Novobiocin. If a zone is present, measure the diameter. A zone larger than 16mm indicates the organism is sensitive to Novobiocin.

WEEK 2 – BOTH GRAM POSTIVE AND GRAM NEGATIVE UKNOWNS

Carbohydrate Utilization – Acid Production (Gas Production for Lactose Broth) Glucose: Red Cap, Lactose: Yellow Cap
Observe the color of the tubes - Yellow indicates acid production (glucose, lactose). Look at the Durham tube – Glucose Only. Partial (more that 0.3 cm) or complete filling of the Durham tube indicates positive gas production. Record your results.

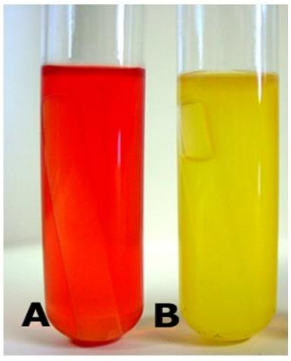


Figure 9.9 Phenol red glucose broth. A: Negative for acid and gas; B: Positive for acid and gas.

Oxygen toxicity- Yellow Cap
Examine the growth patterns of your organism and record a sketch of your tube.

(10 – 12) MICROBIAL GENETICS

(10) TRANSFORMATION OF *ESCHERICHIA COLI* CELLS WITH PLASMID DNA

Escherichia coli strain DH5α (host strain) lacks the ability to ferment lactose (lac^-) and is sensitive to the antibiotic ampicillin (amp^s). The introduction of foreign DNA into a host cell is called transformation. In this exercise the foreign DNA used for transformation of *E. coli* DH5α cells will be either plasmid pUC119 or pRU4x92 (Figure 11-1). Plasmid pUC119 is the parent plasmid from which pRU4x92 was made by inserting foreign DNA into the multiple cloning site of pUC119. In this case the foreign DNA is "foreign" because it is not part of the plasmid pUC119. Both plasmids contain ampicillin resistance genes; we will use this gene for **selection** of transformed cells. Only pUC119 has an intact *lacZ* gene conferring the ability to ferment lactose and produce acid; we will use this gene, or the lack of it, to **differentiate** cells transformed with either pUC119 or pRU4x92. The DH5α cells are first treated with $CaCl_2$, making them competent (competent to take up DNA), followed by a "heat shock". The heat shock makes cells more permeable and uptake of foreign DNA is more likely.

After a cell recovery period, the transformation mix and control DH5α cells will be spread plated onto appropriate media. Next week based on the appearance of the plates, you will determine whether you and your partner were given pUC119 or pRU4x92.

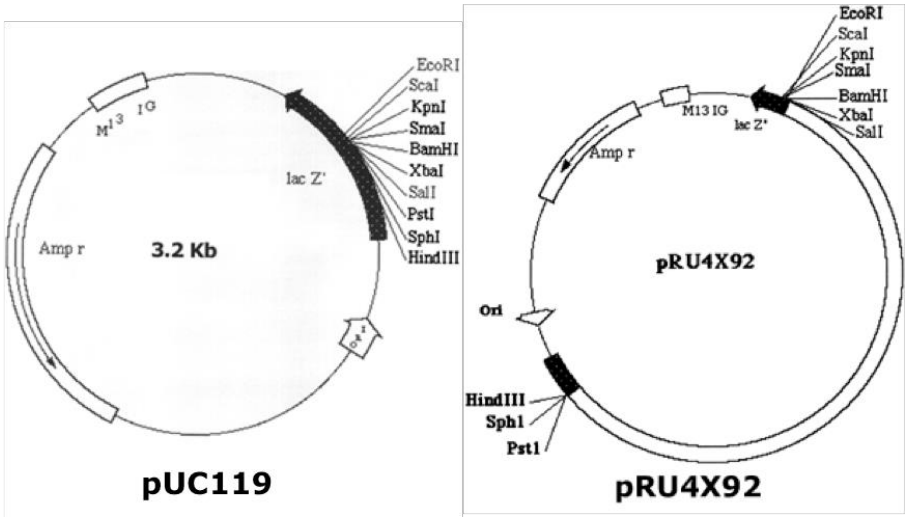


Figure 10.1 Plasmid Maps

E. coli DH5α was grown in LB broth overnight (25 ml/250 ml flask). This culture (about 1 ml) was then used to inoculate LB broth, which was incubated, with shaking, at 35˚C for about 3 hrs (which is well into the exponential phase of growth). After incubation, the cells were pelleted (from about 3 ml of culture - two runs of 1.5 ml each in a microfuge tube) by microcentrifugation. The supernatant from the pelleted cells has been discarded for you.

MATERIALS

Escherichia coli DH5α (lac^- and $ampicillin^s$)
pUCll9 plasmid (lac^+ and $ampicillin^r$) **or** pRU4X92 (lac^- and amp^r) - See Fig. 10.1
MacConkey agar plates without and with ampicillin (50 ug/ml)
Luria broth (LB), about 4 ml per tube (16x100mm tubes)
Microfuge tubes
0.1 M $CaCl_2$ (sterile) - ice cold
Micropipette and sterile tips

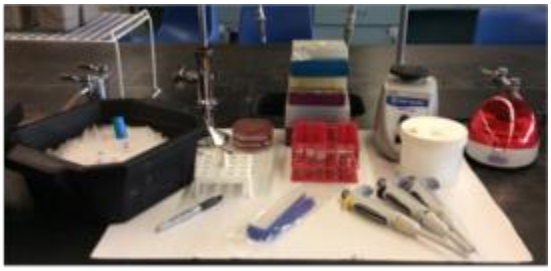


Figure 10.2 Molecular Microbiology Experiment Set Up

1. **Resuspend** the cells in 1 ml 0.1 M $CaCl_2$. Use a vortex mixer to get a smooth suspension. Place the tube on ice for about 10 min. See Appendix D for additional help with pipettes.
2. Pellet the cells in a microfuge for 2 min. Discard the supernatant, and drain the liquid from the pellet by inverting the tube over a paper towel.
3. **Resuspend** the cells in 100 µl cold $CaCl_2$. Use a vortex mixer again to get a smooth suspension. At this point, the cells should be "competent" and able to take up plasmid DNA from solution. Check that all the cells have been resuspended and none are still on the bottom of the microfuge tube. Add the 5 µl plasmid solution (~50 ng DNA) to the cell suspension. Mix with a flick or by pipetting up and down gently and place on ice for 30 min.
4. After the ice incubation period, place the microfuge tube in a 42˚C water bath for exactly 90 sec. This "heat shock" treatment will enhance the cells uptake of plasmid. Then cool the suspension by placing the tube on ice for 2 min.
5. To facilitate cell recovery from transformation, add growth medium - 0.5 ml LB broth - to the cell suspension. Mix gently by inverting the tube and incubate at 35˚C for 40 min.
6. After incubation, spread plate 0.1 ml of the cell suspension on each of the two formulations of MacConkey agar plates, one with ampicillin and one without. Plate 0.1 ml of the original culture on MacConkey-Ampicillin plates.
7. Incubate the cultures at 35˚C for 20 hrs until colonies are visible. If lactose is fermented colonies will be maroon; if not, colonies are colorless or nearly colorless. Neutral red, the pH indicator used in MacConkey agar, is maroon below pH 6.8 and amber above pH 8.0. When lactose is fermented, acid is

produced and the pH goes below 6.8. Organisms that cannot ferment lactose are still able to grow on MacConkey agar; however, they must use a different carbon source. The only other carbon source is the carbon skeletons of the amino acids provided by the peptone. The amino acids are deaminated making their carbon skeletons available. The amino groups, released as ammonium, cause the pH to increase. Using this information, explain the color changed observed.

WEEK TWO

8. Record and interpret your results.

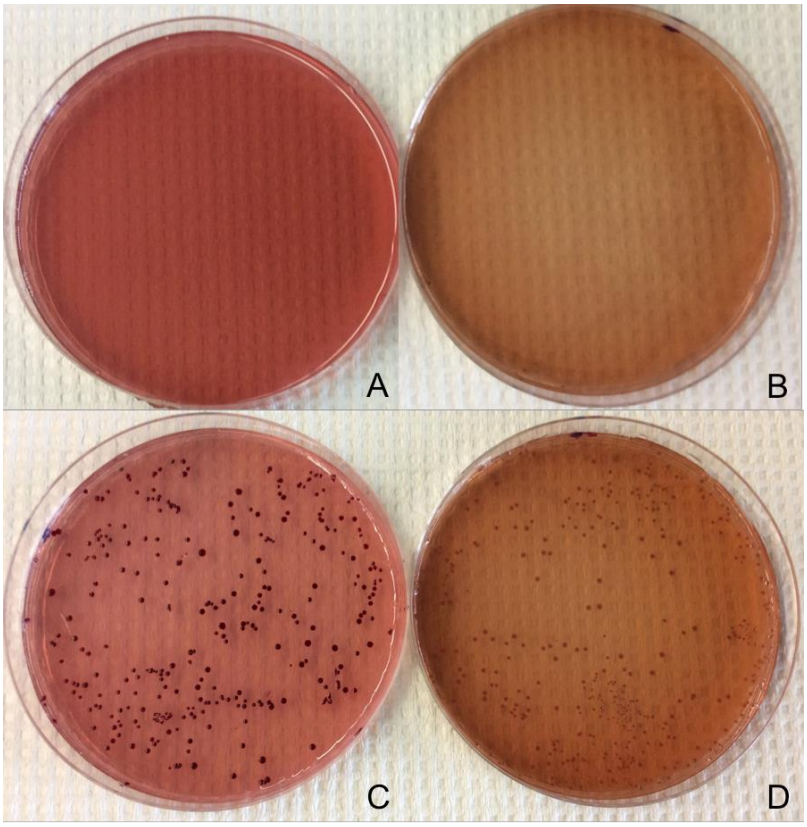


Figure 10.3 MacConkey Agar. A: Unioculated MacConkey Agar; B: MacConkey Agar withour Ampicillin; C and D: MacConkey with Ampicillin. Which cells were inoculated on Plates B, C, and D?

(11) ISOLATION OF PLASMID DNA

A plasmid (pRU4X92 or pUC119) will be isolated from DH5α cells. The plasmid pRU4X92 was made by inserting foreign DNA into plasmid pUCll9. The plasmid pUC119 carries several genes, the most important for our experiment are the genes responsible for ampicillin resistance and for lactose fermentation. The *lacZ*' gene (codes for part of the enzyme beta-galactosidase) contains a multiple cloning site (MCS), an innovation of the pUC series of plasmids. The *lacZ*' gene has been manipulated cleverly to encompass many restriction enzyme sites (MCS). These characteristics contribute to the usefulness of the plasmid as a cloning vector. Ampicillin in the growth medium acts as a selective agent as well as acting to prevent the spontaneous loss of plasmid from the cell; Lac^+ cells (b-galactosidase) ferment lactose to produce acid generating maroon colonies on the MacConkey medium we are using. [Note: These cells will also cleave the chromogenic substrate (blue X-gal or Bluo-gal) to yield galactose and a blue dye, thus generating blue colonies. This blue/white differentiation is analogous to the maroon/white differentiation in our experiment except that it is much more expensive.] When pieces of foreign DNA are cloned into the *lacZ*' gene of the plasmid, the ability to ferment lactose is lost. Cells transformed with such a plasmid (e.g. pRU4x92) yield white colonies (i.e. no acid production) and no maroon color on MacConkey agar.

In this part of the exercise, your first objective will be to rupture the cell wall and membranes using alkaline lysis, allowing chromosomal DNA and plasmid DNA to spill out into the suspending medium. Plasmid DNA, the subject of this exercise, is a minor component, and will be separated from the large amounts of chromosomal DNA, proteins, and other cell debris by differential precipitation with alcohol.

This has already been done for you: Transformed cells were harvested from 1.5 ml of the 24-hr culture of *E. coli*: centrifuged for 1 min in a microfuge tube and the supernatant discarded, retaining the pellet.

MATERIALS

***Escherichia coli* DH5α with pUC119 or pRU4X92 plasmid** - 24-hr culture in Luria-ampicillin (50 µg/l) broth.

Solution I (suspending medium)-50 mM glucose (osmotic support); 25 mM Tris-HCl pH 8; 10 mM EDTA (ethylenediaminetetracetic acid), pH 8

Solution II (lysis solution) - 1% sodium dodecyl sulfate (SDS) in 0.2 N NaOH.

Solution III (neutralizing solution) - potassium acetate buffer (29.4 g potassium acetate, 12 ml glacial acetic acid, water to 100 ml).

Ethanol, 100% and 70%.

Micropipettes and sterile tips.

Sterile microfuge tubes.

1. **Resuspend** the pelleted cells in 150 µl solution I. Use a vortex mixer to make sure the cells are suspended evenly.
2. Add 300 µl Solution II (SDS-NaOH) to the cell suspension. Invert the tube rapidly five times to mix (**do not vortex**). Place the tube on ice for 5 min. As the cells lyse, the suspension will become clearer. (The combination of SDS and NaOH causes the bacterial cells to lyse).
3. Add 225 µl Solution III (potassium acetate buffer, the neutralizing solution). Mix

the tube contents gently by inverting the closed tube a few times times.

4. Centrifuge for 10 min to separate the precipitated chromosomal DNA and cell debris from the soluble plasmid DNA. (In this neutralizing step, potassium acetate causes plasmid DNA to reanneal rapidly and remain soluble while most of the chromosomal DNA and bacterial proteins precipitate, as does the SDS, which forms a complex with potassium).
5. Transfer 400 µl (with a micropipette) of the supernatant solution containing the plasmid DNA into a clean sterile microfuge tube taking care not to disturb the bulky gelatinous precipitate. Discard the old microfuge tube along with this precipitate.
6. Precipitate the plasmid DNA from solution with 800 µl of 100% ethanol (800 µl = 2X the 400 µl of plasmid, this is often written "add two volumes"). Mix tube contents by inverting several times and then leaving undisturbed for five min at room temperature.
7. Centrifuge for five min to collect the precipitated plasmid in the bottom of the tube. Position the hinge on the cap in the "up" position; this way you can tell where the plasmid precipitate will collect. It will be on the "top" (hinge side) of the tube. **Carefully** decant and discard the supernatant solution.
8. Wash the precipitated plasmid with 200 µl 70% ethanol. To do this mix gently by inverting the tube a few times after adding the 70% ethanol. Centrifuge for five min to re-pellet the plasmid. Decant the ethanol wash carefully without disturbing the plasmid pellet. Invert the open tube on a paper towel to remove the last traces of ethanol. Allow the pellet to dry at room temperature until the next laboratory period.

Figure 11.1 Tube "Hinge Up"

(12) DIGESTION OF PLASMID DNA WITH RESTRICTION ENZYMES AND GEL ELECTROPHORESIS

In this part of the exercise, plasmid DNA isolated in Ex. 12 above will be digested with endonucleases, HindIII and/or EcoRI. The purpose of the restriction digests and subsequent gel electrophoresis is to determine which plasmid (pUC119 or pRU4x92: shown in Figure 11.1) you and your partner isolated last week.

HindIII is produced by the bacterium *Haemophilus influenzae* and recognizes the following sequence:

5′ AAGCTT 3′
3′ TTCGAA 5′

EcoRI is produced by strains of *Escherichia coli* and recognizes the following sequence:

5′ GAATTC 3′
3′ CTTAAG 5′

Both of these sequences are found in the multiple cloning site of the plasmids pUC119 and pRU4X92. The plasmid maps shown below illustrate the sites at which each of the endonucleases can be expected to cleave the circular DNA. As a result of these cleavages, circular DNA becomes a linear piece if one endonuclease is used or two linear pieces if both enzymes are used.

PREDICTIONS

Take a look back at the plasmid maps. How many fragments would arise from each digest? How long would those fragments be?

Plasmid	EcoRI Digest	HindIII Digest	EcoRI and HindIII Digest
pUC 119			
PRUx492			

These DNA pieces can be separated using agarose gel electrophoresis and visualized with ethidium bromide. Electrophoresis can be generally defined as the separation of charged particles in an electric field. It is an extremely useful tool that may be used to separate amino acids, proteins, substituted carbohydrates as well as nucleic acids. Electrophoresis is usually but not always performed in a solid support. Agarose (1%) is an appropriate solid support for DNA fragments of the size that will be generated by our restriction digests.

DNA will move through agarose gel when current is applied at a rate determined largely by the size of the molecule and by its shape (as the charge/mass on any piece of DNA is essentially equal). In general, small molecules move faster than large ones; compact molecules move faster than loose ones. Thus plasmids with closed circular DNA move faster than those of the same size but with open circular

DNA (one strand nicked by an endonuclease). The size of linear plasmid DNA can be estimated by comparison to linear DNA of known size. The direction of movement through the gel is determined by the charge (negative or positive) on the molecule. Since DNA is an anion (negatively charged), it will move toward the anode (positive terminal).

In this exercise, you will prepare agarose gels for electrophoretic analysis of DNA. Samples to be analyzed will be placed in wells in the gel. When current is applied in the presence of electrolyte buffer, the differently sized pieces of DNA in the mixtures will gradually separate into bands. Ethidium bromide (EtBr) will be included in the gel when it is prepared. DNA in the gel will react with the ethidium bromide and fluoresce when exposed to ultraviolet light. DNA will show up as fluorescent bands which can be compared to controls of uncut plasmid DNA and to molecular weight standards. Please study the gel picture on the web site so you have some familiarity with expected results.

MATERIALS – PLASMID DIGEST

Plasmid preparation from Exercise 11.
Sterile H_20
Restriction enzyme HindIII in buffer.*
Restriction enzyme EcoRI in buffer.*
A combination of restriction enzymes HindIII and enzyme EcoRI in buffer.*
Micropipettes and sterile tips.

Figure 12.1 Lab Bench Set Up

*The restriction enzymes have been diluted into appropriate buffer (provided with the enzyme when purchased) so that 10 ml provides appropriate endonuclease activity to digest an equal volume of the plasmid DNA.

PROCEDURE – PLASMID DIGEST

1. Working with your partner, dissolve your isolated plasmid DNA (prepared last week) in 50 µl sterile ddH_2O. To do this, add 50 µl sterile ddH_2O (you will find a microfuge tube containing ddH_2O in the microfuge rack on your mat) to your plasmid tube from last week. Your plasmid tube will look empty – THAT'S OK!
2. After adding the sterile ddH_2O, "pulse spin" the tube to bring all the liquid to the bottom of the tube. Your TA will demonstrate this for you.
3. In your ice bucket you will find three tubes - "E", "H" and "EH" containing 10 µl of digestion reaction mixes for EcoR1, HindIII or a double EcoR1 + HindIII digest. Add 10 µl of plasmid to each of these tubes.
4. After adding the plasmid to the digest tubes, "pulse spin" the tube to bring all the liquid to the bottom of the tube as in #2.
5. Incubate the digest tubes at 37°C for 40-45min.
6. At the end of the incubation time, stop the reaction by adding 2 µl of GLB (gel loading buffer) to each tube.

Figure 12.2 Floatie Incubating in Water Bath

MATERIALS – GEL ELECTROPHORESIS

Plasmid digests
DNA standards (lambda phage digest)
Agarose
10X TAE buffer with l0mM acetate
GLB (Gel loading buffer containing tracking dye, 0.25% bromophenol blue in glycerol or 40% sucrose).
Ethidium bromide IT IS A MILD CARCINOGEN!
Ultraviolet light box and goggles
Electrophoresis equipment - gel boxes and power supplies

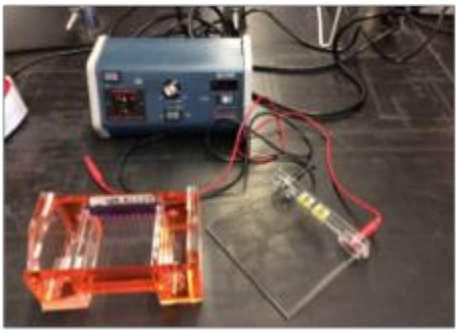


Figure 12.3 Gel Electrophoresis Set Up

PROCEDURE – GEL ELECTROPHORESIS
WEAR GLOVES WHEN HANDLING GEL BOXES!

1. You need to make 350 ml of 1X TAE from the 10X TAE you have been given. This will be enough for pouring and running the gel.
2. There will be one or two pairs of students per gel, depending on the number of students in each section. On each mat there is an Erlenmeyer flask containing the appropriate amount of agarose to prepare 40 ml of a 1% agarose gel. How many grams of agarose are in the flask? Add 40 ml of 1X TAE to the Erlenmeyer flask. Dissolve the gel using the microwave oven. Start with 1 min @ high power - swirl and continue with 10 sec at a time until gel is **completely** dissolved.
3. While the gel is being dissolved set up the casting tray as described by your instructor.
4. Cool the dissolved gel slightly and then ask your instructor to add 5 µl ethidium bromide (EtBr). Adding the EtBr directly to the gel will allow us to follow the progress of the electrophoresis and generate less EtBr contaminated waste.
5. Pour the gel according to instructions. **Don't forget the comb!**
6. After the gel has set, rotate the tray 90°, and place it in the electrophoresis chamber (gel box). **Make sure the gel is oriented correctly.** Remove the comb carefully.
7. Add running buffer (TAE) to cover the gel slab to depth of 2-3 cm.
8. **Prepare uncut plasmid by adding 2 µl of GLB to the remaining 20 µl of your plasmid prep.** You will load ~15 µl of sample. Use a micropipette to deliver each sample, your instructor will demonstrate the proper method. Two groups will be sharing a gel.

Use the following guidelines for well assignments:

Well #	Sample	
1 uncut plasmid	~15 µl	**Samples** – **Group 1**
2 EcoR1 digest	~15 µl	
3 HindIII digest	~15 µl	
4 Double digest	~15 µl	
5 lambda (**I**) DNA size markers	5 µl	(Loaded by your instructor)
6 uncut plasmid	~15 µl	**Samples** – **Group 2**
7 EcoR1 digest	~15 µl	
8 HindIII digest	~15 µl	
9 Double digest	~15 µl	

9. After the samples have been placed in the gel, attach the terminal from the gel box unit to the DC power supply, red to red and black to black. Your instructor will help with starting the gel.
10. Count the bands for each sample and note their relative location. Compare your results the predictions table and identify your isolated plasmid.

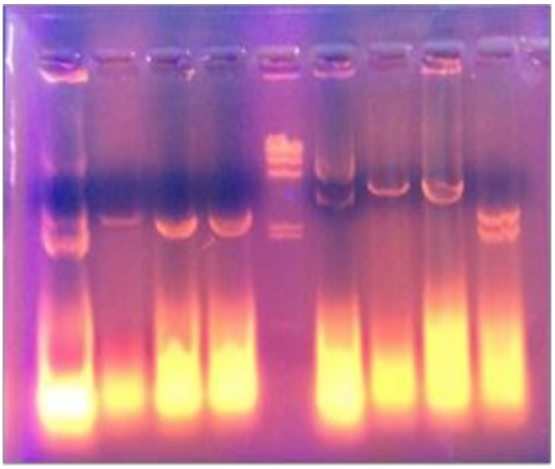


Figure 12.4 Results for Plasmid Isolation. Can you tell which plasmids were isolated?

<u>CAUTION</u>: High Voltage. Do not touch the equipment while it is plugged in and running.

<u>CAUTION</u>: Wear UV protective goggles.

APPENDIX A

List of Risk Level 1 Organisms Used During the Semester

Bacillus subtilis
Bacillus atropheaus
Citrobacter freundii
Escherichia coli DH5α
Escherichia coli K12
Enterobacter aerogenes
Enterobacter cloacae
Enterococcus durans
Geobacillus stearothermophilus
Halobacterium salinarum
Micrococcus luteus
Micrococcus roseus
Pseudomonas fluorescens
Rhodococcus rhodochrous
Saccharomyces cerevisiae
Sarcina aurantiaca
Serratia marcescens
Spirillium volutans
Staphylococcus epidermidis
Staphylococcus saprophyticus
Streptococcus salivarius

APPENDIX B

Microbiology Vocabulary

Aseptic Technique: A technique used to handle a sterile substance without introducing contamination

Colony: Visible mass of bacteria, derived from one single cell or colony forming unit

Colony Forming Unit (CFU): Each colony consists of the progeny of a single cell.

Isolated Colony: A colony that is NOT touching any other colonies on the growth medium

Culture: Bacteria or other living organisms growing in a tube, plate, flask, bottle or any format of medium

Mixed Culture: Culture with a mixed population of microorganisms

Pure Culture: Culture that contains only one type of microorganism

Subculture: A new culture made by transferring cells (colony) from a previous culture to fresh growth medium

Incubate: To hold a culture (solution) UNDER CONTROLLED CONDITIONS

Inoculate: To purposely introduce bacteria or other organisms into sterile medium in order to grow and study them

Medium (Media): Surface/Liquid that provides nutrients for growth of bacteria; contains ions, amino acids, carbohydrates, fats, vitamins, water (liquid, solid or semi-solid form), may also contain other "enriching" ingredients

General Purpose Medium: Provides general nutrition and supports a wide range of microorganisms.

Selective Medium: Used to **SELECT (ISOLATE)** specific groups of bacteria (Include substrates to enhance the growth of one type, but inhibit the growth of another type of microorganism.) Selects for the growth of a group of organisms with a common trait e.g. antibiotic resistance.

Differential Medium: Used to **DIFFERENTIATE (TELL APART)** different types of related bacteria**;** Includes compounds that produce a characteristic change in the appearance of bacterial growth and/or the medium surrounding the colonies.

Agar: A complex polysaccharide that can be added to any kind of medium to make it solid**.** Remains solid at higher temperatures and few bacteria can digest it.

Fluid Thioglycolate Broth: Used to cultivate anaerobic microorganisms. Strict aerobes tend to grow in a thin layer at the surface of the medium; obligate anaerobes will only grow in the portion below the oxidized layer.

MacConkey Agar: A selective and differential medium used for the isolation (selection) of Gram-negative organisms and differentiation of lactose-fermenting (red colonies) and lactose-nonfermenting organisms (clear colonies)

Metabolism: The Sum of all reactions taking place in an organism.

Aerobic Respiration: A chemical process found in many microorganisms; uses oxygen as the final electron acceptor.

Anaerobic Respiration: A chemical process found in many microorganisms that does NOT use oxygen as the final electron acceptor; e.g. Arsenic, Selenium, Nitrate, etc. may be used as final electron acceptors.

Fermentation: A survival mode in the absence of oxygen to continue to make reduced amounts of energy.

Plasmid: Piece of extrachromosomal DNA, usually circular, that replicates autonomously and is not essential for growth.

Plasmid map: Map of known restriction sites within the DNA sequence of a plasmid

Restriction Enzymes: An enzyme that cuts DNA at a specific recognition site (restriction site).

Restriction Site: Location on a DNA molecule that contains the specific sequences of nucleotides recognized by a restriction enzyme.

Restriction digest: Cutting plasmid DNA within specific restriction site

Resazurin: An indicator for the presence of oxygen (will turn pink) used in microbiological media, e.g. thioglycollate broth.

Species: A collection of microbial strains that share many properties and differ significantly from other groups of strains

Strain: A population of microbes descended from a single organism; different strains represent genetic variability within a species.

Transformation: Transfer of genetic material (Free DNA – Plasmids) into living cells

Triphenyltetrazolium Chloride (TTC): Redox indicator that is added to medium as a visualization aid. Bacterial cells will metabolize nutrients in the medium, reducing TTC and producing a maroon color.

APPENDIX C

Alphabetical Listing of Culture Media (All measurements in g/L). Recipes taken from the Difco Manual (5).

Luria Broth (LB)

Tryptone	10
Yeast Extract	5
Sodium Chloride	5
Agar	15

1. Dissolve 20 g of the powder in 1 L of purified water: mix thoroughly.
2. Heat the agar medium with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes.

MacConkey Agar

Bacto Peptone	17
Protease Peptone, Difco	3
Bacto lactose	10
Bacto Bile salts No. 3	1.5
Sodium Chloride	5
Bacto Agar	13.5
Bacto Neutral red	0.03
Crystal Violet	0.001

1. Suspend 50g in 1 liter distilled water.
2. Heat to boiling with gentle swirling to dissolve completely.
3. Sterilize in the autoclave for 15 min @ 15 PSI (121˚C). Avoid overheating of the medium.
4. Cool to 45-50˚C and aseptically dispense in approximately 20 ml amounts into sterile Petri dishes.
5. Note: For MacConkey Agar + amp, add filter sterilized ampicillin to cooled agar for a final concentration of 50 mg/ml.

Methyl Red - Voges-Proskauer Medium (MR-VP)

Buffered Peptone	7
Di-potassium phosphate	5
Bacto Dextrose (glucose)	5

1. Dissolve 17g in 1 liter distilled or deionized water.
2. Distribute into test tubes in 10 ml amounts.3. Sterilize in the autoclave for 15 min @ 15 PSI (121˚C).

Mueller Hinton Agar

Beef Extract Powder	2.0
Acid Digest of Casein	17.5
Starch	1.5
Agar	17.0

1. Suspend 38 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve powder.

3. Autoclave at 121°C for 15 minutes. DO NOT OVERHEAT.
4. Pour cooled Mueller Hinton agar into sterile Petri dishes on a level, horizontal surface to give a uniform depth of about 4 mm (60-70 mL of medium for 150 mm plates and 25-30 mL for 100 mm plates) and cool to room temperature.4
5. Check prepared medium to ensure the final pH is 7.3 ± 0.1 at 25°C.
6. Test samples of the finished product for performance using stable, typical control cultures.

Mycophil

Papaic digest of soybean meal	10
Dextrose (glucose)	10
Agar	18

1. Suspend 38 g in 1 liter distilled water and heat to boiling to dissolve completely.
2. Sterilize in autoclave for 15 min @ 15 PSI (121°C).
3. Cool the medium to 45-50°C and dispense into sterile Petri dishes.

Final pH 4.7 +/- 0.2 @ 25°C.

Nutrient Agar

Beef extract	3
Peptone	5
Agar	15

1. Suspend 23 g in 1 liter distilled or deionized water and heat to boiling to dissolve completely.
2. Sterilize in the autoclave for 15 min @ 15 PSI (121°C).
3. Dispense as desired.

Final pH 6.8 +/- 0.2 @ 25°C.

Phenol Red Lactose/Glucose/Mannitol Broth

Pancreatic digest of casein	10
Lactose/Glucose/Mannitol	5
Sodium chloride	5
Bacto Phenol Red	0.018

1. Suspend 20 g of Phenol Red Lactose/glucose broth in 1 liter distilled water and stir to dissolve completely.
2. Dispense into tubes containing Durham (fermentation).
3. Sterilize in the autoclave for 15 min @ 15 PSI (121°C). The minimum amount of heat required for complete sterilization is preferred. By packing tubes loosely in the autoclave to allow free circulation of steam the time required may be appreciably shortened, provided the temperature in the autoclave is 121°C.

SIM Medium (Sulfide, Indole, Motility)

Pancreatic Digest of Casein	20.0
Peptic Digest of Animal Tissue	6.1
Ferrous Ammonium Sulfate	0.2
Sodium Thiosulfate	0.2
Agar	3.5

1. Suspend 30 g in 1 liter distilled water and heat to boiling to dissolve completely.

2. Heat with frequent agitation and boil for 1 min to completely dissolve the powder.
3. Dispense and autoclave for 15 min @ 15 PSI (121°C).
4. Test samples of the finished product for performance using stable, typical control cultures.

Simmons Citrate Agar

Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Agar	15
Bromothymol Blue	0.08

1. Suspend 24.2 g in 1 liter distilled or deionized water and heat to boiling to dissolve completely.
2. Sterilize in the autoclave for 15 min @ 15 PSI (121°C).
3. Medium is usually prepared as agar slants. Final pH 6.8 +/- 0.2 @ 25°C.

Trypticase Soy Agar (TSA)

Tryptone (pancreatic digest of casein)	15
Soytone (papaic digest of soybean meal)	5
NaCl	5
Agar	15

1. Suspend 40 g in 1 liter distilled or deionized water and heat to boiling to dissolve completely.
2. Sterilize in the autoclave for 15 min @ 15 PSI (121°C). Cool to 45-50°C.
3. Dispense as desired.
   Final pH 7.3 +/- 0.2 @ 25°C.

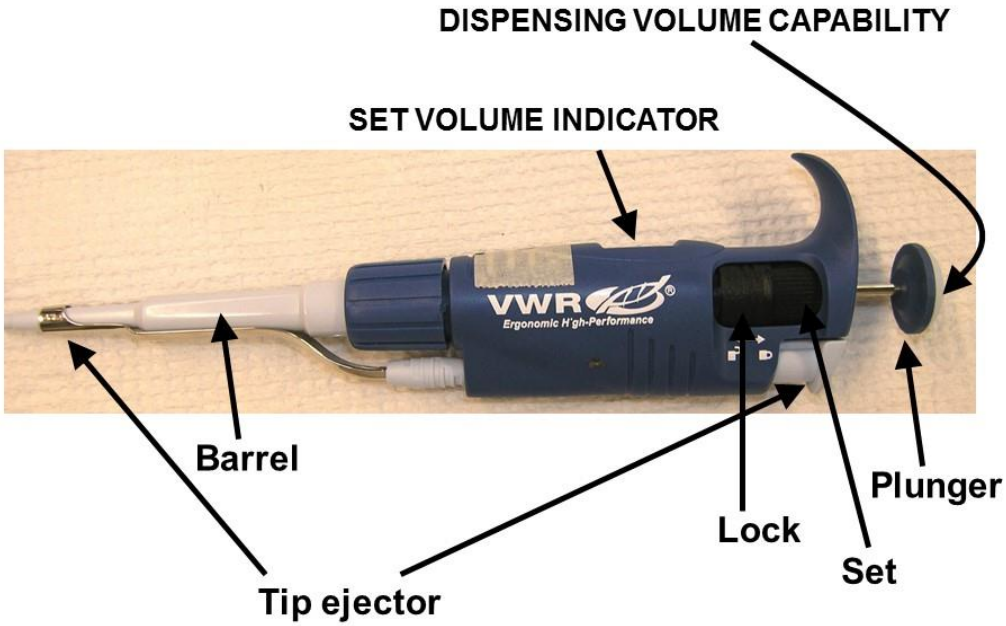
Trypticase Soy Broth (TSB)

Pancreatic digest of casein	17
Enzymatic digest of soybean meal	3
NaCl	5
Dextrose	2.5
Dipotassium phosphate	2.5
Final pH 7.3 +/- 0.2 @ 25°C.	

1. Dissolve 30 g in 1 liter of distilled or deionized water. If necessary, warm slightly to dissolve completely.
2. Dispense as desired.
3. Sterilize in the autoclave for 15 min @ 15 PSI (121°C).

APPENDIX D

GENERAL FEATURES OF MICROPIPETTES

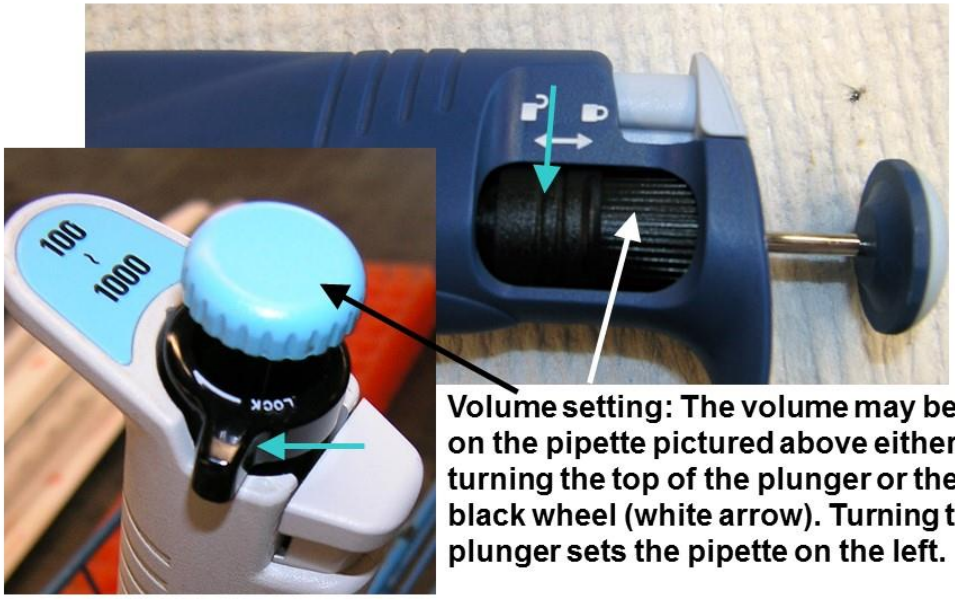


DISPENSING VOLUME CAPABILITY

Volumes are always shown in microliters (µl). The symbol used by the manufacturer - squiggly line & division sign on the micropipettes on the left – are arbitrary. Both micropipettes in the picture on the left dispense from 100 – 1000 µl.
The pipette below dispenses 5 – 50 µl.

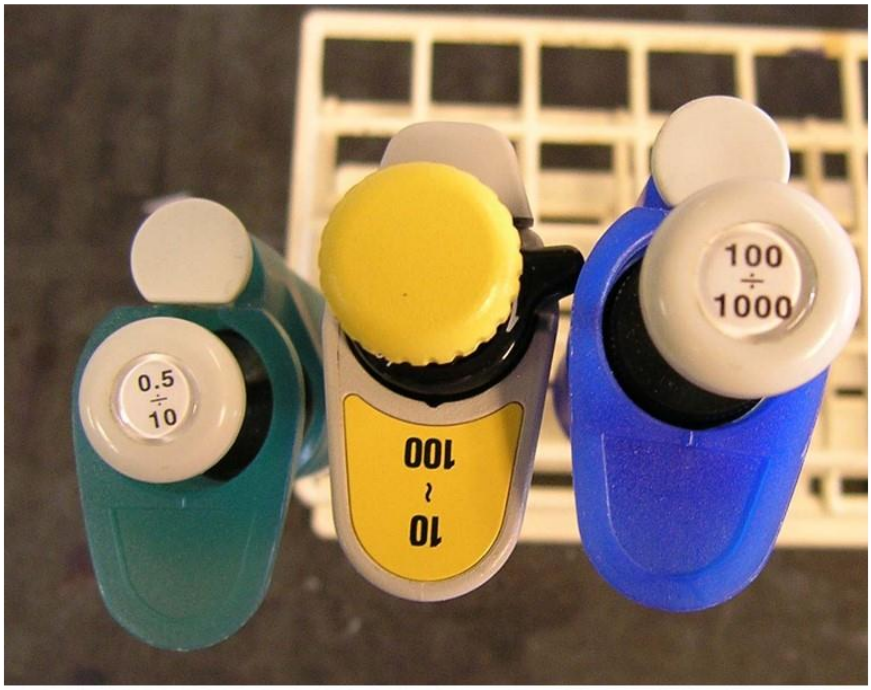
Locking Mechanisms

Both micropipettes shown have locking mechanisms (small blue arrows). This prevents the volume from changing while pipetting. You must unlock before you can set the volume. Once set the pipette is locked.



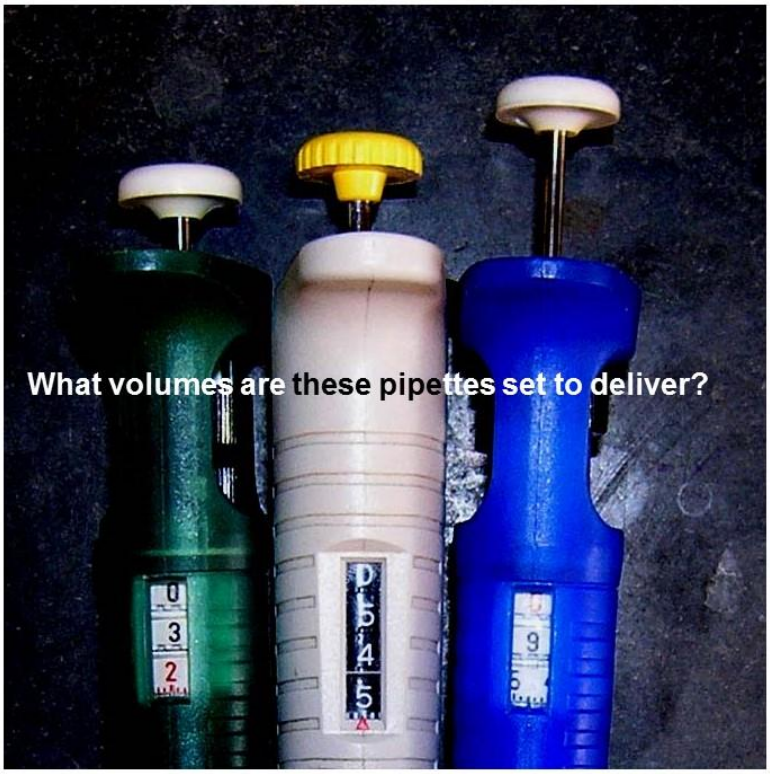
Volume setting: The volume may be set on the pipette pictured above either by turning the top of the plunger or the black wheel (white arrow). Turning the plunger sets the pipette on the left.

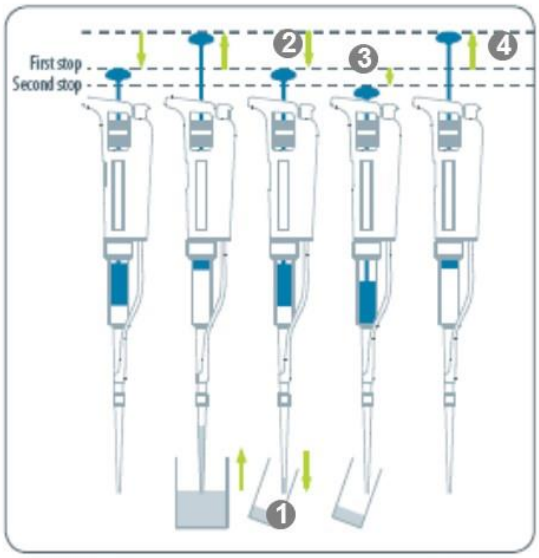
SETTING VOLUMES: What volume range is each of the micropipettes pictured designed to dispense?



The micropipettes on the left are set to dispense the maximum volume each pipette is able to deliver – 10 µl, 100 µl and 1000 µl – from left to right. Note the plunger rods are long when the pipette is at or near maximum volume.

The pipettes on the right are set to dispense the minimum volume each pipette is able to deliver – 0.5 µl, 10 µl and 100 µl – from left to right. Note the plungers are short when the pipette is at or near minimum volume.

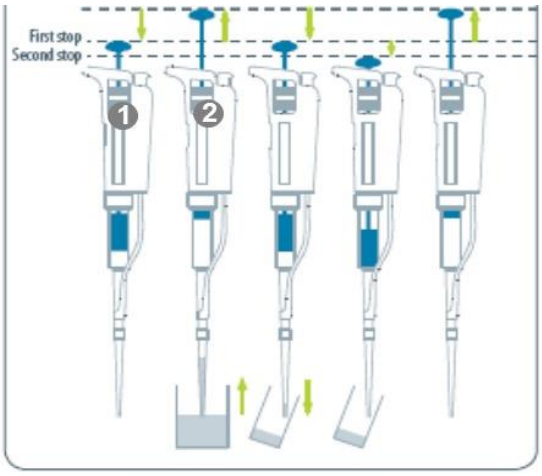




pipetman CLASSIC™

Dispense

1. Place the end of the tip against the inside wall of the recipient vessel (at an angle of 10° to 40°).
2. Press the push-button slowly and smoothly to the **first stop**.
3. Wait for at least a second, then press the push-button to the **second stop** to expel any residual liquid from the tip. Keep the push-button pressed fully down and (while removing the pipette) draw the tip along the inside surface of the vessel.
4. Release the push-button, smoothly.



pipetman CLASSIC™

Aspirate

1. Press the push-button to the **first stop** (this corresponds to the set volume of liquid).
2. Hold the pipette vertically and immerse the tip in the liquid (see immersion depth table, page 7). Release the push-button slowly and smoothly (to top position) to aspirate the set volume of liquid. Wait one second (time depends on model, see table) ; then withdraw the pipette-tip from the liquid. You may wipe any droplets away from the outside of the tip using a medical wipe, however if you do so take care to avoid touching the tip's orifice.

Micropippette instructions (above) from Gilson's Pipetman® P User's Guide (6).

APPENDIX E

Microscopy Pocket Guide

Brightfield

Focus

1. Place slide securely into slide clip.
2. Rotate nosepiece to 10X objective lens; click into place making sure edge of coverslip (or stained image) is in place under objective opening.
3. While looking AT the microscope, using the COARSE focus, raise stage carefully until the stop setting.
4. Raise condenser to the highest position, set condenser dial to “0” and close the condenser diaphragm.
5. While looking through the microscope, slowly lower the stage (using coarse focus) until image comes into or nearly into focus. NOTE: To lower the stage, turn coarse focus toward you.
6. Use fine focus to sharpen image.

Maximum Resolution

7. While looking through the microscope close down field diaphragm until edges come into view.
8. Adjust condenser height to sharpen the edges of the diaphragm (sharp decagon visible). Center as necessary.
9. Open field diaphragm until the edges of the decagon are at the edge of the field.
10. Adjust fine focus as necessary.
11. Adjust condenser diaphragm and dim light as necessary.

Increasing Magnification

12. While looking at the microscope, rotate nosepiece so that the 40X objective is in place.
13. While looking through the microscope, focus image: minimal adjustment of the fine focus should be required. NOTE: If image looks cloudy or nothing appears to be in the field of view, clean the oil off the 40x objective and repeat step 13.
14. Once in focus at 400X total magnification (40X objective), rotate nosepiece so no objective is in place.
15. Place a small drop of oil on slide; rotate nosepiece until 100X objective is in the oil and clicked into place.
16. Use fine focus to sharpen image.

PHASE CONTRAST

Focus

1. Place slide securely into slide clip.
2. Rotate nosepiece so that 10X objective lens clicks into place – make sure edge of coverslip is in place under objective opening.
3. While looking AT the microscope, using the COARSE focus, raise stage carefully until the stop setting.
4. Raise condenser to the highest position; set condenser dial to “10” or “PH1.”
5. While looking through the microscope, slowly lower the stage (using coarse focus) until image comes into or nearly into focus. NOTE: To lower the stage, turn coarse focus toward you.
6. Use fine focus to sharpen image.

Maximum Resolution

7. While looking through the microscope, close down field diaphragm until edges come into view.
8. Adjust condenser height to sharpen the edges of the diaphragm (sharp decagon visible).
9. Open field diaphragm until the edges of the decagon are at the edge of the field.
10. Adjust fine focus as necessary.

Increasing Magnification

12. While looking at the microscope, rotate nosepiece so that the 40X objective is in place.
13. Change the condenser dial setting to “40” or ‘PH2.”
14. Minimal adjustment of the fine focus only should be required. NOTE: If image looks cloudy or nothing appears to be in the field of view, clean the oil off the 40x objective and repeat step 13.
15. Once in focus at 400X total magnification (40X objective), rotate nosepiece so no objective is in place.
16. Place a small drop of oil on slide; rotate nosepiece until 100X objective is in the oil and clicked into place.
17. Change the condenser dial setting to “100” or ‘PH3.”
18. Use fine focus to sharpen image.

APPENDIX F

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4. Waksman S. 1947. What is an Antibiotic or Antibiotic Substance? Mycologia 39:565-569.
5. Becton Dickinson. The Difco Manual. http://www.bd.com/ds/technicalCenter/inserts/difcoBblManual.asp.
6. Gilson. 2009. Pipetman ® P User's Guide, France.

Supplemental Material:

- Nikon Microscopy U, The Source for Microscopy Education.https://www.microscopyu.com/techniques/phase-contrast
- Olympus Microscopy Resource Center.http://www.olympusmicro.com
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- Gottschal, J.C., W. Harden, and R.A. Prins. 1992. *Principles of Enrichment,Isolation, Cultivation, and Preservation of Bacteria.* In A. Balows, H.G. Truper, M.Dworkin, W. Harden, and K.H. Schleifer. *The Prokaryotes.* 2d ed. New York:Springer-Verlag.
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