Biology 325: Introductory Microbiology Laboratory Manual

Centre for Science
Athabasca University

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Introduction

Microbiology is the study of organisms that are not visible with the unassisted eye. Over time, various techniques and processes have developed to gain information about their structure, growth, and metabolic functions in humans, animals, and the environment. As such, results from observations obtained by applying skills in the laboratory help us to understand how microorganisms interact with the world around us, such as health and disease, and nature. As you proceed through the laboratory exercises in this course, you will learn to use various essential applied skills that will contribute to the understanding of microbiology.

The exercises presented in this manual will provide you with necessary information that will assist you in practising basic microbiological techniques. You will also learn of the importance of conducting safe practice in the laboratory. More information on biosafety is presented in the following section of the manual. As you proceed through the various exercises, you will find that there are specific skills that are unique to this area of science. However, there are also techniques that you can take with you, should you proceed in other fields of study in science.

It is important to take the time to read the laboratory manual carefully before working through the exercises. This will allow you to plan each exercise in advance, and consult your textbook and Laboratory Atlas to familiarize yourself with the techniques and organisms.

In addition to this laboratory manual, please bring the following items: the Biology 325 required textbook, the Colour Atlas, and a calculator. If you have an electronic device that can take pictures, you can also bring that in for recording results of certain experiments. You are not required to bring a lab coat as we will provide this for you.

Learning Outcomes

After completing these labs, you will

1. Demonstrate familiarity and competency with a wide variety of microbiological laboratory techniques, including transfer, culture, isolation and identification, growth rates and antibiotic sensitivity.
2. Define immunity and understand the mechanisms of the immune response.
3. Describe the effects of microorganisms on the environment.
4. Outline the applications of microorganisms in the food industry, biotechnology, industrial processes, and the development of medical treatments.

Biosafety Laboratory Practice and Procedures

The Athabasca University teaching laboratory is a licensed facility which is regulated by the Public Health Agency of Canada (PHAC). The laboratory is classified as a Level 2 Containment facility. This means that the facility handles and stores organisms that are classed as Risk 2 organisms. According to the Canadian Biosafety Standard (Third Edition)[1], 2022, the organisms in this group can cause moderate risk to an individual and low public safety risk. It is therefore imperative that appropriate measures are in place to ensure that the safety of individuals who work in the laboratory is maintained. The laboratory staff are well-versed in compliance of biohazard control and will ensure the safety of students taking these labs.

As part of ensuring awareness of laboratory safety, students are asked to undertake an online WHMIS (Workplace Hazardous Materials Information Systems) course before engaging in experiments in the laboratory. You will also be informed of additional information and demonstrated safety skills at the beginning of the laboratory session and throughout each session.

Should there be an accident at any time during the laboratory sessions, it is imperative that you inform a member of the teaching staff.

[1] https://www.canada.ca/en/public-health/services/canadian-biosafety-standards-guidelines.html

Laboratory Safety Guidelines

Because of their propensity to grow and proliferate in different environments, microorganisms can easily contaminate surfaces, equipment, or people. It is therefore important that when you work with microorganisms, you conduct yourself in the laboratory safely and wisely. This will ensure that risk of potential infection or contamination will not occur.

Please carefully read the following laboratory safety guidelines below. If you require any clarification on these guidelines, please consult the laboratory staff.

1. Do not smoke, eat, or drink in the laboratory, or place any object on the laboratory bench which you could later transfer to your mouth. Keep your books, laboratory manual, and workbook at a reasonable distance from your work area or use the pullout shelf at your workspace. Keep electronic devices such as cell phones or iPads away from your work area. If you use these devices in the laboratory, make sure you decontaminate them by wiping with a tissue soaked in 70% ethanol before leaving the laboratory.
2. Practice good aseptic technique by performing the following before starting each class:

a. Wash your hands thoroughly with soap and water before starting your exercises.

b. Long hair should be tied back.

c. Wear closed footwear to protect the feet.

d. Wear a buttoned protective laboratory coat.

e. Never place any instruments or materials into your mouth.

f. Do not under any circumstances pipette by mouth. Use a pipette bulb or automatic pipet-aid provided.

g. Use disposable nitrile gloves as indicated by your instructor.

h. Decontaminate your work bench by applying the antiseptic wash provided for you.

3. Wear protective goggles (provided) when dealing with chemicals that are heated.
4. Contain contaminated spills by placing a paper towel soaked in 70% ethanol immediately. Keep the towel on the spill for 20 minutes. Inform your instructor of the spill. Place the towel in the autoclave bag provided. Ensure that you wash your hands immediately after dealing with the spill.
5. Note the location of the first aid kit, fire extinguishers, and emergency showers. Note the emergency exits in the laboratory.
6. Dispose pipettes that are used during class immediately after use into labeled containers of disinfectant.
7. Dispose glass slides that you have made yourself in the glass disposal boxes assigned to your work bench.

   Do not discard any demonstration slides.
8. Collect used bottles, tubes, and cultures and place them in the designated area for autoclaving.

9. Place contaminated waste (e.g., culture plates, swabs) into autoclave bags for disposal. All other waste can be disposed of in appropriately labeled containers or bins.
10. Do not place any hazardous or infectious materials in the sink. Do not dispose of solid material in the sink.
11. All materials required for incubation or refrigeration should be appropriately labeled and placed in the relevant containers or trays provided.
12. At the conclusion of each class clean the microscope provided for you. Use lens tissue and cleaner to clean the oil immersion lens (100X) and leave the microscope standing with the low power lens in place (this is called the park position). Avoid bumping or pushing the microscope on the bench.
13. At the conclusion of each session, tidy your laboratory bench and disinfect the work area using disinfectant or 70% ethanol.
14. Before leaving the laboratory, remove your lab coat and hang it on the back of your chair. Wash your hands thoroughly with soap and water.

Practical Class Schedule

DAY	EXERCISE	TOPIC
1	0	Biosafety practices and procedures
	1	Introduction to the microscope
	2	Microscopic slide techniques
	3	Streak plate method and bacterial isolation
	4	Aseptic technique
	Project (Day 1)	**Bacterial Identification Project**
2	5	Selective, enrichment, and differential media
	6	Differential tests
	7	Differential staining techniques
	Project (Day 2)	**Bacterial Identification Project**
3	8	Determination of bacterial growth
	9	Sterilization
	10	Chemical control of bacterial growth
	11	Bacterial transformation
	Project (Day 3)	**Bacterial Identification Project**
4	12	Environmental and food microbiology
	13	Medical microbiology
	14	Immunology
	Project (Final)	**Bacterial Identification Project**

Grading

Your final laboratory mark constitutes 20% of your final grade in the course. Your laboratory report constitutes 75% of your final lab grade. Your laboratory report will consist of your bacterial identification project, i.e., the tests you performed, the results of these tests, and your conclusions, including which bacterial species you identified. For the other 25% you will be marked on participation, completion of the exercises in your lab manual (10 marks), and lab skills. The lab skills you will be marked on will include Gram stain technique (5 marks), streak plate technique (5 marks), and set up of Köhler illumination (5 marks) of your microscope.

Bacterial Identification Project

The next few pages contain information needed to complete your bacterial identification project and write your lab report. The lab report consists of your bacterial identification project and the results you obtained. Students will receive a tube of two unknown bacteria on Day 1 (one Gram positive species and one Gram negative species) which they will identify over the course of the 4 days using several selective and differential media and biochemical tests, as determined by consulting the previous exercises, Tables 1 and 2, and the Dichotomous Key described in the following pages.

Bacterial Identification Project (Lab Report)

This project involves the isolation and identification of a mixed culture containing two unknown species of bacteria to the genus level. You will be required to go through the process of performing basic laboratory techniques to isolate a pure culture of each bacterial species, and to adopt a key according to the principles of classification of bacteria using Bergey's Manual of Determinative Bacteriology (9th edition) to identify each bacterial genus. Differential tests and media will be provided for you based on your reasoning and subsequent consultation with the lab instructor. You will continue with the project until the final day of the lab session.

The total mark for the report comprises 75% of your final lab mark and will be marked out of 100. The lab report should contain the following criteria:

1. **Abstract:** A summary of the project (200-250 words) that provides an overview of the results achieved and conclusions drawn from the exercise. **/10 marks**
2. **Introduction:** A brief introduction that clearly states the aim of your study. **/5 marks**
3. **Materials and Methods:** Outline the methods used to isolate each species and obtain a pure culture of each. You can cite the Laboratory Manual for listing of materials. **/10 marks**
4. **Results:** A written results section that outlines the interpretation of Gram stain reaction of each species, the streak plate results, colony morphology on the plate, microscope morphology, and the results of all of the differential tests and selective media that you obtained. **/50 marks**

   Use figures and tables where possible to illustrate the results. Figures may be hand drawn or taken from online resources if they are cited and referenced appropriately. Figures and tables should be numbered and titled and referred to in the text where they are described.
5. **Discussion:** A conclusion stating the names (genus, and species, if possible) of the two bacteria identified, a summary of important results according to the keys provided in your manual, and a brief description of each bacterium and their relevance or applications (i.e., medical, industrial, etc.) **/15 marks**
6. **References:** A reference list according to the American Society for Microbiology (ASM) format and citing of your sources in the text. Students are required to use a

minimum of 3 references (lab manual, Color Atlas, and textbook). Additional references may be used and are particularly helpful when writing about the relevance of each species identified. **/5 marks**

7. **Spelling/Grammar:** Students will be marked on scientific language, spelling, sentence, and paragraph structure, and using italics to indicate bacterial genus and species names. **/5 marks**

Examples of how to apply references using ASM format:

http://journals.asm.org/

Follow the link titled "Author Centre", "see all", and then "Writing your paper." There are good tips on format, style how to display figures and tables, and citation of references used in your report.

The lab manual should be referenced as:

Carter, L. and S.L. Zenteno. 2022. Biology 325 Laboratory Manual. Centre for Science. Athabasca University. Athabasca, Alberta, Canada.

The lab report will be due in 2 weeks following completion of the lab. You will be informed of the due date during the lab session. Lab reports are to be uploaded onto the Biology 325 Course Moodle Site. This site accepts a maximum of 10 MB so any reports larger will not be uploaded. Please try to compress any images or not include images of all results so that your file is not too large. If you are unable to upload the lab report please contact your Academic Expert (AE) or the course professor by emailing:

fst_success@athabascau.ca

Bacterial Identification Project (Procedure)

The purpose of this project is to provide you with the skills to follow an identification key to classify the species of two unknown bacteria. You will be provided with a mixture of two different microorganisms, one Gram positive organism, and one Gram negative organism. You will follow through the process logically and come to conclusions based on your results and the information provided in the identification key. You will perform streak plate techniques, Gram stain reactions, differential staining, and biochemical testing to identify your bacteria. You should record your observations and results each day. This information will be required for the identification process as well as for your lab report. Please note there will be time set aside in the afternoon of each day to work on your project.

Materials

Suspension of two unknown bacteria (note: one organism is Gram positive, and the other is Gram negative)
Nutrient agar plates (NA) and tryptic soy agar plates (TSA)
Slides
Gram stain reagents
Selective and differential media (MAC, EMB, SCA, SS, PIA, MSA)
Biochemical tests and reagents

Procedure

Day 1

1. Obtain a mixed culture tube from the rack assigned to this project. Record the ID number assigned to this tube in your lab manual.
2. Gently mix the tube containing the unknown culture. Make a Gram stain of the culture and examine the slide using your microscope. Observe the two different types of bacteria visible in the stain. Record the microscopic morphology for each organism, which includes the Gram reaction, cell shape, and cell arrangement.
3. Streak the suspension onto one NA and one TSA plate and incubate at 37ºC overnight (18 hours). Make sure you clearly label the plates with your name, tube number, and date.
4. Some students may be required to streak an extra set of plates for incubation at 24ºC (room temperature overnight).

Day 2

1. Examine the colony morphology on the representative plates. Describe the colour, diameter, shape, and edges of each colony type (consult your text or **Colour Atlas** for more information).
2. Select likely colonies that may be different for Gram stain determination and plating (you should have two separate colony types on your plates). **Please consult a lab instructor to verify that you have two different colony types.**

3. Perform Gram stains on a pure colony of each type or a portion of a colony type if

large enough. Record the microscopic morphology for each organism.

4. Streak a pure colony of each type or use the other portion of the colony to streak onto 1 NA or 1 TSA plate (total 2 NA or TSA plates, 1 plate for each colony type) and incubate at the required temperature overnight.
5. Save your culture containing your unknowns in case you require them again. They will be refrigerated overnight.

Note: You will observe a mixed population of Gram-negative and Gram-positive organisms on your plates, therefore, try to look at single colonies located at the final streak line of your plate to isolate individual colonies. Please ask your lab instructor if you have trouble identifying two colony types. You may be required to re-streak your plates. The main objective for Day 2 is to try to determine which colony type corresponds to which Gram stain result (keep track of your results) and to be able to isolate a pure culture of each organism by streaking them out onto separate plates.

Day 3

1. Examine the plates which were incubated overnight. You should have a pure culture of each organism. Perform Gram stains on each culture and streak each one onto 1 NA plate or 1 TSA plate again to confirm that you have a pure culture of each (total of 2 plates). Your Gram stains should confirm the presence of a pure culture of each. Record the microscopic and colony morphology.
2. Examine Tables 1 and 2, and the Dichotomous Key provided in this section to select likely tests that may be able to be performed on the pure culture isolates. **You may be able to conduct some of these tests following consultation with the instructor**.
3. Use streak plate technique to isolate your Gram-positive organism using the following plate: Mannitol Salt agar.
4. Use streak plate technique your Gram-negative organism using the following plates: McConkey (MAC) agar, Eosin Methylene Blue (EMB) agar, Simmon's Citrate agar. Depending on your unknown number you will be asked to either streak onto *Pseudomonas* isolation (PIA) agar or *Salmonella-Shigella* (SS) agar (your lab instructor will indicate which agar to use, and this may change depending on the lab session).
5. If you have a Gram-positive rod you may wish to save an older plate to conduct a spore stain.
6. Please consult your lab instructor to make sure you are on the right track.

Day 4

1. Examine your NA and TSA plates to confirm you have isolated the pure culture of each organism. Perform a Gram stain for each organism once again to confirm the reaction and purity of each. Make sure you record your microscopic and colony morphologies.
2. Examine all your differential and selective media to see the results for each of your organisms. Use Table 1 and Table 2, and the Dichotomous Key provided in

this section to make your conclusions. Make sure you describe all your plate results.

3. Perform any other tests relevant to the organism you may have, and other tables provided). Make sure you record your results and describe them. You should be able to identify each of your two organisms by this point. Please consult your instructor for assistance.
4. You may take photos with your cell phone or iPad of your results to include in the lab report.

At this stage you should have finalized your results and identified your organisms. Please make sure that you have been marked on your lab work and your lab manual. Make sure you have all the information you require to write your lab report.

Observations

Observations

Observations

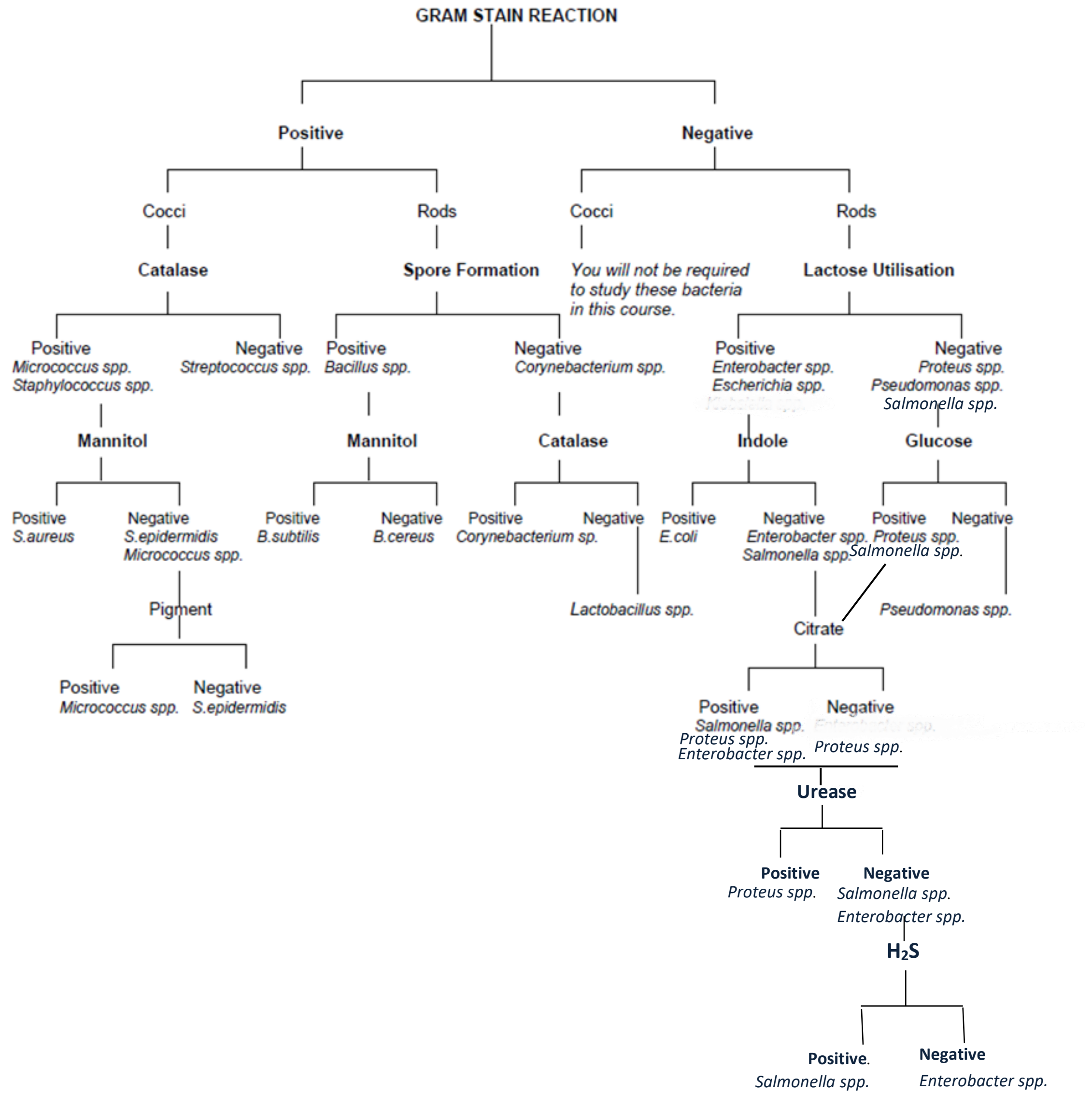
Table 1. Major characteristics of organisms encountered in the Biology 325 laboratory

Organism	Gram stain	Colony morphology	Oxygen requirement	Motility	Spore formation
Micrococcus roseus	+ cocci	Smooth, raised, pink	Aerobic	-	-
Micrococcus luteus	+ cocci	Smooth, raised, yellow	Aerobic	-	-
Staphylococcus aureus	+ cocci	Smooth, raised, golden	Aerobic	-	-
Staphylococcus epidermidis	+ cocci	Smooth, raised, cream	Aerobic	-	-
Corynebacterium xerosis	+ rods (older organisms can be club-shaped)	Smooth, convex, grey or white	Aerobic	-	-
Bacillus cereus	+ rods	Opaque, rough, waxy	Aerobic	-	+
Bacillus subtilis	+ rods	Opaque, rough, waxy	Aerobic	-	+
Escherichia coli	- rods	Smooth, raised, cream	Facultative anaerobe	+	-
Salmonella typhimurium	- rods	Smooth, small, grey	Facultative anaerobe	+	-
Salmonella enteritidis	- rods	Smooth, small, grey	Facultative anaerobe	+	-
Proteus vulgaris	- rods	Spreading, grey	Facultative anaerobe	+	-
Enterobacter aerogenes	- rods	Smooth, raised, white	Facultative anaerobe	+	-
Pseudomonas aeruginosa	- rods	Smooth, cream, green sheen	Aerobic	+	-

Table 2. Biochemical characteristics of organisms encountered in the Biology 325 laboratory.

Organism	H_2S	Indole	MR/VP	Citrate	Urease	Oxidase	Catalase	Gelatinase	Amylase	Glucose
Micrococcus roseus	-	-	NA	-	+	-	+	+(slow)	-	-
Micrococcus luteus	-	-	NA	-	+	-	+	+(slow)	-	-
Staphylococcus aureus	-	-	NA	-	-	-	+	+	-	+
Staphylococcus epidermidis	-	-	NA	-	-	-	+	-	-	+
Bacillus cereus	-	-	NA	-	-	-	+	+	+	+
Bacillus subtilis	-	-	NA	-	-	-	+	+	+	+
Escherichia coli	-	+	+/-	-	-	-	+	-	-	+
Salmonella typhimurium	+	-	+/-	+	-	-	+	-	-	+
Salmonella enteritidis	+	-	+/-	+	-	-	+	-	-	+
Proteus vulgaris	+	+	+/-	+/-	+	-	+	+	-	+
Proteus mirabilis	+	-	+/-	+	+	-	+	+	-	+
Enterobacter aerogenes	-	-	-/+	+	-	+	+	-	-	+
Pseudomonas aeruginosa	-	-	-/-	+	-	+	+	+	-	-

Dichotomous Key of Bacteria Used in this Course



Day 1

Objectives

At the conclusion of this session you should be able to:

1. Identify all major components of a compound light microscope and understand their function in microscopy.
2. Set up the microscope using Köhler illumination.
3. Examine specimens using low power, high power, and oil immersion magnification.
4. Interpret patterns of growth for different colonies.
5. Perform and identify a Gram stain of bacteria.
6. Perform a bacterial streak plate.
7. Aseptically transfer a sterile suspension using a loop to broth cultures or agar plates.

Exercise 1: Introduction to the Microscope

The study of organisms that are not visible to the eye requires the correct use of a microscope, which enlarges specimens for easy viewing. Typically, the compound light microscope is a versatile tool in discerning microscopic structures of cells. This is because, such instruments have different lenses and knobs which aid in magnifying and focusing on samples applied to a glass slide. It is the combination of applying magnification (enlargement) and resolution (contrast) that is an effective technique to observe microscopic specimens.

For the purposes of this course, you should be able to recognize all of the major parts of the microscope and identify their function. Proper understanding of function and setting is essential for maximum resolution of bacterial smears. Read the notes below before proceeding with this exercise.

General Guidelines

1. If you wish to move the microscope to another area, you should always handle it by lifting the microscope using both sides of the arms when using the Olympus CX23 Biological microscope. For all other microscopes that may be used, move the microscope with one hand holding the arm of the microscope and the other hand holding the base.
2. Be sure that the microscope is unplugged.
3. The microscope is a precision instrument. Treat all components with care and ensure that it is kept free of dust. Clean objectives, condenser, and ocular lenses

using quality lens paper (never use paper towels or tissue paper). Ensure that oil is cleaned from the oil immersion objective with the cleaning solution provided after use.

4. Once cleaned, at the end of the day, ensure the stage is lowered and objective lenses moved away from the front (this is known as "park position").

DO NOT LIFT OR MOVE THE MICROSCOPE BY HOLDING THE STAGE OR OBJECTIVES, OR OTHER PARTS OF THE MICROSCOPE.

1.1 Microscope orientation and Kohler illumination.

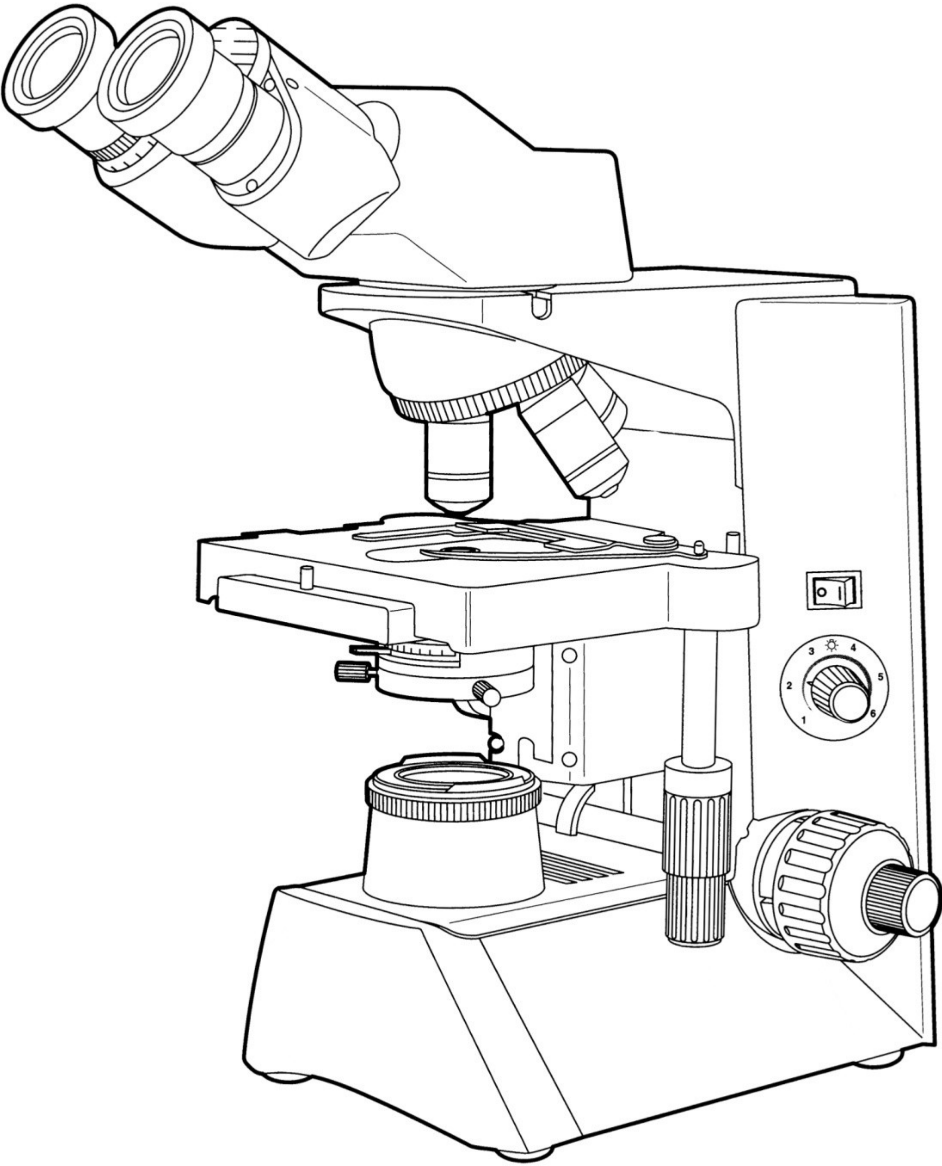
Materials

Compound light microscope
Glass slides
Marker
Immersion oil
Lens paper

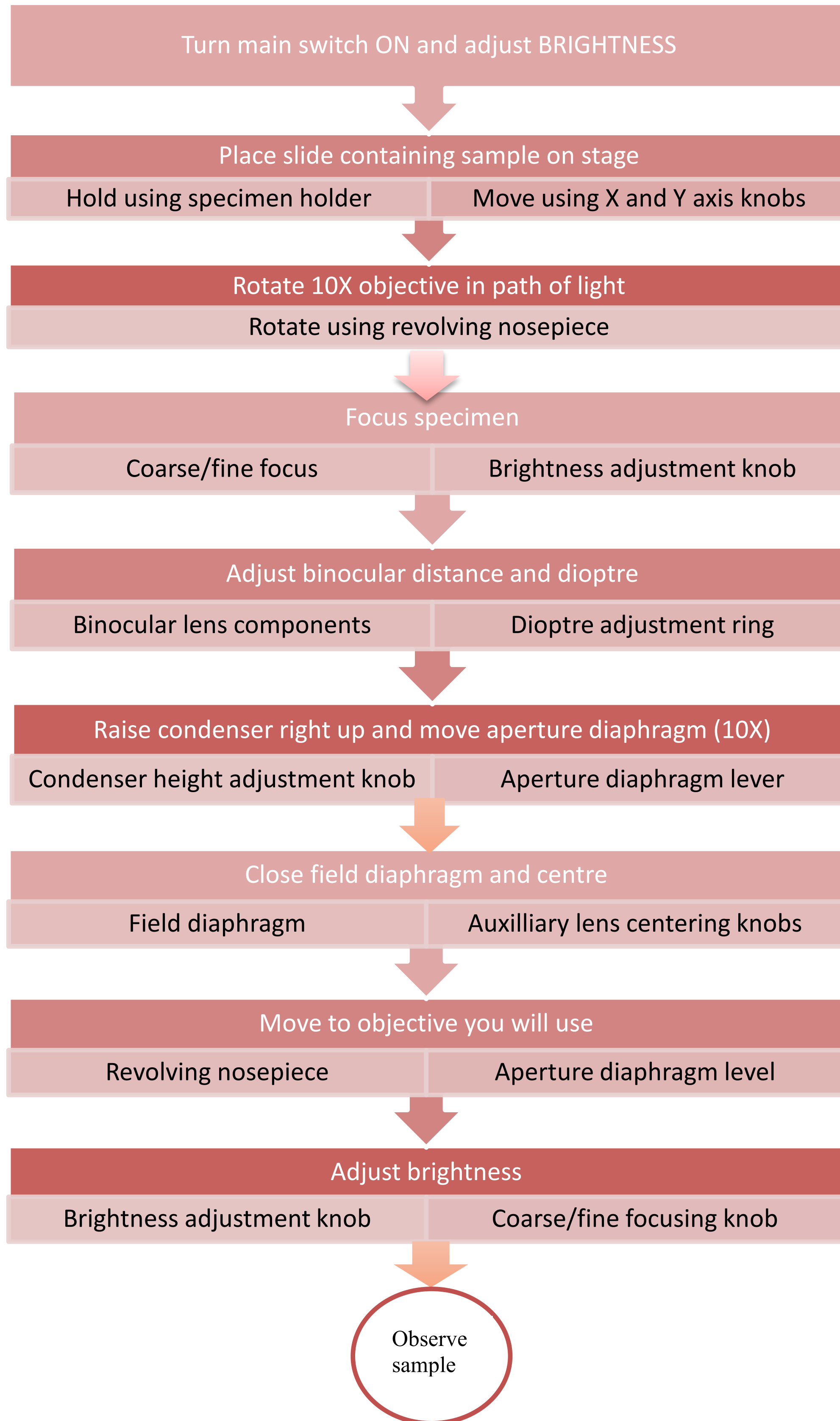
Procedure

1. Remove the cover from your microscope, plug it in, and turn on the light. You should be able to see the light appear from the light source which is at the base of the microscope.
2. Using the diagram provided for you, identify and label all of the components of the microscope. Provide a brief description of the function of these pieces in the table below.

Component	Function
Ocular lenses	
Light adjustment	
Field diaphragm	
Stage	
Condenser	
Aperture (iris diaphragm or condenser diaphragm)	
Coarse focus knob	
Fine focus knob	
Slide adjustment	
10X objective	
100X objective	
Auxiliary lens centering knobs	


3
4
2
5
1
6

Setting up the microscope for sample observation using CX23 Biological Microscope



Köhler Illumination

The CX23 Biological Microscope contains automatic adjustment for Kohler illumination by using the aperture diaphragm lever. This corresponds with the numerical aperture of the condenser related to the numerical aperture of each objective lens. This will allow the best resolution and contrast of the sample observed.

Adjusting ocular lenses

The CX23 Biological Microscope has two ocular lenses. Begin with both lenses positioned horizontally. As you are looking through the lenses, move each right or left until both appear comfortable.

Adjusting the dioptre

Because your eyes are not alike, you can also adjust the dioptre, which is a measure of how powerful the lens can be viewed.

- For each ocular lens, move the dioptre ring to zero ("0").
- Move the objective to 10X magnification and focus using coarse/fine adjustments.
- Then move the objective to 40X magnification and focus.
- Return back to 10X objective. Look through RIGHT eyepiece and move the right dioptre ring until you see the sample perfectly focused. Repeat the same for the left ring.
- Change to 40X objective and focus. Move back to 10X objective and check if it is also focused.

Cleaning and Disinfecting the Microscope

It is important that your microscope is maintained free of materials used during each exercise. In particular the eyepieces and handles are kept clean and free of contamination (stage and focusing knobs, nosepiece). The following is a recommended procedure:

- Wear nitrile protective gloves during cleaning of the microscope.
- Clean the plastic frame of the microscope by wiping major parts with 70% ethanol.
- Clean 100X objective lenses using lens cleaner as follows:
  - Remove any dust by using a blower (if available)
  - Fold a piece of lens paper around your forefinger and add a drop of lens cleaner
  - Wipe lens in spiral motion in clockwise direction

1. Complete table below

Use the following QR Code to link to a video of the features mentioned in this information sheet:

Kohler Illumination for all other microscopes

Kohler illumination is essential for optimizing resolution of a specimen. This is because this technique provides uniform illumination of a sample by concentrating and aligning the pathway of light from its source to viewing through the ocular lenses.

1. Prepare a glass slide by drawing a line with a marker and place the slide on the stage.
2. Turn on the light source and centre the mark over viewing area.
3. Rotate the objective lens apparatus and select the 10X objective. Focus on the sample.
4. Close the field diaphragm (at the base of microscope) and focus on the edges when viewing the sample (this should look like a hexagon).

   Note: (a) if you are unable to see sharp edges of the hexagon of the small circle, raise or lower the condenser to complete focusing on the edges.

   (b) if the hexagon is not in the centre of the field of view, use the centering screws on the condenser to move the orientation of the view.
5. Move the condenser aperture (also known as iris diaphragm) until you see about 60-70% of the specimen.
6. Open the field diaphragm (at the base of the microscope) until it illuminates the viewing area of the sample.
7. Comment on what you observe with the mark on the slide when you perform the following activities described in the table below:

Activity	**Observation**
Kohler illumination	
Use of the 10X objective	
Use of the 40X objective	
Moving the stage back and forth	

Exercise 2: Microscope Slide Techniques

2.1 Wet mount preparation of a specimen for light microscopy

Wet mount preparations are normally used to examine the structure of larger organisms such as fungi, protozoa, and algae. They are normally performed using water as the suspending medium. However, in order that internal structures can be better discerned, a suspending medium usually containing methyl blue stain is used.

Materials

Plate culture of *Rhizopus spp*. and/or *Penicillium spp*.
Pond water containing protozoa and algae
Methyl blue stain
Glass slides
Coverslips
Plastic pipettes
Cotton swabs
Microscope

Procedure

1. Using a clean slide in each case make a wet mount preparation of each sample provided.
2. For the fungal preparation use a sterile wooden stick to gently remove a small amount of the top of the fungal growth on the plate and apply this to a drop of methyl blue stain on your glass slide and gently mix.
3. Cover the sample with a cover slip by placing one side of the cover slip down onto the edge of the suspension at a 45° angle and slowly lowering the coverslip, avoiding the accumulation of air bubbles. View the sample using the microscope's 10X and 40X objectives.
4. Use a pencil to draw a labeled diagram of what you see (spores, hyphae, etc.).
5. For the pond water, apply a drop of the pond water to a clean glass slide and cover with a cover slip as described above.
   When preparing specimens for microscopy, first focus on the specimen with low (10X) magnification using the coarse adjustment knob. Then carefully move the objective to high power (40X) and focus using the fine adjustment knob. Draw what you see in your sample and try to identify the organisms by referring to the charts provided in this class.

Observations

Pond water:

Fungi:

2.2 Microscopic examination of bacteria

Bacteria are too small to be characterized by wet mount techniques which you performed in the previous exercise. Thus, a major technique that aids in the classification of bacteria is the Gram stain. This staining technique uses specific dyes that use knowledge of the principles underlying cell wall components of bacteria. The use of this stain is useful in identification of certain bacterial characteristics according to the way they react to a Gram stain. There are typically two reactions: Gram positive (purple) and Gram negative (pink) organisms. Refer to your **Colour Atlas** and textbook for the underlying principles of the reaction. This exercise will enable you to visualize and distinguish the differences among size, shape, arrangement, and gram reaction of representative bacteria.
This exercise will involve observation of two Gram positive and two Gram negative organisms which have been prepared for you.

Materials

Prepared slides of Gram-positive and Gram-negative bacteria
Microscope
Immersion oil
Lens tissue

Procedure

1. Place the slide containing the stained bacterial smear onto the microscope stage.
2. Focus on the slide as outlined in Exercise 1. First focus on the sample using 100X magnification (low power) and then focus on the sample using 400X magnification (high power).
3. Move the objective lens away from the slide and position the 100X objective in preparation for examining the specimen using oil immersion.
4. Place a small drop of immersion oil onto the slide at the position the objective would normally be placed.
5. Carefully move the 100X objective to centre the specimen. Slowly move the fine adjustment forward and backward until the lens is coated evenly with oil. Focus on the specimen until a sharp image of the smear is evident. The final magnification here will be X1000.
6. Upon completion of examination of the slide, remove the slide and wipe the objective with lens tissue.
7. Record your observations of each Gram stain according to Gram reaction (positive or negative), shape of the organisms (cocci or rods), and arrangement (clusters, single, paired, chains) in the table below. You may refer to your **Colour Atlas** for assistance. For each of the two Gram reactions, try to select and record an example of each type of bacteria: e.g. gram-positive coccus, a gram-positive bacillus, a gram negative coccus, and a gram-negative bacillus species.

Note: once you have examined representative slides, remove oil from the slide with lens tissue and return it to the slide box.

Results

Organism	Gram reaction	Shape	Arrangement	Drawing

2.3 Bacterial Gram stain technique (two methods)

The Gram Stain method is integral to viewing bacteria using a compound microscope. To prepare smears on slides from agar plate surfaces or broth cultures, the bacteria must firmly adhere to the slide surface, involving fixation procedures of bacterial smears on the slide. As indicated earlier in this laboratory, Athabasca University ensures it is in compliance with the safe handling of organisms that are considered to be pathogenic. As such, the University has adopted protocols that ensure that all techniques used are conducted safely. The exercise presented here will teach you two protocols using Gram stain reaction for bacterial specimens. As you proceed through the various exercises in this course, these techniques require some practice in order to produce consistent results with your samples. Please refer to your textbook and the **Colour Atlas** to review the principles of the Gram reaction.

Procedure

1. Prepare Gram stains for all three samples provided.
2. Examine slides first at 10X (low power), then 40X (high power) and then 100X (oil immersion). Draw labeled diagrams of what you see for each sample and note the specific Gram reaction, shape, and arrangement of the bacteria.

Preparation of a cell smear (to use for Methods 1 and 2)

1. Obtain a clean glass slide. Handle the slide only by the edges so that grease and fingerprints are not transferred to its surface.
2. Sterilize the slide by passing it through the flame of the Bunsen burner. In order to do this, turn on the Bunsen burner and ensure that the flame is at its hottest setting. This means that the central flame will appear blue, and its centre will be golden-red. The junction where the gold-red is replaced by blue at the centre is the hottest part of the flame. Hold the edge of the slide with a slide holder or forceps and quickly pass it through the Bunsen flame three times. Allow the slide to cool on a clean surface.
3. Using a marker or wax pencil draw a large circle on the slide and flip the slide over.
4. Sterilize a loop by placing its wire at the hottest part of the flame of the Bunsen burner. Allow the loop to turn red (this takes several seconds) and remove from the flame, but do not set down on the bench as this will contaminate the loop. You may set the loop upright on the stand provided.
5. To make a bacterial cell smear from a broth culture, first gently swirl the flask containing the bacterial culture. Remove the cap from the tube (as demonstrated by your instructor) and sterilize the lip of the tube by passing through the flame of the Bunsen burner. Insert the loop into the broth and remove a sample of the suspension. Return the lid of the tube before proceeding further.
   Note: The loop itself will look like the surface of a drum when it contains the sample. If there does not appear to be fluid in the loop, repeat the above process.
6. If a smear is taken from an agar plate, immerse the loop first into some sterile water, and place the flat of the loop at the centre of the slide (within the circle) so that a

droplet of water forms on the surface of the slide. Sterilize the loop again, allow it to cool and then pick up a small portion of a bacterial colony with the side of the loop. Note that only a very small amount is necessary, as each colony represents millions of bacteria. If you apply too much, it will be difficult to discern single bacteria on the slide. Gently mix the bacteria in the water droplet to make the same bacteria smear described above.

7. Sterilize the loop and allow it to rest on the support.
8. Allow the slide to air-dry by placing it on a clean surface of the bench. Do not speed up this process by attempting to dry the slide over the flame as this will distort the cell wall components and give an inconsistent Gram reaction.

Gram Stain Method 1: Risk Group 1 bacteria

This protocol is used for organisms that are classified to cause low risk and unlikely to cause human or animal diseases.

Materials

Broth culture of a mixture of *Escherichia coli* and *Micrococcus roseus*
Plate culture of *Bacillus subtilis*
Plate culture of *E. coli*
Forceps
Glass slides
Gram reagents
Bunsen burner
Inoculation loop
Sterile water
Microscope, immersion oil, lens paper

Once the slide has dried, the smear must be heat fixed by quickly passing it through the flame two or three times as demonstrated by your instructor. The slide is now ready for staining.

1. Prepare a fixed cell smear as described above.
2. Place the smear (cell side up) on a staining rack over the sink. Flood the smear with crystal violet by applying sufficient stain to completely cover the entire slide. Leave for 1 minute.
3. Tip the slide with forceps to allow the stain to run off. Turn the cold water tap on and gently pass the water hose over the slide to rinse it. Gently shake off the excess water from the slide.
4. Add iodine solution to cover the smear, tip the slide to drain the iodine, and then reapply with another application of iodine. Allow this to react 1 minute. Note: iodine serves as a mordant in this reaction. This means that the type of reaction occurring on the bacterial cells will prevent excessive decolourization due to the insoluble binding of the iodine with the crystal violet stain.
5. Pour off the iodine, gently rinse with water, and flood the slide with decolourizing solution (90% ethanol), while at the same time rocking the slide back and forth.

This action allows the stain to decolourize uniformly. Note that as the slide decolourizes, granules from the stain will be removed from the cell smear.

Continue to add the decolourizer drop by drop, and slowly rock the slide. Decolourize for approximately 20 seconds or until you observe that the stain no longer leaches from the smear. Wash the slide thoroughly with a gentle stream of water.

6. Flood the slide with safranin and leave for 30 seconds. This provides a counterstain for the slide preparation.
7. Blot dry the smear by applying blotting paper or paper towel. You may also rinse the slide with water before blotting.

Note: Bacterial smears that have been stained for routine identification do not require a coverslip. After examining the slide, the oil may be wiped off with a soft tissue. The slide can be stored in a clean, dust-free box for later viewing.

Gram Stain Method 2: Risk Group 2 bacteria

Risk Group 2 bacteria are classified as infectious agents that can pose a risk to human or animal health. Therefore, handling of such organisms requires careful attention to following appropriate protocols. Studies have shown that using the usual heat fixation technique to adhere bacterial smears on the slide produces variability in viable and non-viable numbers of organisms. Therefore, a different process is used to ensure that the bacteria are fixed accordingly to ensure that they are all non-viable before conducting the Gram stain. This protocol describes the Gram stain method using methanol as a fixative agent for Risk Group 2 (RG2) bacteria.

Materials

Broth culture of a mixture of *Staphylococcus aureus* and *Enterobacter aerogenes*
Plate culture of *Pseudomonas aeruginosa*
Conklin jar containing 95% methanol
Forceps
Glass slides
Gram reagents
Inoculation loop
Sterile water
Microscope, immersion oil, lens paper

Fixation of cell smear using Method 2

1. Before starting this process, ensure that you are properly equipped with protective personal equipment (buttoned lab coat, protective gloves, and safety glasses).
2. Follow steps 1-8 of the section "Preparation of a cell smear."
3. Ensure the cell smear is completely dry.
4. Using forceps or slide holder, immerse the slide in a Conklin jar that contains 95% methanol for two minutes.
5. After two minutes, remove the slide with forceps or slide holder and gently wick the slide by tapping a corner of it on a paper towel.

6. Allow the slide to completely dry before proceeding with the Gram stain in steps 1-7 described above.

Results

Draw and describe using a label as to what you see using high power (400X) and oil immersion (1000X) magnification.

Exercise 3: Streak Plate Method of Bacterial Isolation

The aim of this technique in microbiology is to obtain single colonies without achieving contamination of organisms from extraneous sources (eg. your fingers, mouth, surface bacteria). This technique is important in the initial stages of bacterial identification. Individual colonies from mixed cultures of bacteria may be separated, and their characteristics, such as shape, size, colour, and distinguishing features may be observed. The streak plate method also allows the microbiologist to confirm and maintain pure cultures of bacteria.

3.1 Performing a streak plate from a plate culture and a broth culture.

Materials

Nutrient agar plates (3)
Plate culture of *E. coli*
Nutrient broth cultures of *E. coli* and *S. aureus*

Procedure

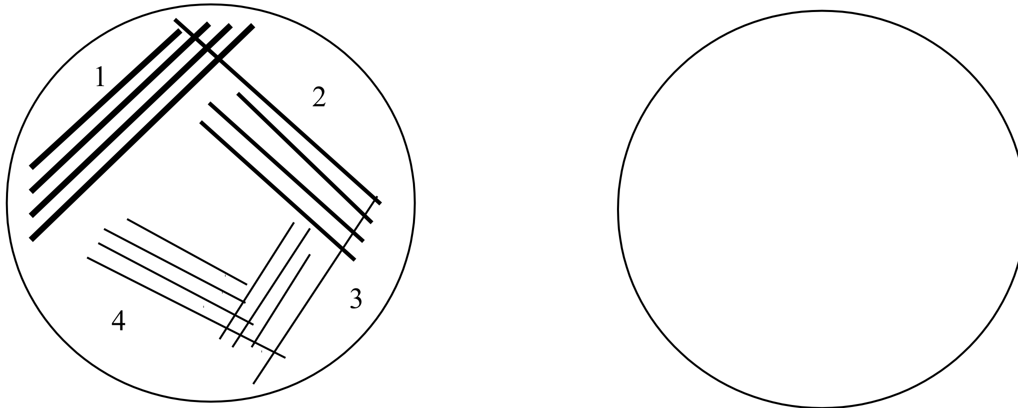
1. Sterilize the inoculation loop and sterilize the lip of the tube containing the broth culture as demonstrated by your instructor. Ensure that the culture is suspended uniformly by swirling the tube gently. Insert the loop into the tube and remove a sample of the culture. Again, ensure that the loop contains the broth culture. Sometimes when loops are too hot and are immersed in broth they will evaporate the suspension removed from the tube. To prevent this you may cool your loop on the inside of the tube before you take out the broth.
2. In order to begin streaking an agar plate place the nutrient agar plate provided for you with the base of the plate facing up. With the nutrient agar plate now inverted remove the base with one hand and have the agar surface facing you at a 45° angle. Ensure that the plate is kept within the vicinity of the Bunsen flame. This will protect the plate from possible contamination.
3. Place the loop at the uppermost end of the plate and apply a concentrated streak to the plate by spreading the side of the loop back and forth four or five times along the length of the uppermost part of the plate. Following this, place the plate onto its lid and rotate it 90°. Sterilize the loop and at this new position, streak back and forth again using the side of the loop. Repeat this process again.

   ALWAYS MAKE SURE YOU STERILIZE YOUR LOOP BETWEEN EACH STEP!

Note: The purpose of sterilizing the loop before streaking each new sector is to safeguard that you will not be transferring the same numbers of bacteria to each sector of your plate. This will enable the formation of isolated colonies, which is the objective of this exercise. Thus, the microbiologist may examine the characteristics of individual

colonies, and also separate colonies which may exist in a mixed group of bacteria. If the loop is not sterilized at each point, there will be a confluence of bacteria with colonies running into one another, thereby making identification processes very difficult.

A diagram of a typical streak plate showing the sequence of streaking is shown below. Using the other circle provided, practise the streaking technique using a pencil.



4. Label the base of the plate with your name, the date, and exercise number, invert it, and place it in the container that will be collected for incubation.
5. Repeat the same process for the plate cultures of bacteria. In this case, sterilize the loop as described above. Remove a fraction of one colony from the plate and streak on a fresh agar plate as described above.
6. Invert all plates and store in the container provided for incubation. Incubate overnight (18 hours) at 37°C.

REMEMBER TO PROPERLY LABEL THE *BASE* OF EACH PLATE WITH YOUR NAME, DATE AND DESCRIPTION OF EXERCISE.

Results

1. The next day, draw the plate conformation you have observed after incubation. Describe in your own words what you see on all plates. Measure the approximate diameter of colonies from each plate using a ruler, and describe the colour, shape, and edges of typical isolated colonies.

Drawing	Colony Diameter range	Colour	Edges

Exercise 4: Aseptic Technique

Aseptic technique is a necessary procedure that ensures bacteria inoculated to a broth or agar plate are preserved from unwanted organisms. This method therefore allows the study of specific characteristics of the organisms in a sterile environment. This exercise will involve the transfer of substances in broth culture or agar plates. For this exercise you will first perform the aseptic transfer of sterile broth into tubes of sterile broth. You will then transfer sterile broth to a nutrient agar plate.

Aseptic transfer involves a systematic delivery of material from one receptacle to another in an organized procedure whereby the transferring apparatus (in this case a loop but may involve the use of sterile pipettes) between each transfer. If you follow the procedure correctly, you will be able to ensure that you are transferring nothing but what is contained in the original tube.

Materials

Nutrient agar plates (1)
Tubes of sterile nutrient broth (4)

Procedure

4.1 Transfer of nutrient broth to tubes.

1. Label 4 nutrient broth tubes #1 to #4. Tube #4 will serve as a control. This tube will not be opened and will be used to compare the presence or absence of bacterial growth in other tubes. Light the Bunsen burner and sterilize the loop. If you are right-handed, pick up the first tubes with your left hand. Remove the cap of the tube with your fourth and fifth fingers without touching the underside of the cap. Hold the cap by wrapping your fourth and fifth fingers around it while you are working. Note, the caps of the tubes should not be placed on the bench surface and should be held as described above during inoculation.
2. If the tube is a sterile glass tube, sweep the lip of the tube through the flame two or three times to ensure sterility of the tube.
3. Take the loop (which has been sterilized) into your right hand (remember you are still holding the cap), and carefully insert it into the broth. Remove the loop, pass the lip of the tube through the Bunsen flame again, and then place the cap back onto the tube. **ENSURE THAT YOU HAVE A VISIBLE BROTH SUSPENSION CONTAINED WITHIN THE LOOP.**
4. Pick up the second tube containing sterile nutrient broth and repeat the process of uncapping and sterilizing the tube as before. In this case you are holding the loop containing the broth from the first tube. Once you have sterilized the lip of the second tube, insert the loop into the second tube, give it a little shake, and remove the loop.
5. Sterilize the loop again, insert it into Tube #2, and proceed to Tube #3 as described above.

Notes:

This procedure should be performed carefully and quickly, thereby avoiding the possibility of contamination.

In some cases, caps may not be used. For example, you may be working with large flasks containing broth cultures. These may be sealed with non-absorbent cotton wool plugs. In this case, you MUST hold the plug between your fourth and fifth fingers while you are working. These plugs may easily become contaminated if placed on the work bench.

In some laboratories, the use of glassware has been dispensed with and replaced with sterile plastics. If sterile plastic tubes (eg. polystyrene) are used, then omit the procedure of sterilizing the lip of the tube. Otherwise, the plastic will melt.

4.2 Transfer between agar plates

1. In preparation for this exercise, place Tube #3 of nutrient broth in front of you, and appropriately labeled nutrient agar plate. Place the plate near you in the inverted position (lid side resting on the bench).
2. Sterilize the loop and proceed to remove a sample of broth culture as described in the previous exercise. Replace the cap on the tube and pick up the base of the first nutrient agar plate, with agar side facing you (as described in the previous exercise). Proceed to streak the plate as described previously.
3. Place the plate and tubes in the appropriate area for incubation.
4. A control plate will be incubated for the entire class to review.

Results

4.1 Transfer of nutrient broth to tubes.

Complete the table below to record your results. Record the presence (+) or absence (-) of bacterial growth (as measured by the presence of turbidity as compared to the control tube).

Sample	Tube 1	Tube 2	Tube 3	Control tube
Growth				

4.2 Transfer to agar plate.

Record your observations for original broth culture and plate cultures.

Sample	Tube 3	Plate 1	Control plate (Unopened NA plate)
Growth			

Question

What conclusions can you draw about your aseptic technique from the above two exercises?

Day 2

Objectives

At the conclusion of this session, you should be able to:

1. Identify differences in morphology of bacterial colonies.
2. Distinguish the type of bacteria and growth patterns that occur on differential, selective, and enrichment media.
3. Determine and perform differential stains and tests according to given properties of bacteria.

Exercise 5: Selective, Enrichment, and Differential Media

The streak plate technique is an important technique to isolate a pure culture of one organism by identification of single colonies for observation and study. You have already been introduced to general purpose media, such as nutrient agar. This media does not distinguish between organisms which have special metabolic needs, or ability to grow well on this media, and it is not possible to distinguish a mixture of cultures from each other, other than generic descriptions. Therefore, various products and indicator dyes have been incorporated in some media to promote the growth of bacteria that utilize them and inhibit the growth of unwanted organisms for study. The term used for this media is “selective” media.

To examine the growth characteristics of bacteria on selective media, chemical indicators are added to the media. Thus, in addition to the media being selective for growth of certain organisms, one may also “differentiate” between those organisms that use the special components in the media from those that do not use these components. This media is classified as both selective and differential. In some cases, the growth of certain bacteria may be enhanced by the addition of specialized components, such as blood or serum. This type of media is known as “enrichment” media. Please refer to your **Colour Atlas**, which described in detail the different types of selective, differential, and enrichment media that is available.

5.1 Isolation of bacteria on selective, enrichment, and differential media

Materials

Suspensions in sterile distilled water of:
Escherichia coli
Salmonella typhimurium
Staphylococcus aureus
Staphylococcus epidermidis
Inoculation loop
Sheep blood agar plates (2)
Mannitol salt agar plates (2)
Nutrient agar plates (4)
MacConkey agar plates (2)

Eosin methylene blue agar plates (2)

Procedure

5.1.1 Blood agar as a differential enrichment medium.

1. Using a marker, clearly label each plate with your name and date.
2. Label one plate *S. aureus* and the other *S. epidermidis*.
3. Using the broth cultures provided, streak organisms on a blood agar plate according to the protocols described in Exercise 4.
4. Invert each plate and store for incubation at 37ºC overnight.

5.1.2 Mannitol salt agar as a selective and differential medium.

1. Repeat the same procedure as described above using *S. aureus* and *S. epidermidis*.
2. Incubate plates.

5.1.3 MacConkey agar as a selective and differential medium.

1. Follow the procedure described above, except streak one plate with *E. coli* and the other with *S. typhimurium*.
2. Incubate plates as described above.

5.1.4 Eosin methylene blue agar as a selective and differential medium.

1. Streak one plate with *E. coli* and the other with *S. typhimurium* as described above.
2. Incubate plates.

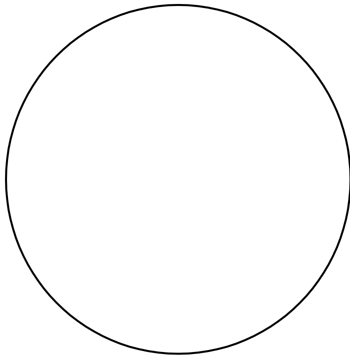
5.1.5 Nutrient agar as an all-purpose growth medium.

1. Repeat the same procedure using all four organisms provided.
2. Incubate plates.

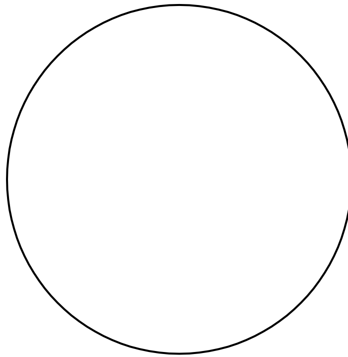
Results

5.1.1 Sheep blood agar

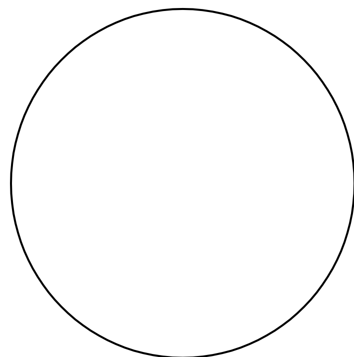
1. Draw the typical isolated colonies seen in both plates. Describe what you see in each case. Compared to the control plate, how has the agar changed? Are the results different for *S. aureus* compared to *S. epidermidis*?



S. aureus



S. epidermidis

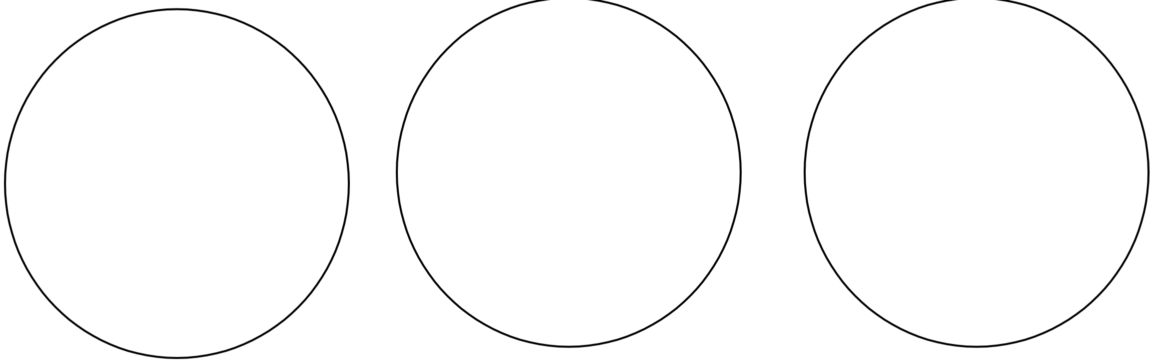


Control

2. Explain how the hemolysis on this agar differs in appearance between the two organisms. Name the two types of hemolysis observed in the plates.

5.1.2 Mannitol salt agar

1. Draw and label typical isolated colonies for each bacterial species streaked on the test plates.



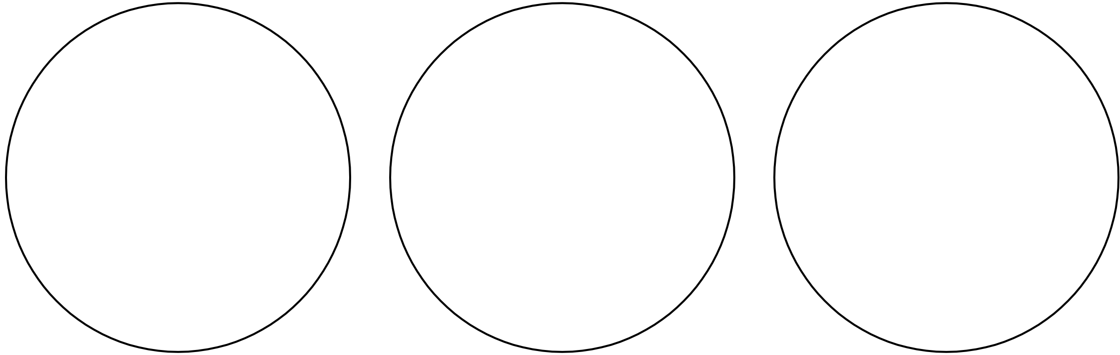
S. aureus ***S. epidermidis*** **Control**

2. How does the growth of each bacterium differ on this media?

3. Why are the colony colours different?

5.1.3 MacConkey agar

1. Describe the differences in growth patterns among the two bacteria streaked on the test plates.

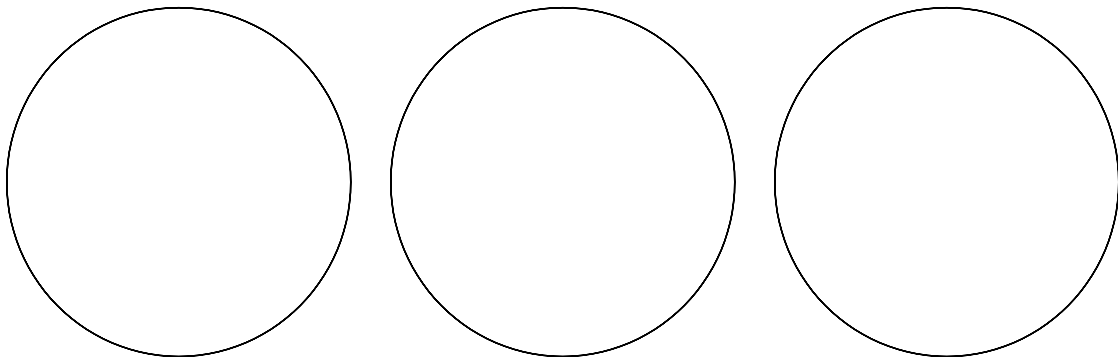


E. coli ***S. typhimurium*** **Control**

2. Do the organisms tested ferment lactose? How is this evident?

5.1.4 Eosin methylene blue agar

1. Describe the differences in growth patterns between the two types of bacteria streaked on this medium.

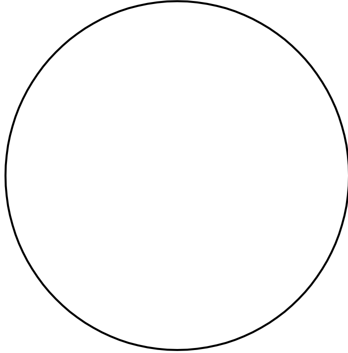


E. coli ***S. typhimurium*** **Control**

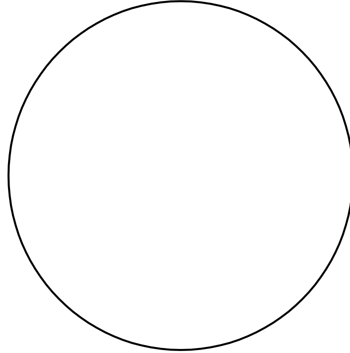
2. Which of the two organisms ferments lactose more vigorously? What colour indicates avid, or vigorous, lactose fermentation with this medium?

5.1.5 Nutrient agar

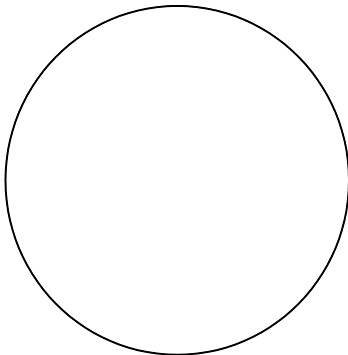
1. Describe and draw the growth of each of the four organisms on this media.



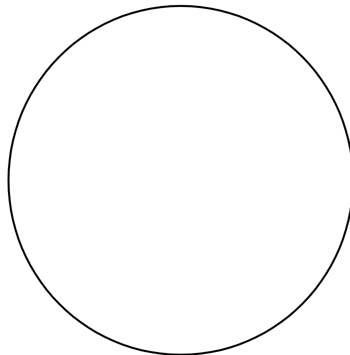
S. aureus



S. epidermidis



E. coli



S. typhimurium

5.2 Demonstration of selective and differential media

Materials

Prepared plates of bacteria grown on:

- Eosin methylene blue (EMB) agar
- Mannitol salt agar
- Sheep blood agar
- Salmonella-Shigella (SS) agar
- MacConkey (MAC) agar
- Simmons citrate agar
- Nutrient agar

Procedure

1. Examine the plates and describe the types of colonies observed according to the colour (include single colony diameter for organisms grown on the nutrient agar plates as this may help you with your unknown project). Note the reaction and colony growth of each species for each type of media. Enter your observations in the tables provided.

Results

EMB Agar

	S. aureus	***E. coli***	***S. typhimurium***
Colony colour			
Colony diameter			
Media colour			
Reaction (lactose)			

MacConkey Agar

	E. coli	***Shigella spp.***	***S. typhimurium***
Colony colour			
Colony diameter			
Media colour			
Reaction (lactose)			

Mannitol Salt Agar

	S. aureus	*S. epidermidis*
Colony colour		
Colony diameter		
Media colour		
Reaction		

Sheep Blood Agar

	S. aureus	*S. epidermidis*	*S. pyogenes*
Colony colour			
Colony diameter			
Media colour			
Reaction (hemolysis)			

SS Agar

	S. sonnei	*S. typhimurium*
Colony colour		
Colony diameter		
Media colour		
Reaction (H_2S production)		

Simmons Citrate Agar

	S. typhimurium	*E. coli*	*Enterobacter spp.*
Colony colour			
Colony diameter			
Media colour			
Colony growth (appearance)			

Nutrient Agar

	S. aureus	*S. epidermidis*	*S. pyogenes*
Colony colour			
Colony diameter			
Colony growth (appearance)			

	E. coli	*S. typhimurium*	*S. enteritidis*
Colony colour			
Colony diameter			
Colony growth (appearance)			

	S. sonnei	*Enterobacter spp.*
Colony colour		
Colony diameter		
Colony growth (appearance)		

2. For the following agar media used, list their **significant components** and explain how they react. Provide one example of a bacterium that will show change to the components (positive), and one example of a bacterium that will not show change or grow on the medium (negative). Consult your **Colour Atlas** for assistance.

Agar Medium	**Components**	**Function/Reaction**	**Positive Example**	**Negative Example**
Nutrient Agar				
Mannitol Salt Agar				
MacConkey Agar				
EMB Agar				
Simmons Citrate Agar				
SS Agar				
Sheep Blood Agar				

Exercise 6: Differential Tests

Microbiologists use specific identification tools to separate bacteria according to knowledge of how bacteria utilize several different metabolites. Biochemical testing is a critical step in the classification of microorganisms. This is used in conjunction with bacterial growth on differential and/or selective media as shown in Exercise 5. Taken together, these procedures form the basis of differentiation between species according to Bergey's Manual. As you proceed through the following tests please consult your **Colour Atlas** for interpretation.

6.1 Tests that demonstrate the activity of some extracellular enzymes

6.1.1 Starch

Agar Materials

Plate culture of starch agar of:
E. coli
B. subtilis
Iodine solution

Procedure

1. Examine the plates provided for you. Flood each plate with iodine solution.
2. Describe your observations in the table below. Indicate in the table if there was a colour change to the colonies or the media and if the organism is positive or negative for the ability to degrade starch.

Results

Iodine	***E. coli***	***B. subtilis***
Before adding iodine		
After adding iodine		

1. Describe the rationale used for this test: What enzyme does the organism possess if it is positive for starch utilization?

6.1.2 Gelatin hydrolysis

Materials

Nutrient gelatin stab tubes of:
Staphylococcus aureus
Staphylococcus epidermidis
Bacillus subtilis
Proteus vulgaris
Sterile stab (negative control)

Procedure and Results

1. Examine the tubes provided for you. Record you observations in the table.

Sterile stab (nil)	***S. aureus***	***S. epidermidis***	***B. subtilis***	***P. vulgaris***

2. What enzyme does this test for? What does this enzyme hydrolyze? How is this reflected in the test?

6.2 Tests that demonstrate the effects of some intracellular enzymes.

6.2.1 Carbohydrate fermentation

Materials

Semisolid agar tubes containing glucose and a green-yellow pH indicator, inoculated with:
Escherichia coli
Salmonella typhimurium
Staphylococcus aureus

For each sample two tubes were inoculated. One tube was incubated for 18 hours and the other for 48 hours. Two uninoculated controls have been included as controls. Your **Colour Atlas** does not describe the same test, however the principle behind the other fermentation tests (purple broth and phenol red broth) is the same as the glucose fermentation test that you will observe.

Procedure and Results

1. Examine each sample and record your observations in the table below. (Yellow indicates a lowered pH).

Organism	**Observations 18 h**	**Observations 48h**	**Acid/Gas formation 18h**	**Acid/Gas formation 48h**
Nil				
E. coli				
S. typhimurium				
S. aureus				

2. What conclusions can be made about the biochemical pathways utilized by organisms that caused the media to turn yellow in the main tube and yellow in the inner tube and produced gas in the inner tube? What are the oxygen requirements of these organisms?

3. What metabolic pathway are these organisms using? What are the oxygen requirements of these organisms?

6.2.2 Kligler's Iron Agar (Hydrogen Sulfide Test)

Materials

Tube slants incubated at 37°C for 24 and 48 hours inoculated with:

Escherichia coli
Shigella flexneri
Proteus vulgaris
Salmonella enteritidis

Procedure and Results

18 hours

Organism	**Colour of slant**	**Colour of butt**	**Gas production**	**H_2S production**
Nil				
E. coli				
S. flexneri				
P. vulgaris				
S. enteritidis				

48 hours

Organism	**Colour of slant**	**Colour of butt**	**Gas production**	**H_2S production**
Nil				
E. coli				
S. flexneri				
P. vulgaris				
S. enteritidis				

6.2.3 Catalase reaction

Materials

Nutrient agar plates inoculated with the following organisms and incubated at 37ºC overnight:
Staphylococcus aureus
Micrococcus luteus
Enterococcus spp.
Bacillus subtilis
Staphylococcus epidermidis
Streptococcus spp.
3% hydrogen peroxide
Pipette

Procedure and Results

1. Place a drop of 3% hydrogen peroxide directly onto a small area of bacterial growth on an agar plate.
2. Observe the results. The production of bubbles indicates catalase production.
3. Record your observations in the table.

Catalase production

Organism	Catalase production (+) or (-)
S. aureus	
M. luteus	
Enterococcus spp.	
B. subtilis	
S. epidermidis	
Streptococcus spp.	

1. What metabolic function does the catalase test represent?

2. What is the reaction that this enzyme catalyzes?

3. Can anaerobic bacteria perform positive catalase reactions? Explain your answer.

6.2.4 Urease test

Materials

Overnight (18 hour) cultures of urea broth containing:
Escherichia coli
Proteus vulgaris
Salmonella enteritidis

Procedure and Results

1. Examine the broth cultures and record your observations in the table.

Urease

Organism	Observation	Urease (+) or (-)
Nil		
E. coli		
P. vulgaris		
S. enteritidis		

1. Describe the metabolic process involved in the urease test.

2. What indicator is used in the urease broth?

6.2.5 Oxidase reaction

Materials

Nutrient agar plates incubated at 37°C for 48 hours previously with the following organisms:

Salmonella enteritidis
Escherichia coli
Pseudomonas aeruginosa

Oxidase reagent
Inoculation loop or sterile cotton swab
Filter paper

Procedure and Results

1. Using a sterile cotton swab, pick lots of colonies of bacteria from an agar plate.
2. Add one or two drops of oxidase reagent to the bacterial sample on the swab.
3. Observe whether a colour change occurs (if the sample turns purple).
4. Record your observations in the table.

Oxidase

Organism	**Observations**	**Oxidase (+) or (-)**
S. enteritidis		
E. coli		
P. aeruginosa		

1. What metabolic process does the oxidase reaction demonstrate?

6.2.6 Identification of *Enterobacteriaceae* (IMVic test)

The term "IMVic" denotes the four key tests used to identify members of the family *Enterobacteriaceae*. The letters abbreviated above represent the following tests: **I**ndole reaction, **M**ethyl red test, **V**ogues-Proskauer, and **C**itrate utilization.

Please consult your **Colour Atlas** for the principles behind these reactions. Your instructor will explain these tests to you.

As you have seen with the previous tests, each can be performed individually with specific media and chemicals. This demonstration provides some tests used for the Family *Enterobacteriaceae*.

Materials

The following organisms were applied to media or broth and incubated at 37ºC for 18-24 hours (see Table 1):
Pseudomonas aeruginosa
Proteus vulgaris
Escherichia coli
Salmonella typhimurium

Procedure and Results

1. Your instructor will direct one member from the class to perform an indole test in the appropriate tube.
2. Observe the reactions and record the results below. You may consult the **Colour Atlas** to see how the positive and negative reactions for the MR/VP tests should appear, as these are presently not performed in this class. You will use a different indole reagent (cinnemaldehyde) than that described in the **Colour Atlas**. The principle of the test is the same, but determination of a positive indole test is green, not red.
3. Using Table 1 below, record the reactions you observed.

Table 1. Summary of Results for IMVic Reaction of three members of *Enterobacteriacae* and one member of *Pseudomonadaceae*.

S.t.	*E.c.*	*P.v.*	*Ps.a.*	
				Glucose
				Gas
				H_2S
				Indole
				Lactose
				Urea
				Citrate

Legend: Ps. a.- *Pseudomonas aeruginosa*

P.v.- *Proteus vulgaris*

E.c. – *Escherichia coli*

S.t. – *Salmonella typhimurium*

Exercise 7: Differential Stains

The ability to stain bacteria is extremely important in assisting their classification and identification. As demonstrated in Exercise 2, the Gram stain enables one to categorize bacteria according to the specific characteristics of their cell wall components. In addition, properties such as shape and arrangement of bacteria on the slide preparation also help with the first steps of identification and classification of bacteria. Because bacteria come with a myriad of different characteristics, the use of dyes can enable detection of specific features that aids in providing clues to their classification.

This exercise will draw from knowledge of special features of bacteria, and some stains will be used to examine these features. Please note that the staining techniques you will be undertaking only represent a fraction of the diverse stains that exist in microbiology.

7.1 Gram stain

You have already been introduced to this staining technique. While the Gram stain enables you to discern bacteria that comprise differences in their cell wall components (Gram positive versus Gram negative), there are also some additional clues that can be used to determine special features in bacteria. In this section you will compare the morphological characteristics of several bacteria using the Gram stain technique.

Materials

Nutrient agar plate cultures of the following:
S. aureus
B. subtilis (overnight culture: about 18 hours)
B. subtilis (4-day culture)
Gram stain reagents
Inoculation loop
Glass slides

Procedure

1. Prepare slides of each sample according to Risk Group protocols:
   S. aureus – use methanol fixation of cell smears
   B. subtilis – use heat fixation of cell smears

2. Label each slide to ensure identification of each sample.
3. Prepare Gram stains of each.

Results

1. Record the microscopic observations in the table below.

	S. aureus	*B. subtilis* (18h)	*B. subtilis* (4 day)
Drawing of organisms observed using microscope			
Cell arrangement			
Gram reaction			
Are spores visible?			
Other observations?			

7.2 Schaeffer-Fulton spore stain

When conditions are ideal for bacteria, they will metabolize nutrients and exist as vegetative cells. However, as a mechanism of survival, some bacteria have the capability to remain dormant during extreme conditions. They do this by forming endospores, which are tough, intracellular structures that contain all the genetic material within the cells. Should conditions become even more hostile to the bacteria, they will release these structures, which in their free form are called spores. In either case, these structures remain impermeable armor, retaining all the genetic material required to proliferate at a more favorable time. These endospores can resist the damaging effects of heat, cold, radiation, chemicals, and dessication.

Materials

18-24 hour and 4-day nutrient agar plate cultures of:
Bacillus subtilis
Malachite green stain
Safranin stain
Hot plate
Glass slides
Microscope and immersion oil

Procedure

1. Make a new smear of each sample, air dry, and heat fix the slides.
2. Flood smears with malachite green and steam slides by placing on a hot plate.

3. Note: **DO NOT BOIL THE SMEAR**, as this will destroy the bacteria. Ensure that stain is always present during this period. You may need to add more stain if it appears to be evaporating from the surface of the slide.
4. Steam the slides for 5 minutes.
5. Remove the slide from the hot plate, cool, and rinse in running tap water.
6. Counterstain with safranin for 30 seconds.
7. Blot dry and examine microscopic characteristics using oil immersion.

Results

1. Record the microscopic observations of each slide as described below. Compare this with the Gram stain slides processed earlier. Indicate the location of spores as terminal, central, or subterminal.

	Spore stain *B. subtilis* (18-24h)	Spore stain *B. subtilis* (4 day)
Drawing of organisms observed under microscope		
Are spores visible?		
Colour of spores		
Vegetative cell colour		
Location of endospore		

1. Why is it necessary to use heat during the preparation of the spore stain?

2. What are the major differences between the vegetative cell and the spores?

3. Is the Gram stain a reliable stain that shows the presence of spores? Why or why not?

Day 3

Objectives

At the conclusion of this session, you should be able to:

1. Demonstrate and understand the methodology for determining microbial growth.
2. Construct a growth curve for *E. coli* and calculate bacterial generation time.
3. Understand the principles of various sterilization techniques.
4. Evaluate the minimal inhibitory concentration (MIC) testing of bacteria.
5. Interpret the practice and principles of bacterial transformation.

Exercise 8: Quantifying Bacterial Growth

There are many areas in microbiology where quantifying microorganisms is essential. For example, in the food industry, testing of possible microbial content in foods is a necessary component of quality control. Also, in research settings, such as pharmaceutical laboratories, the efficacy of antimicrobial agents on growth of microorganisms is commonly tested. Thus, accuracy in determining exact microbial growth is of importance to the outcome of scientific investigations. The experiment described below will demonstrate two ways in which the growth and/or viability of a bacterial culture is determined.

8.1 Bacterial growth curve

Materials

Nutrient broth containing a pure culture of *E. coli* previously incubated for 18 hours at 37ºC
Conical flask containing 100 ml of nutrient broth
Shaking water bath adjusted to 37ºC
Spectrophotometer and cuvettes
Sterile test tubes for dilutions
Sterile water for dilution
1 ml, 5 ml, and 10 ml pipettes
Eppendorf pipettes and tips
Nutrient agar plates
70 % ethanol in beakers
Glass spreader (hockey stick)
Vortex

Procedure

1. For the two techniques used in this exercise you will be required to follow the growth of *E. coli* in nutrient broth. **This experiment involves working in a group.**
2. Using aseptic technique, remove 3 ml of the overnight culture and use this to inoculate 100 ml of sterile nutrient broth contained in a conical flask.
3. Gently swirl the flask, clearly label it with your Group name, and place the flask in a shaking water bath. It is from this flask that you will remove a sample at each time point for turbidity measurement and quantitation of viable bacteria.

8.1.1 Turbidometric determination of bacterial growth (Method 1)

In this first technique, you will examine growth of *E. coli* incubated in broth to determine changes in turbidity over time (4 hours). The spectrophotometer is an apparatus that is used to measure a certain wavelength of light of a liquid solution. For microbiologists, this method is extremely useful, as it is related to the cells which interfere with the light in the solution. As cell numbers increase in the suspension over time, there will be more opportunities for light applied to the sample to scatter and increase the measure of optical density.

1. Aseptically remove a 6 ml sample of your suspension into a sterile empty test tube, Five ml of this sample will be used for determination of turbidity or **optical density**. The remaining portion of the sample (1.0 ml) will be used to perform serial dilutions and a spread plate technique to measure viable cells.
2. Your instructor will demonstrate the use of the spectrophotometer. Before you read your sample, set the spectrophotometer to a wavelength of 600 nm. Pipette 5 ml of nutrient broth only (without *E. coli*) into a cuvette and insert into the appropriate chamber in the spectrophotometer (this is called a blank). Adjust the spectrophotometer so that the reading will be zero. You have now calibrated the apparatus so you can accurately read your sample containing *E. coli*. You will not be required to adjust the spectrophotometer for each timepoint once you have calibrated the blank at this point.
3. Using aseptic technique, remove 5 ml of the sample from the first tube into a cuvette and measure the optical density of the suspension.
4. Record the optical density measurement of your sample for each time point. The time points will be at 0, 1, 2, 3, and 4 hours.
5. Once you have read the optical density of your sample, you may discard the sample into a beaker containing bleach next to the spectrophotometer workstation. Rinse the cuvette with distilled water so that you can use it during the course of the experiment. **Do not discard the cuvettes.**

Results

1. Record the optical density readings for your group in the table.

Time (hours)	Optical Density OD_{600} (nm)
0	
1	
2	
3	
4	

6. Using the graph paper provided draw a growth curve based on the optical density measurements at each timepoint. Clearly label the axis, and indicate the phases of growth (lag, log, stationary, and death phases), where applicable.

8.1.2 Determination of viable cell numbers using spread plate technique (Method 2)

Although the presence of microorganisms in a solution may contribute to the optical density, or physical viewing of total numbers, this does not indicate whether the cells are alive or dead. Thus, a precise measure of viability can be determined by a viable spread plate count technique. It is this method which will reflect numbers of cells that are viable as they multiply over time.

Refer to your Colour Atlas for details on serial dilutions and spread plate technique.

Serial Dilutions

As bacterial cells grow over time, the original inoculum placed in the flask will become progressively more turbid. If you were to simply remove a sample from the original flask and spread it over agar, it will be difficult to count colonies following incubation, as there would be colonies too numerous to count. Thus, it is necessary to dilute a fixed volume of a sample removed from the flask into successive tubes containing a fixed volume of diluent, so that colonies spread onto an agar plate can easily be counted following incubation. This technique is known as "serial dilution."

1. To perform a serial dilution, you will use 4.5 ml sterile water added to a series of tubes that are assigned for each timepoint. You will first make a ten-fold dilution by adding a portion of sample (0.5 ml) removed from the original inoculum and dispensing to the first tube. You will then progressively remove a sample from this tube to the next tube (and so on), thus diluting the sample ten-fold as you progress along the tubes assigned for the timepoint.
2. The process of making each serial dilution is described in detail as follows. At each timepoint, aseptically transfer 0.5 ml of suspension from a sample removed from the original flask into 4.5 ml sterile distilled water (tube #1). This constitutes a 10^{-1} dilution.
   - Using a vortex, gently mix tube #1 containing the 10^{-1} dilution and remove the cap by holding it between your fourth and fifth finger. Do not place the cap on the bench.
   - Take a sterile 1 ml pipette and remove 0.5 ml of the sample and directly place into 4.5 ml of sterile water into tube #2 (this will then be a 10^{-2} dilution). Discard the pipette immediately after use into a waste bucket containing bleach solution. Gently mix the tube by using a vortex, and remove a 0.5 ml sample using another sterile 1 ml pipette, and place into sterile tube #3 containing 4.5 ml of water (10^{-3}).
   - **Note: if you do not use a clean pipette for each of these dilutions, you shall be transferring the original suspension each time, thereby carrying organisms over from the previous dilution, which will result in inaccurate counts.**
   - Repeat serial dilutions of samples until a final (10^{-6}) dilution is achieved for the assigned timepoints**.**
3. Once all serial dilutions are completed, arrange labeled agar plates for each timepoint. The plates should be placed in order from the lowest dilution (e.g. 10^{-6} for the 4 hour

timepoint) to highest dilution (10^{-1}). Starting with the tube that is most dilute (10^{-6}) and mix the sample by vortexing and aseptically remove 0.1 ml (100 μl) of the sample and dispense into the centre of an appropriately labeled agar plate.

- Remove the hockey-stick cell spreader from the beaker containing 70% ethanol and flame the it to burn off residual ethanol. Spread the sample of bacteria evenly over the surface of the agar plate. The process of spreading will be demonstrated by your instructor. Allow the sample to dry before inverting the agar plate.
- Use the following table as a guide for conducting serial dilutions and spreading of samples on agar:

Timepoint	Serial tube dilutions	Plate the following dilutions	Number of plates
0	Original sample (10^0) 10^{-1} 10^{-2}	10^0 10^{-1} 10^{-2}	3
1	Original sample (10^0) 10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}	4
2	Original sample (10^0) 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}	10^{-2} 10^{-3} 10^{-4} 10^{-5}	4
3	Original sample (10^0) 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}	10^{-3} 10^{-4} 10^{-5} 10^{-6}	4
4	Original sample (10^0) 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}	10^{-3} 10^{-4} 10^{-5} 10^{-6}	4

Note: the original sample will be 6 ml of each suspension removed at each timepoint. Five ml are used for tubidity measurement and the remaining one ml will be used to perform serial dilution measurements.

- Incubate plates at 37ºC overnight (18 hours).
- The next day, count all colonies observed on the agar. Note: count the dilutions that have about 30-300 colonies.

Results

1. Tabulate the number of viable cells and record in the table below. Remember that each colony is equivalent to 1 colony forming unit (CFU) or 1 cell. Because you plated only 0.1 mL of sample per plate, you must also take into account a plating factor (PF) of 10. To calculate the number of cells per ml use this formula:

colonies on plate X 1/dilution X PF = #cells/ml

Eg. 33 cells on the 10^{-5} plate would be:

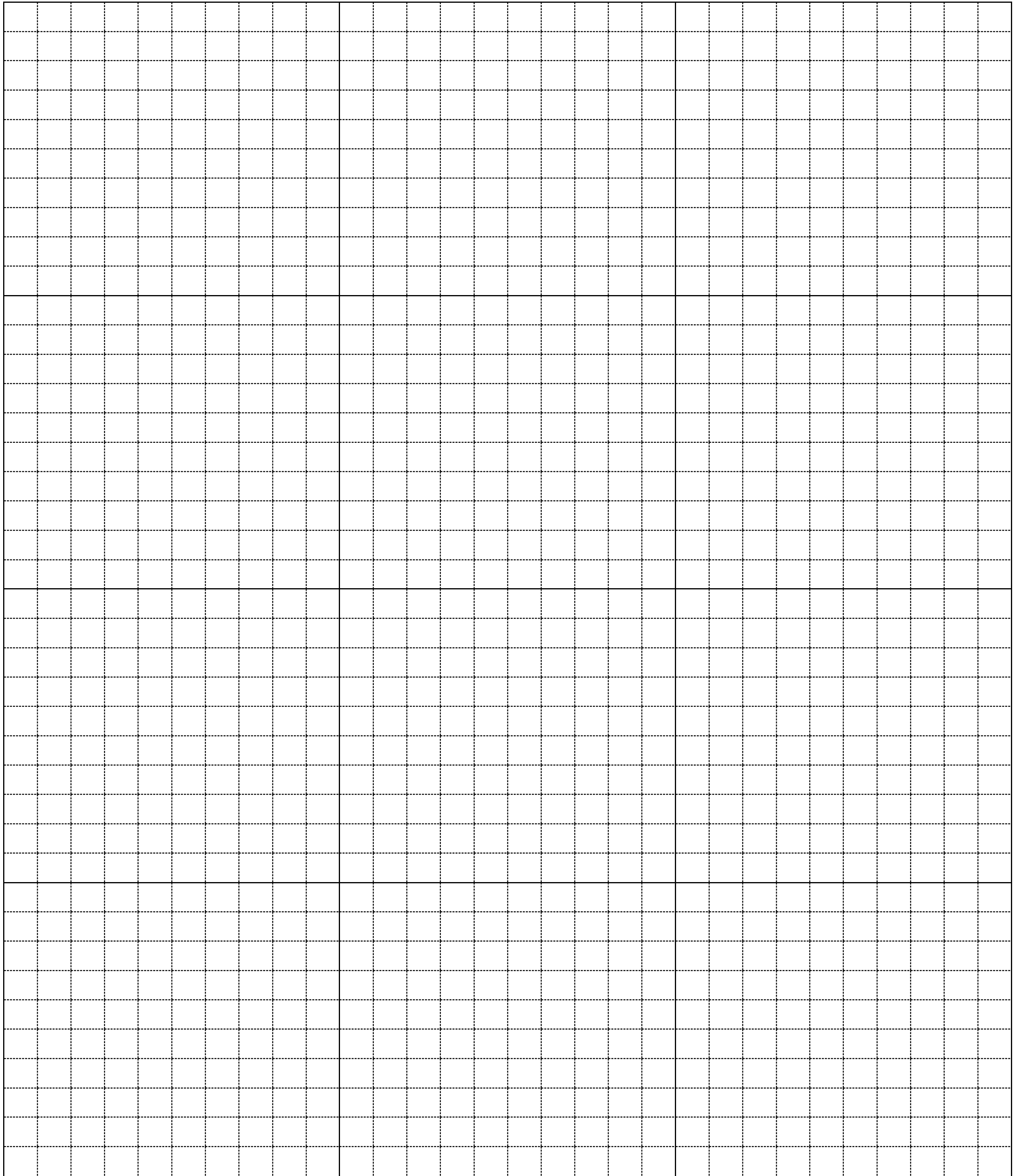
$33 \times 10^{5} \times 10 = 3.30 \times 10^{7}$ cells/ml (33 000 000 cells/ml)

Time (h)	**# CFU**	**Dilution used**	**# cells/ml**	**Log #cells/ml**
0				
1				
2				
3				
4				

2. Using the original graph of turbidity, plot the log number of viable cells/ml for each time point.
3. Determine the generation time by using the following formula:

$$\text{Generation time (h)} = \frac{\Delta t \log 2}{\text{Log } n - \log N}$$

Where: N = number of bacteria at a particular time point during log phase n = number of bacteria at a second time point during log phase
Δt = time



Questions

1. Describe the differences between methods determining turbidity measurements and viable cell numbers.

2. Would you expect the exponential (log) phase of growth to vary if twice the number of organisms were used to originally inoculate the broth culture?

3. How would the growth curve vary if a second microorganism were added to the broth culture?

Exercise 9: Sterilization

Sterilization techniques are important in many areas, such as health care, industry, biotechnology, to name a few examples. From a microbiological perspective, sterilization ensures that all microorganisms (including spores) are eliminated. In addition, it is preferred that materials are not deformed or altered as a result of the sterilization process (unless they are discard materials). The demonstrations below highlight some of the important principles of sterilization techniques. You can consult your textbook regarding these procedures.

9.1 Sterilization demonstration.

There will be a display of materials that have been sterilized by different techniques. The table below lists a number of different items that can be sterilized. Complete this table by checking the appropriate box or boxes that correspond to a sterilization technique.

	Autoclave- Liquid Cycle	Autoclave- Dry Cycle	Filtration	Gamma rays
Plastic syringes				
Glass bottles				
Gauze bandage				
Glucose solution				
Agar broth				
Antibiotic disc				
Plastic Petri dishes				

9.2 Effects of boiling on bacterial viability

The examples provided in the demonstration above represent some physical methods used to sterilize solids and liquids, plastics and materials that can be degraded in certain situations. This exercise examines the viability of some organisms when exposed to varying temperatures when left for a certain period in a water bath. Boiling is a simple technique that uses high heat to break down cell structures. This method however is not considered to sterilize all things, such as endospores.

Materials

Boil-proof culture tubes containing:
S. aureus
E. coli
B. subtilis
Nutrient agar plates (3)
Foam floating tube rack
Thermometer
Sterile cotton swabs
Water bath
Bunsen burner
Inoculation loop

Procedure

1. Use one nutrient agar plate per organism. Divide each plate into four sections. Label each section with one of the four temperatures that will be used:

   25, 50, 75, and 100°C. Also label the plates with the individual names of the organisms.
2. Use a sterile applicator to dip into a previously mixed tube containing one of the bacterial species and spread thoroughly in the sector labeled 25°C. This sector serves as observation of growth of the original inoculum. Repeat for the remaining bacterial cultures.
3. Transfer the tubes labeled 25°C to a water bath heated to 50°C using a floating tube holder. Leave the tubes in the bath and maintain for 10 minutes.
4. Carefully remove each tube and inoculate the plate labeled 50°C with each of the three organisms as described above.
5. Repeat steps 3 and 4 for the remaining temperatures (75°C and 100°C).
6. Incubate the plates for 18-24 hours at 37°C.

Results

Tabulate the growth patterns for each organism and temperature as follows:

+++ (heavy growth)
++ (moderate growth)
+ (poor growth)
- (no growth)

Organism	**25°C**	**50°C**	**75°C**	**100°C**
S. aureus				
E. coli				
B. subtilis				

Questions

1. Which of the above organisms showed the greatest resistance to boiling? Which showed the least? Why?

2. Which organism was the most temperature sensitive? Suggest an explanation for this result.

Exercise 10: Chemical Control of Bacterial Growth

Antimicrobial agents can also be used to kill or inhibit the growth of bacteria. These agents are usually chemicals that have profound effects on microbial growth. While bacteriostatic agents are chemicals that will stop the growth of bacteria without killing them, bactericidal agents can kill bacteria. Bacteriolytic agents will not only kill bacteria, but will also lyse them. The experiments outlined below feature some of the effects of these agents. The exercises in this section show three different techniques that are used to demonstrate the inhibitory effects on bacterial growth in the presence of an anti-microbial agent.

10.1 Agar diffusion technique

Materials

Sterile absorbent discs Forceps
95% alcohol
Overnight nutrient broth cultures of
Staphylococcus aureus
Bacillus cereus
Escherichia coli
Nutrient agar plates (3)
Sterile cotton swabs
Beakers containing the following disinfectants:
tea tree oil
10% H_2O_2
Dettol®

Procedure

1. Label three plates to identify each culture.
2. Divide the plate into three sections, one for each chemical and label.
3. Immerse a separate sterile cotton swab into a nutrient broth culture of *each organism and perform spread plate on each plate.* Spread the culture by uniformly moving the swab back and forth, first horizontally, and then vertically.
4. Sterilize forceps by dipping ends into 95% alcohol and passing briefly through a Bunsen flame.
5. Carefully pick up a disc with sterile forceps, immerse in tea tree oil, and blot gently on absorbent paper. Place disc onto the plate approximately 2.5 cm from the perimeter of the plate and gently press down.
6. Repeat the above procedure for the remaining discs and test organisms. Ensure that the discs are sufficiently separated so as not to interfere with each other.
7. Invert plates and incubate at 37°C for 24 hours.

8. The next day, use a ruler to determine the zone of inhibition around each disc. This can be done by measuring the radius, the distance from the middle of the disc to the edge of where the bacteria have stopped growing (area of clearing).

Results

Measure and record the zone of inhibition as described above in the table below.

Organism	**Tea tree oil**	**10% H_2O_2**	**Dettol®**
S. aureus			
B. cereus			
E. coli			

Questions

1. Which of the above test organisms showed greatest sensitivity to the antimicrobial agents? Which showed the least?

2. Describe an experiment that would use the above technique to test the minimum inhibitory concentration of an antibiotic.

3. What factors should one consider for testing antimicrobial agents?

10.2 Measurement of antimicrobial activity (minimum inhibitory concentration)[2]

Materials

7 sterile tubes with lids
Sterile nutrient broth (for making dilutions)
Nutrient broth culture of *Staphylococcus aureus* incubated at 37ºC 24 hours previously
Disinfectant solutions:
vinegar, household cleaner, Presept, benzalkonium chloride
(one disinfectant per group so results can be compared between groups)
Pipettes for dilutions (10 ml and 5 ml pipettes)

Procedure

1. Label 7 sterile tubes from 1 to 7.
2. Add nutrient broth using aseptic technique to each tube as follows:

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7
12 ml	10 ml	8 ml	6 ml	4 ml	2 ml	0

3. Dispense the disinfectant solution selected for each tube as follows:

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7
0 ml	2 ml	4 ml	6 ml	8 ml	10 ml	12 ml

4. Add 1.0 ml of a broth suspension of *Staphylococcus aureus* to each tube.
5. Gently vortex each tube. **LOOSEN ALL CAPS**
6. Incubate tubes at 37^0C for 24 hours.
7. Determine the minimum inhibitory concentration (the tube which does not show turbidity).

Results

1. Record your observations in the table below according to the presence (+), or absence (-) of growth. Vortex the tubes before observing them.

Disinfectant Used:

Tube	1	2	3	4	5	6	7
Disinfectant Dilution	Nil	0.15	0.31	0.5	0.6	0.8	1.0
Growth (+ or -)							

[2] Adapted from Wikipedia en.m.wikipedia.org/wiki/minimum_inhibitory_concentration

2. Determine the minimal inhibitory concentration (MIC), as judged by the first tube to show no detectable growth.

3. Does the above experiment provide information as to whether the disinfectant is bactericidal or bacteriostatic? Explain your response.

4. How would you determine minimal bactericidal concentration (MBC)?

Exercise 11: Bacterial Transformation

Bacterial transformation is defined as the horizontal gene transfer of "naked" (foreign) DNA from the environment. In nature this often occurs when bacteria die and fragments of their genetic material are taken up by other bacterial species in the environment and incorporated into the chromosome. This is termed "natural competence", which is the ability of an organism to take up foreign DNA in its genome or plasmid. A typical plasmid is a small, circular segment of DNA that replicates independently of bacterial chromosomal DNA. Only a few bacterial species are naturally competent, such as *Haemophilus*, *Neisseria*, and some *Streptococcus spp*. To make other organisms competent (e.g. *E. coli*), the use of chemicals or other techniques such as electropulsing, must be used.

Transformation of bacteria can be utilized in the laboratory in several applications, such as producing DNA copies, cloning techniques, and expression of enzymes or other proteins. A DNA fragment of interest (that encodes for a protein of interest) can be ligated (integrated) to a plasmid and then introduced into *E. coli* in order to produce many copies of the gene, or to produce the protein of interest. Since *E. coli* is not naturally competent, one of two procedures can be used to transfer the plasmid: electroporation and chemical transformation using $CaCl_2$. For this exercise, we will follow a chemical transformation protocol in order to introduce a plasmid into *E. coli*.

11.1 Transformation of a *GFP*-containing plasmid into *E. coli.*

The jellyfish, *Aequorea victoria*, can produce light due to its gene which expresses a green fluorescent protein (GFP). This gene is important in the study of molecular biology. GFP has been isolated and incorporated into a plasmid. This engineered plasmid is known as pGLO. Expression of GFP will occur following the principles of an operon system, where pGLO allows inducible expression of GFP.

In this experiment we will transform pGLO, a plasmid containing the GFP gene, into *E. coli*. The plasmid contains a gene that confers ampicillin resistance, which is expressed following transformation. This will ensure that only the transformed cells will be able to grow on media that contains ampicillin. To express the GFP, the plasmid also contains a promoter that is stimulated in the presence of the sugar, arabinose. We will plate potential transformants on agar containing arabinose to "turn on" transcription of the GFP gene. We will then be able to determine if the transformation was successful by looking for light production in the transformed colonies (transformants).

Materials

Purified plasmid DNA (pGLO)
E. coli culture on an LB plate
Transformation solution (contains $CaCl_2$)
LB broth (called Luria-Bertani broth, this
medium permits excellent growth of *E. coli*)
LB agar plates with ampicillin (2)
LB agar plate with ampicillin and arabinose (1) LB agar
plate (1), LB amp plates (2), LB amp arab plates (2)

Sterile microcentrifuge tubes (2)
Microcentrifuge tube racks
Pipettes and pipette tips
Sterile microcentrifuge tubes
Sterile plastic loops
Sterile spreaders
Water bath
Ice
UV light box

Procedure[3]

1. Your instructor will provide you with two microcentrifuge tubes, each labeled +pGLO and –pGLO. Each tube will contain 250 µl of transformation solution. Keep the tubes on ice until step 5.
2. Use a sterile plastic loop to pick up a ½ loop of *E. coli* from an LB plate. Dip the loop in the +pGLO tube to transfer bacteria into the tube. Repeat this step to add bacteria to the –pGLO tube. Put the tubes back on ice.
3. Add 10 µl of pGLO to the +pGLO tube. Mix tubes well. Incubate both tubes on ice for 10 min.
4. Heat shock the cells by placing both tubes at 42ºC in a water bath for 30 seconds.
5. Remove from the bath and place cells on ice for 2 min.
6. Add 250 µl of LB broth to each tube and incubate cells for 20 min to 1 hour at 37˚C. This is the "recovery" period.
7. Plate 200 µl of the +pGLO sample onto each of the LB plates labeled +pGLO/amp and +pGLO/amp/ara.
8. Plate 200 µl of the -pGLO sample onto each of the LB plates labeled -pGLO/amp and –pGLO. Incubate all 4 of the plates overnight at 37˚C.
9. The next day, examine the colonies for light production using a UV light box. If the plasmid was taken up by the *E. coli* and the GFP gene expressed, the colonies will produce light.

Results

1. Record your observations for each of the four plates that you have.

Questions

1. Why was arabinose included in the growth medium?

2. Why must antibiotics be incorporated into the growth medium?

3. What was the purpose of the –pGLO samples plated on LB agar with ampicillin and LB agar without ampicillin?

4. What are the two other methods of genetic transfer in bacteria?

5. Name one application of genetic engineering.

[3] Adapted from Biotechnology Explorer™, pGLO™ Bacterial Transformation Kit

Day 4

Exercise 12: Environmental and Food Microbiology

Microorganisms are used in a variety of ways in industrial applications. For example, they may be harvested to produce a specific product, such as protein. Or they may be used to make certain foods and beverages, such as yogurt, cheeses, and beer. On the negative side, some microorganisms may have deleterious effects where, for example, they may contribute to the spoilage of food products. Sometimes, these organisms may cause changes to the taste or consistency of the food without having ill effects on human consumption. At other times, contaminating bacteria may be pathogenic in nature, and can produce toxins, which may be responsible for causing fever, diarrhea, and vomiting. The experiment described here will explore some of the effects microorganisms may have on food.

12.1 Food microbiology

Materials

Sample of contaminated food
Gram reagents
Prepared plates from food sample
Fixed yogurt smears for examination

Procedure

1. Examine a fixed yogurt smear and draw a few representative cells.
2. Prepare and examine a Gram stain of the contaminated food sample.
3. Examine samples of the contaminated food on agar plates and Gram stain one of the colonies.

Results

1. Record your observations:

   Yogurt smear:

Contaminated food:

2. Can you identify the species present in the contaminated food? If yes, what characteristics allowed you to conclude what this organism is?

3. Is this organism a food pathogen? If so, what disease does it cause?

4. How are bacteria used in making yogurt? What are the benefits of eating yogurt?

12.2 Bacterial flora in the environment

This experiment will be performed on Day 2 or 3.

Materials

Nutrient agar plates (2)
Sterile cotton wool swabs
Sterile distilled water

Procedure

1. Wet one sterile cotton swab in sterile water. Obtain a swab sample of a surface (eg. telephone handle, doorknob, pen, computer keyboard). Streak one nutrient agar plate by spreading the swab across the plate horizontally and vertically.
2. For the remaining plate (1), place the lid of the nutrient agar plate on the bottom half of the plate, and leave the plate exposed to the air at a particular location (eg. bathroom, laboratory, refrigerator, for at least three hours). After three hours, cover the plate with the lid.
3. Incubate all plates at 37ºC for 24 hours.

Results

1. Record your observations in the table that follows of the types of colonies, and appearance of predominant cells by performing wet mounts and Gram stains if applicable. Draw two to three of the typical organisms observed in each location (use the table on the next page).

Location	Observation of colonies	Wet mount	Gram reaction

Questions

1. Can you predict which types of microorganisms are present at the different localities? Is there an abundance of one type over others in any one particular location?

2. In terms of the concept of nosocomial contamination, what types of organisms would you expect to find if this experiment was performed in a hospital setting?

Exercise 13: Medical Microbiology

Objectives

At the conclusion of this session, you should be able to:

1. Recognize normal bacterial flora of the body.
2. Understand the principles of nosocomial infections.
3. Examine medically important bacteria.
4. Determine antibiotic sensitivity of some microorganisms.

Bacteria play a critical role in medical microbiology. The exercises in this section will demonstrate some of the important properties of medically relevant bacteria. The role of bacteria in disease will be considered.

13.1 Nosocomial infections: the effects of washing hands on bacterial load

Materials

Lawn culture of *Micrococcus roseus*
Nutrient agar plate (1)
Choice of the following treatments or alternatives:
Hand soap
70% ethanol
Purell
Antibacterial hand soap

Procedure

1. Draw a line down the centre of the bottom half of the nutrient agar plate. Label one half "control" and the second half "washed".
2. Press down the fingertips of your hand onto the plate of *M. roseus*.
3. Press these fingers onto the "control" section of your nutrient agar plate.
4. Wash your hands with one of the treatments available and press fingertips onto the section of the plate labeled "washed".
5. Incubate your plate in inverted position at 37ºC for 24 hours.

Results

Compare your results with others and record the observations in the table below.

Treatment	Growth	Comments
Hand soap		
70% ethanol		
Antibacterial soap		
Purell		

Questions

1. Was there any treatment that appeared to be more effective?

2. Is proper hand washing technique as important as the disinfectant that is used?

3. Suggest reasons why hand washing is important in a clinical situation.

13.2 Normal bacterial flora

This experiment will be performed on Day 2 or 3.

Materials

Nutrient agar (NA) plates (2) Sheep's
blood agar (SBA) plates (2) Sterile
cotton swabs

Procedure

1. Immerse one swab in sterile water and thoroughly swab one part of your anatomy (eg. scalp, behind the ears, armpit, foot). Prepare a lawn plate on one NA plate and one SBA plate as described previously.
2. Use a *new* cotton swab to swab inside of your mouth and prepare a lawn plate on one NA and one SBA plate.
3. Incubate plates for 24-48 hours.
4. Examine the plates for morphological appearance of colonies and perform Gram stains and wet mounts (if applicable) of selected colonies. You may also perform a catalase test to confirm catalase positive organisms.

Results

Record your observations in the table below:

Site	Appearance of colonies	Microscopic Appearance

1. What is the role of normal flora in human health?

13.3 Sensitivity to Antibiotics

Demonstration of the E-test for determination of MIC (minimal inhibitory concentration).

The E-test strip is a rapid and accurate method for the determination of MIC of a particular organism for an antibiotic and yields an exact MIC value without having to calculate the concentration of the antibiotic or measure the zone of inhibition.

Materials

Overnight broth cultures of the following organisms:

Staphylococcus aureus
Escherichia coli
Pseudomonas aeruginosa

E-test strips of antibiotics from 4 major groups:

Ampicillin (Am) - β lactam, semi-synthetic penicillin, inhibitor of cell wall synthesis.
Chloramphenicol (Cl) – inhibitor of protein synthesis
Tetracycline (Tc) – inhibitor of protein synthesis
Gentamicin (Gm) – aminoglycoside, inhibitor of protein synthesis
Clarythromycin (Ch) – macrolide, inhibitor of protein synthesis

Procedure

Each organism was swabbed onto an agar plate (Mueller-Hinton) to form a lawn of confluent growth. An E-test strip was then added aseptically onto the surface of the plate and the plates were inverted and incubated at 37ºC for 18-24 hours. The MIC value is considered to be the point at which growth around the E-test strip stops. Sensitive organisms will have a zone of inhibition around the strip, resistant organisms will not.

Results

1. Record the MIC values in the table below.

Organism	**Am**	**Cl**	**Tc**	**Gm**	**Ch**
S. aureus					
E. coli					
P. aeruginosa					

2. Describe the resistances of the different organisms (i.e. which organisms were resistant to which antibiotic)?

3. Were there any differences between the Gram positive and the Gram negative organisms in MIC values for each antibiotic?

4. Suggest why some organisms are more resistant than others to various antibiotics.

13.4 Microbial Virulence Factors

Microorganisms possess several cellular structures or enzymes that contribute to their ability to colonize a host and/or cause disease. These structures maybe be cell- associated or secreted and may allow the bacteria to adhere, be mobile, degrade tissues, or prevent phagocytosis by host cells.

Materials

Prepared slides of flagella, capsule.

Procedure

View images of flagella and capsule on the microscope under the 100X oil immersion objective and draw what you see in the space below. Then answer the questions that follow.

Results

1. What is the function of flagella and capsule respectively? Are these structures cell-associated or secreted?

2. Name two other bacterial virulence factors, outline their function, and indicate if they are cell-associated or secreted.

Exercise 14: Introduction to Immunology

Immunology is the study of the immune response. This involves the interaction between antigens, antibodies, and cells. Immune responses are generated by the body to consider a substance to be “foreign”. Depending on the type of recognition, the immune system will usually react in a particular sequence of events, culminating in the removal of the foreign material.

Microorganisms can generate different immune responses. Depending on the species and their characteristics, the numbers present, and their location on the human body, the immune system will mobilize an active process of removal and destruction of these organisms. This exercise will explore some of the basic principles of immunology.

14.1 Antigens and antibodies

Direct ELISA to identify an unknown antibody

Materials

96-well microtitre plate
Antigens to three known intestinal pathogens
Primary antibody: patient sera
Secondary antibody: linked with horse radish peroxidase enzyme
Blocking solution
Coating solution
Wash solution
Substrate for horse radish peroxidase

Solution Preparation:

- Coating Solution: Antigen or antibody are diluted in coating solution to immobilize them to the microplate. Commonly used coating solutions are: 50 mM sodium carbonate, pH 9.6; 20 mM Tris-HCl, pH 8.5; or 10 mM PBS, pH 7.2. A protein concentration of 1-10 µg/ml is usually sufficient. This lab will use PBS- Tween.
- Blocking Solution: Commonly used blocking agents are: BSA, nonfat dry milk, casein, gelatin, etc. Different assay systems may require different blocking agents. This lab will use PBS-Tween.
- Primary/Secondary Antibody Solution: Primary/secondary antibody should be diluted in 1X blocking solution to help prevent non-specific binding. A concentration of 0.1-1.0 µg/ml is usually sufficient.
- Wash Solution: Use 0.1 M Phosphate-buffered saline or Tris-buffered saline (pH 7.4) with a detergent such as Tween 20 (0.02%-0.05% v/v).

Scenario

A young child was admitted to emergency with the following symptoms: diarrhea, vomiting, nausea, and fever. The child had attended a picnic earlier that day. Although biochemical testing and plating on differential media can be used to help identify the cause of the girl's infection, an ELISA using antigens of known intestinal pathogens will be conducted immediately to identify more rapidly what the child has contracted, so that physicians can initiate treatment.

You will be provided with a sample of serum from the patient and antigens of three known intestinal pathogens: *Shigella sonnei*, *Escherichia coli*, and *Salmonella typhimurium*.

Protocol[4 5]

Step 1. Apply Antigen (This will be completed for you)

1. Add 50 µl antigen from the known pathogens diluted in coating solution to rows A-C and columns 1-12 in the microtitre plate
2. Incubate up to 1 hour at room temperature.
3. Empty plate and tap out residual liquid.

Step 2. Block Plate

1. Add 300 µl blocking solution (PBS-Tween) to each well.
2. Incubate 5 min, empty plate and tap out residual liquid.

Steps 1 and 2 were performed in advance; please proceed from this step on.

Dilute and React Primary Antibody

1. Add 50 µl of PBS/Tween to rows A-C and columns 1-12 in the microtitre plate.
2. Add 50 µl of the patient serum to row A, column 2 only. Similarly, add 50 µl of patient serum to row B, column 2, and 50 µl of patient serum to row C, column 2. (Do not add patient serum to column 1, as it will be a negative control). Dilute the patient serum for each row by transferring 50 µl from column 2 into column 3, and then 50 µl from column 3 into column 4 and so on. The dilutions are indicated in the following table.

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[4] This procedure is adapted from http://www.chemicon.com/techsupp/Protocol/indirectELISA.asp and the Biotechnology Explorer™ ELISA Immuno Explorer™ Kit Instruction Manual
[5] For a simulated ELISA experiment please see: http://www-micro.msb.le.ac.uk/labwork/LabWork.htm

		1	2	3	4	5	6	7	8	9	10	11	12
Shigella sonnei	**A**		1/2	1/4	1/8	1/16	1/32	Etc.					
Escherichia coli	**B**												
Salmonella typhimurium	**C**												

3. Incubate for 10 min at room temperature.
4. Empty the plate, tapping out residual liquid on a stack of paper towels.

Wash Procedure

1. Fill each well with PBS-Tween (plastic squeeze bottle).
2. Invert plate to empty and tap out residual liquid.
3. Repeat 3 to 5 times.

Add Secondary Antibody Solution

1. Add 50 µl diluted secondary antibody to each well.
2. Incubate for 10 min at room temperature.
3. Empty plate, tap out residual liquid.
4. Wash using PBS-Tween (plastic squeeze bottle).
5. Invert plate to empty and tap out residual liquid.
6. Repeat 3 to 5 times.

React Substrate

1. Dispense 50 µl substrate into each well.
2. If desired, after sufficient color development add 100 µl of the appropriate stop solution to each well. (**We will not do this step**).
3. The microtitire plate could be read with an automated reader. However, the colour change may also be detected with the naked eye.

Results

1. Which species of bacteria was the girl infected with? How do you know?

2. At what dilution of primary antibody was the reaction no longer visible (refer to the column number)?

3. How could diluting the primary antibody in series be helpful in a clinical setting?

CONGRATULATIONS! YOU HAVE COMPLETED THE LAB.

BEFORE YOU LEAVE PLEASE MAKE SURE YOUR INSTRUCTOR HAS MARKED YOUR:

1. **Streak plate**
2. **Kohler illumination**
3. **Gram Stain**
4. **Lab book**