EDVOTEK & PLTW Experiment #460

PLTW Isolating and Identifying Bacteria

(PBS 3.1.6)

Experiment Objective:

In this experiment, students will isolate bacteria from a mixed culture and utilize Gram staining to examine the morphological characteristics and gram status.

See page 3 for storage instructions.

Version 460.240226

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Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

Component	Storage	Check (✓)
• *Micrococcus luteus* LyphoCells™	4°C (with desiccant)	❑
• *Escherichia coli* LyphoCells™	4°C (with desiccant)	❑
• *Serratia marcescens* LyphoCells™	4°C (with desiccant)	❑
• *Bacillus subtilis* LyphoCells™	4°C (with desiccant)	❑

This experiment is designed for 10 lab groups.

REAGENTS AND SUPPLIES

Store all components below at room temperature.

- Bottle of ReadyPour™ Nutrient Agar ❑
- Growth Medium ❑
- Large Petri Plates ❑
- 10 mL Sterile Pipet ❑
- Sterile Loops ❑
- Transfer Pipets ❑
- Microscope slides ❑
- Gram's Crystal Violet ❑
- Gram's Iodine ❑
- Gram's Safranin ❑
- Sterile water ❑
- Microcentrifuge tubes ❑
- Wax pencil ❑

Requirements *(Not included with this kit)*

- Microwave
- Pipet pump (optional)
- Incubator (recommended)
- Timers
- Slide holders or clothes pins
- Bunsen burner or alcohol lamp
- Lab marker
- Beakers, flasks, disposable cups, or other liquid containers (25 mL or bigger)
- Small tray or beaker for trash
- Squeeze bottles, large beakers (100 mL or bigger), or sink access for all groups
- 95% ethanol
- Distilled Water
- Bleach or other lab disinfectant
- Paper towels or blotting paper
- Gloves, Goggles, Lab coat
- Microscopes (total magnification of 100x or higher)
- Microscope cover slips and oil (if required)
- Pencils

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Background Information

Bacteria may be Earth's most successful organisms. Scientists estimate that there are around one nonillion ($1x10^{30}$) bacteria on our planet! To put this into perspective, the biomass of one nonillion bacteria is more than all other living animals and plants on the planet. These bacteria are found in an abundance of habitats such as soil, water, hot springs, mountain tops, deep ocean vents, and even radioactive waste. Bacteria also live in symbiotic (beneficial) and parasitic (detrimental) relationships within plants and animals - including us!

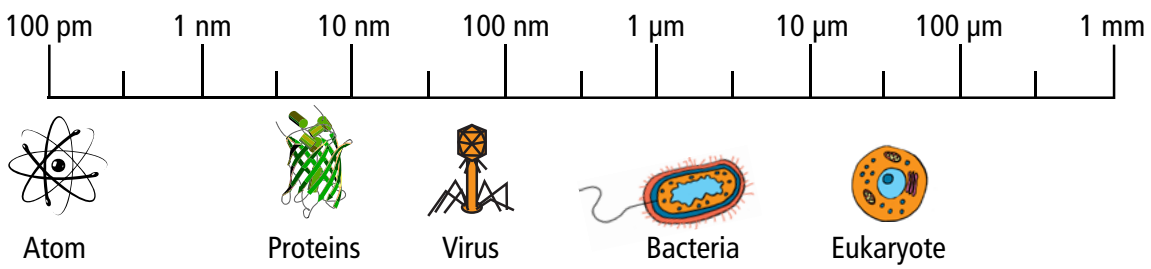


Figure 1: Average bacteria size.

A large and highly diverse group of single cell organisms, bacteria lack membrane bound organelles (like mitochondria, chloroplasts, Golgi apparatus, etc.) In other organisms, these organelles are needed to effectively take in nutrients and extract waste. However, bacteria's small size allows them to do this without these organelles. Bacteria have organelles different from eukaryotes such as the nucleoid which is a centralized threadlike mass containing DNA and sometimes circular plasmids which also organize and maintain the cells DNA as well as enable DNA exchange. In addition to these structures, bacteria also have ribosomes for protein assembly and most have cell walls. Some bacteria cells also have capsules or "slime layers" that help prevent viral infections, phagocytosis, and desiccation. Others have external extensions called flagella or pili that allow them to move or attach to other cells.

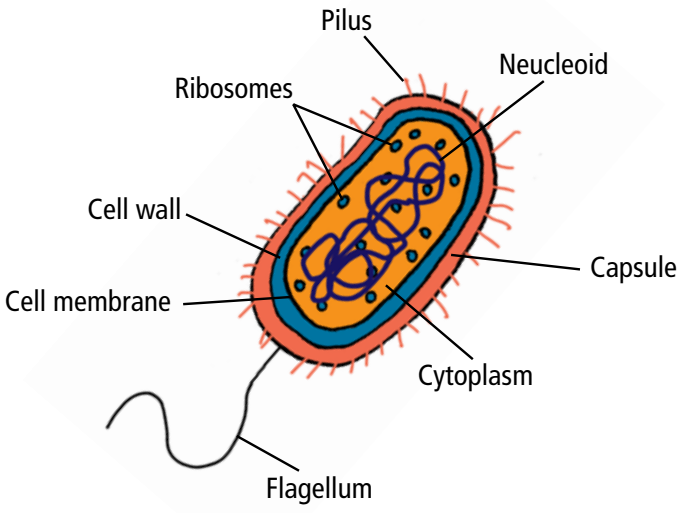


Figure 2: A bacteria cell.

Bacteria's success can be attributed to a variety of factors such as their cellular simplicity, small size, and short generation time. It may also be due to evolutionary strategies, such as horizontal gene exchange, that allow for fast genetic changes. However, these traits - along with the immense diversity that they have created - also make studying bacteria challenging, specifically classification. Classification is the practice of naming and organizing a group of organisms based on similar traits. This practice enables scientists to better understand and describe the diversity of a group and to quickly identify particular species. In bacteriology, different classification methods are used for different purposes. For example, biologists

studying the evolution of bacteria primarily use DNA sequences to describe the relationship between multiple species. Scientists that monitor the safety of drinking water and food often use more accessible but less precise tests that change color when specific bacterial proteins are present. In this setting, bacteria are grouped by their ability to produce certain proteins. Clinicians and clinical biologists that need to quickly identify pathogenic species (or beneficial species) use microscopes to observe key morphological traits and then classify bacteria based on these observations. Gram staining is one of these microscopic techniques and is now a cornerstone of bacteriology.

GRAM STAINING

Gram staining is a quick, effective, and inexpensive identification test that has become one of the most essential tools in bacterial classification. It was originally developed by the Danish physician HC Gram (1853-1938). Gram was searching for a way to stain bacteria cells in tissue samples without also staining the human cells. However, what he found was even more useful - a way to quickly sort bacteria into two major groups based on their ability to absorb and retain crystal violet stain. Today these groups are known as gram-positive and gram-negative.

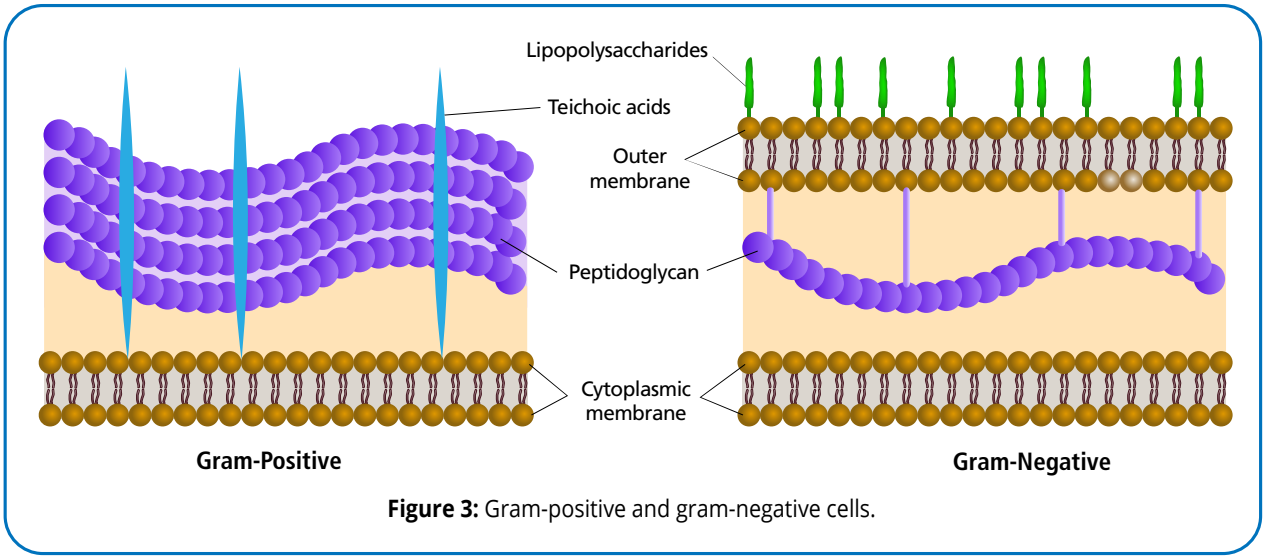


Figure 3: Gram-positive and gram-negative cells.

One reason that Gram staining has withstood the test of time is that the division of bacteria into positive or negative groups is based on large - and medically significant - differences in the cell walls of these two groups. Gram-positive bacteria have thick cell walls (15-80 nanometers) composed mostly of a molecule called peptidoglycan. These molecules form net-like sheets which are then tightly cross-linked to each other. Gram-positive bacteria also have a group of molecules known collectively as teichoic acids that run perpendicular to the peptidoglycan sheets. Gram-negative bacteria have thinner cell walls (10-12 nanometers), a single layer of peptidoglycan, and no teichoic acids. However, they do have a highly permeable outer membrane which contains proteins, phospholipids, and the endotoxin lipopolysaccharide.

Gram staining allows scientists to quickly visualize the different cell walls of gram-positive and negative cells by turning the former purple and the later pink in a six-step process (Figure 4). The procedure begins by fixing the bacteria to the slide which can be accomplished either by exposing the cells to an organic solvent or by gently heating the cells and slide. This both preserves the cells and ensures that they do not get swept away during any of the subsequent steps.

Once the bacteria are firmly attached to the slide the crystal violet (CV) stain is added. On the slide, crystal violet dissociates into positive and negative ions (CV+ and CV-) which quickly permeate the outer cell membranes of most cells. However, it is the positively charged CV+ ions that are responsible for dyeing cells a purple-blue. At this point in the procedure all cells - gram positive, gram-negative, and even nonbacterial - are this color. This step is completed by washing off excess crystal violet either by briefly dipping the slide in a beaker of distilled water or by gently running water down the slide.

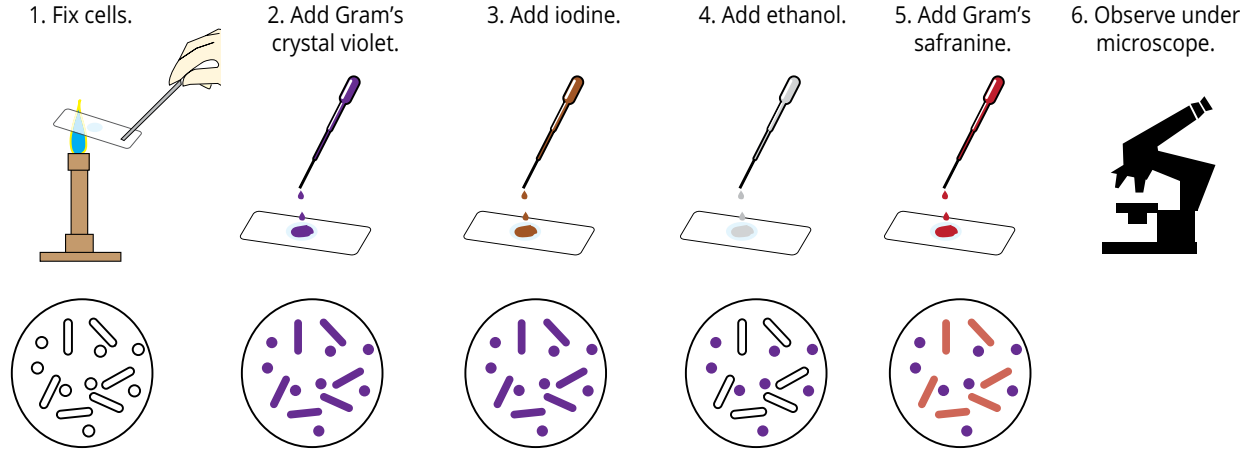


Figure 4: Gram staining.

Following staining, an iodine solution is added to trap the stain within a cell's wall. Iodine accomplishes this by binding to the CV+ ions and creating a large crystal violet-iodine complex. As with the addition of crystal violet, this step also includes washing away any excess iodine with water.

Next, a high percentage alcohol is added. This solution interacts with the cell wall of the two bacteria types very differently. In gram-negative cells, the alcohol strips away the outer membrane and then degrades the single layer of peptidoglycan. This makes gram-negative cells "leaky". In contrast, alcohol causes the multiple peptidoglycan layers in gram-positive cells to become more tightly crosslinked which further entraps the colorful crystal violet-iodine complexes. When the slide is washed again gram-negative bacteria - as well as any eukaryotic cells - lose most crystal violet stains while gram-positive cells maintain the stain. Because of its effect on gram-negative and non-bacteria cells, this step is called a "decolorization" step.

After decolorization, another stain is added to the slides so that the gram-negative cells can also be seen under the microscope. The stain often used in this step is safranin which stains bacteria cells a red-pink. The use of safranin is partly tradition but also because this stain is dark enough to make the gram-negative cells easy to see but light enough to not change the distinct blue-purple coloring of the gram-positive cells.

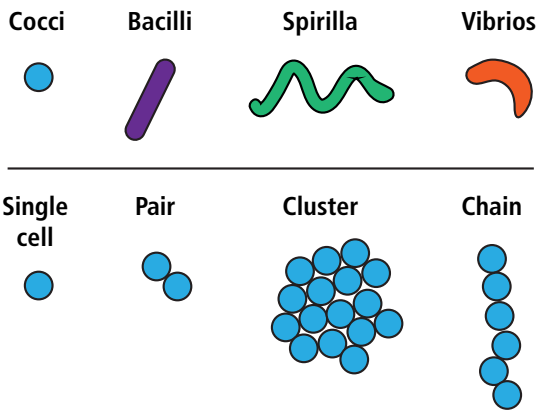


Figure 5:
Common shapes and cellular arrangements.

Finally, the cells are examined under a microscope and their color recorded. During this step, microbiologists will also observe and record the colony morphology, or the visual characteristics of the bacterial colony on an agar plate. These characteristics include shape, size, margin, elevation, and color and help to further classify a bacteria sample - sometimes down to species. The four most common shapes that bacterial cells take are spherical, rod-like, spiral, and comma-shaped. These correspond to the bacterial groups cocci, bacilli, spirilla, and vibrios (Figure 5). Different species of bacteria also tend to have characteristic cell arrangements - some exist as single cells while others grow together in pairs, clusters, or long chains. Many species can be quickly identified based on the combined observation of their gram stain color, shape and arrangement. In other cases, additional information - such as cell size or whether or not the cells require oxygen to survive - may also be needed to rule out similar species.

There are also cases where alternative testing is needed. A few bacteria, such as those species belonging to the group Mycoplasma, lack cell walls. These species do not properly absorb and maintain the crystal violet or safranin stains and so are called gram indeterminate. There are also a small number of gram variable bacteria. In these species, the number of peptidoglycan layers changes rapidly as the cell grows which causes the cell to sometimes appear pink and other times appear purple. Sometimes an indeterminate or variable classification can still help narrow down a bacteria's identity. However, because these results can also be due to experimental mistakes, additional testing is still needed.

MEDICAL APPLICATIONS OF GRAM STAINING

In medicine, gram staining is often used to both confirm a bacterial infection as well as to identify the offending bacteria or bacterial group. Many health conditions can be caused by a bacteria, a virus, a larger parasite, or a non-infectious trigger. When the presenting symptoms are not enough to determine a treatment, doctors use gram staining as a first round diagnostic test. For example, meningitis is a potentially fatal condition that occurs when the protective membrane around an individual's brain and spinal cord becomes swollen. This swelling is usually caused by a viral or bacterial infection but can also be triggered by some fungal infections, an adverse reaction to certain drugs, or cancer. When a patient comes in with meningitis symptoms (headache, stiff neck, and fever) doctors order a lumbar puncture. While multiple tests are performed on the recovered cerebrospinal fluid, gram staining is fast and accurate and therefore often guides treatment decisions.

Staining quickly reveals whether or not bacteria are present. A doctor will then prescribe antibiotics based on which type of bacteria are found. Gram-negative bacteria have an outer membrane and additional enzymes that protect these cells from many popular antibiotics. Furthermore, the outer membrane also contains dangerous endotoxins that can cause sepsis in a patient. Doctors will prescribe specialized antibiotics that combat these defenses and monitor the patient for signs of septic shock. Gram positive bacteria tend to be easier to treat as they are more susceptible to common antibiotics. However, some species are known to form spores which can lead to reinfection unless addressed in initial treatment.

HOSPITAL INFECTIONS

If an illness, injury, or infection proves severe enough to need further monitoring the patient may be admitted to the hospital for a longer period of time. However, in a new environment surrounded by people fighting illnesses of their own and with a potentially already weakened immune system, that person is more susceptible to contracting an infection independent of the original infection or illness. This is what is known as a nosocomial infection or a hospital-acquired infection. A nosocomial infection is categorized as any infection contracted while receiving medical care. This infection must not have been present at the time admission and develops within 48 hours of being admitted. The most common nosocomial infections are urinary tract infections which include symptoms such as painful urination, abdominal pain, and fever. Surgical site infections are also common nosocomial infections in which bacteria can enter the surgical site and cause skin redness, tenderness, and surgical site drainage. Doctors can use gram staining to differentiate the bacteria causing the existing infection and the bacteria causing the nosocomial infection. By identifying the bacteria in question doctors can then use this information to prescribe antibiotics, treat other infected patients, and trace the issue back to the source. The risk of nosocomial infections can be minimized by using proper protective equipment such as gloves, gowns, and masks, frequent hand washing, limiting patient to patient contact, and routine disinfection and sterilization.

In this lab, you will identify four bacteria species based on their gram stain, shape, and size. In addition, your class will observe the different colony morphology of each species when grown on agar plates. You will then use this information to determine which bacteria is causing a nosocomial infection amongst the three hospital patients.

Experiment Overview

EXPERIMENT OBJECTIVE

Gram staining is one of the most popular and important procedures in microbiology! Bacterial infections can be deadly to patients if not treated quickly and effectively. Antibiotics are often the best treatment option for bacterial infections. Healthcare professionals often use gram staining to identify the bacteria causing the infection in order to prescribe the best antibiotic to treat the patient. In this experiment, students will isolate bacteria from a mixed culture and utilize Gram staining to examine the morphological characteristics and gram status of four unknown bacteria.

LABORATORY SAFETY

Wear gloves and safety goggles

1. Exercise caution when working with the open flame used to heat your slides.
2. The bacteria used in this experiment are not considered pathogenic, but it is still important to follow simple safety guidelines. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant before and after the experiment, wash hands thoroughly with soap and water after working in the laboratory, and disinfect material that has come in contact with the bacteria before disposing them.
3. Wear safety goggles and gloves.

LABORATORY NOTEBOOKS

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

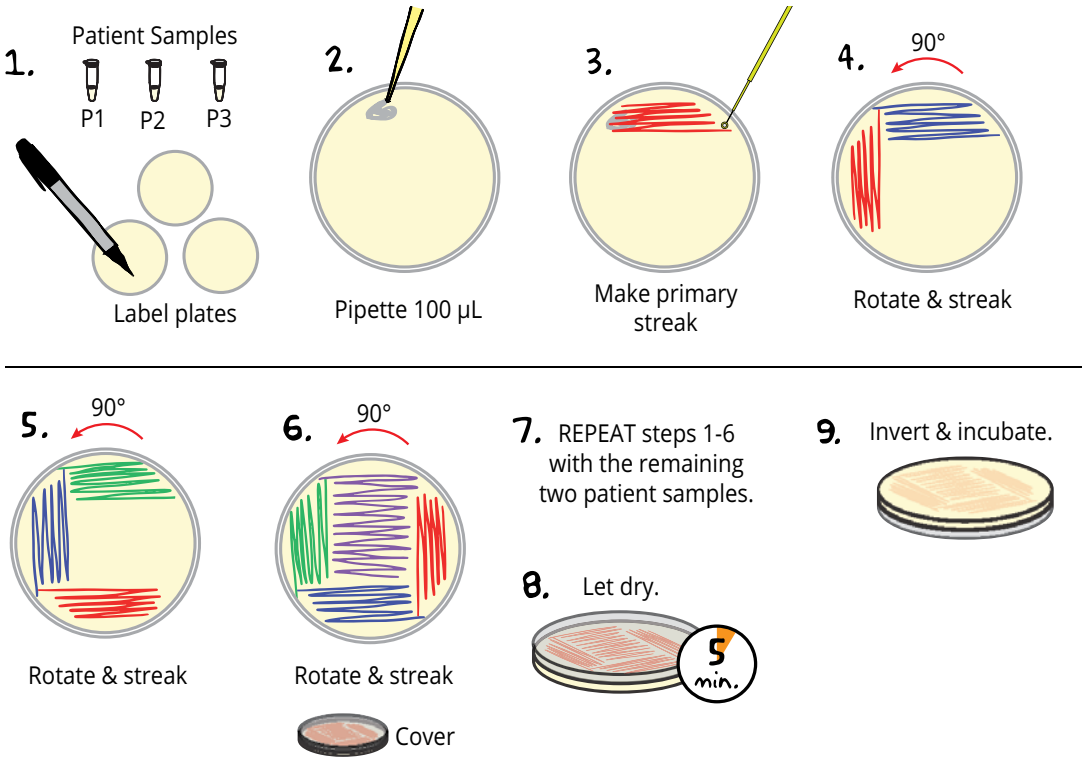
Before starting the Experiment:
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:
- Record your observations.
- Record any challenges faced while performing the experiment.

After the Experiment:
- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Module I-A: Streaking for Isolation

The first step in identifying the bacteria involves creating culture on agar plates. In Module I, you will quadrant streak the patient samples and incubate them for 48 hours to allow the bacteria to grow.



1. **COLLECT** three patient samples (P1, P2, and P3) and three agar plates from your instructor. **LABEL** the agar plates with your group's initials and the patient number of the sample that will be grown on that plate (P1, P2, and P3).
2. Starting with patient sample P1, **MIX** the bacteria by gently pipetting your sample up and down several times. **PIPETTE** 100 µL of the sample onto one corner of the corresponding plate.
3. Using a fresh sterile loop, **STREAK** back and forth at the top of the plate to make the primary streak. Try not to gouge the loop into the medium.
4. **ROTATE** the plate 90°. **DRAG** the loop through the primary streak once, then zig-zag across a clean part of the agar several times to create a secondary streak.
5. **ROTATE** the plate 90°. **DRAG** the loop through the secondary streak once and then across a clean part of the agar several times.
6. **ROTATE** the plate 90° once more. **DRAG** the loop through the third streak and then zig-zag across the remaining clean agar. This should produce isolated colonies. Dispose of loop accordingly. **COVER** plates.
7. **REPEAT** steps 1-6 with the remaining two patient samples (P2 and P3) on the remaining agar plates. ***NOTE: Be sure to use a new sterile loop for each sample.***
8. Let plates sit for 5 minutes to **DRY**.
9. **INVERT** the plate and **INCUBATE** at 30°C for 48 hours.

NOTE:
Keep plates covered with the lid unless adding patient sample and spreading with a loop.

Module I-B: Analysis

SUMMARY: Now that the baterica have grown, you will examine the morphology of the different bacteria on the plate to determine a preliminary identification.

1. **COLLECT** your patient plates from Module I-A.
2. Keeping the lid of the plates closed, **OBSERVE** the different species of bacteria present on each plate. You should be able to identify two species of bacteria on each plate.
3. **RECORD** your observations in your lab notebook or in Observation Table 1, below.

NOTE:
If isolation streak was unsuccessful or if both bacteria are not present on plates, use another group's or the control plates provided by your instructor for observation.

Patient	Color	Form	Margin	Elevation
P1				
P2				
P3				

Module I-B: Analysis, continued

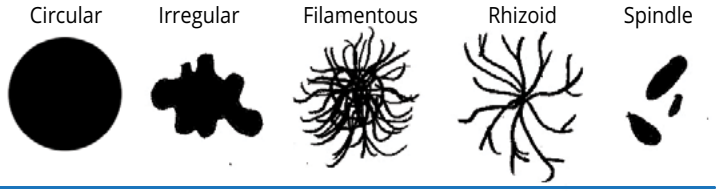
4. Referencing Table A, **IDENTIFY** identify the different species of bacteria present on each plate.

TABLE A

Bacterial Genus and Species	Color	Form	Margin	Elevation
Escherichia coli	Cream	Circular	Entire	Raised
Serratia marcescens	Pink	Circular	Entire	Convex
Bacillus megaterium	Cream	Circular	Entire	Convex
Mycobacterium tuberculosis	Tan	Irregular	Undulate	Undulate
Pseudomonas aeruginosa	Blue-green	Circular	Undulate	Raised
Bacillus subtilis	White	Irregular	Undulate	Raised
Staphylococcus aureus	Yellow	Circular	Entire	Convex
Staphylococcus epidermidis	White	Circular	Entire	Raised

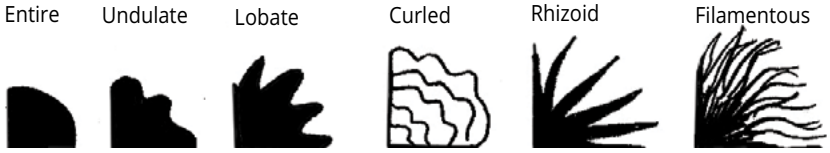
SHAPE/FORM

The overall shape or pattern of the colonies.



MARGIN

The outer border of boundary of each colony.



ELEVATION

The height and shape of the colony off the agar.

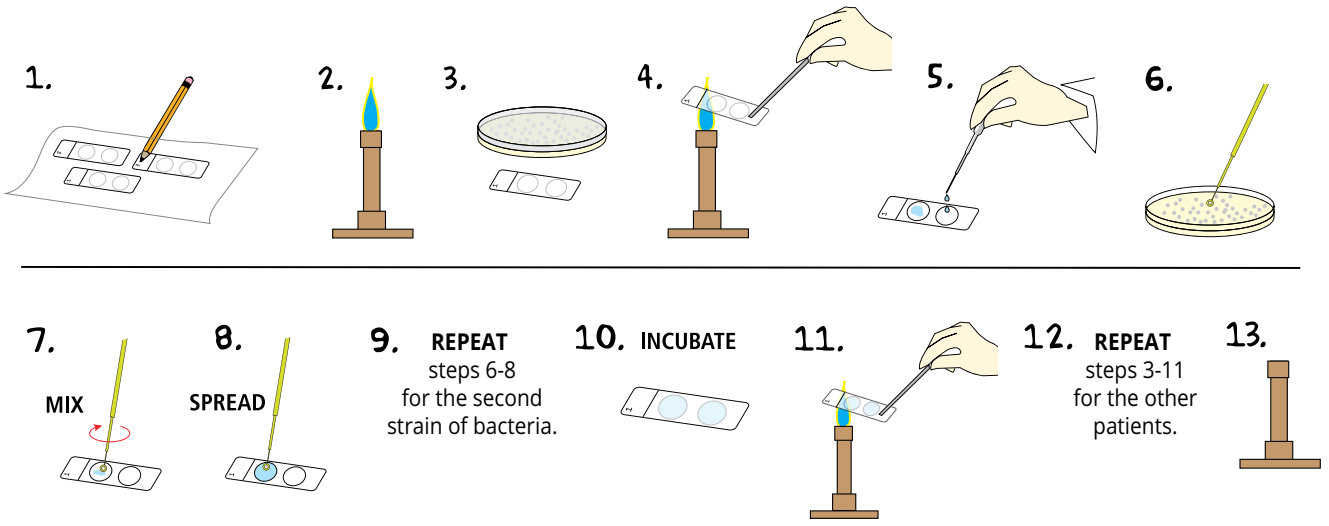
STRAIN #1 STRAIN #2

Plate 1 (P1):_____ _____

Plate 2 (P2):_____ _____

Plate 3 (P3):_____ _____

Module II-A: Preparing the Slides

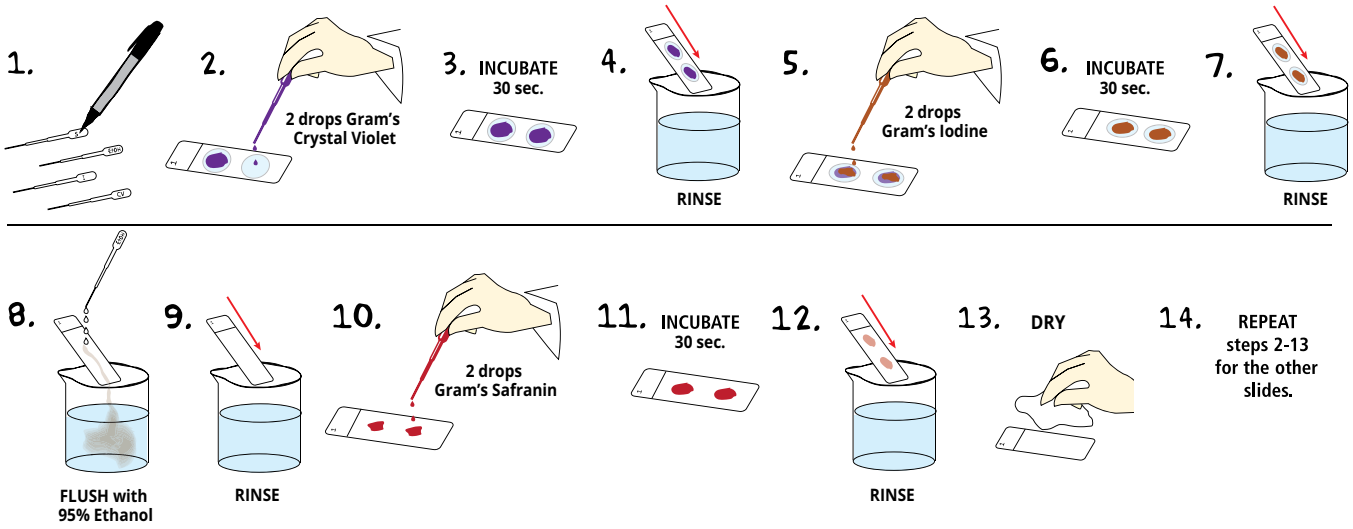


1. **WRITE** your initials or group's ID as well as the numbers 1, 2, or 3 in pencil on the frosted part of three slides. **LAY** these marked slides on a paper towel to prevent scratching. *NOTE: Pencil will not wash off during the following steps.*
2. Carefully **TURN ON** your Bunsen burner or light your alcohol lamp.
3. **COLLECT** your patient sample plates. Then **SELECT** the slide corresponding the plate's number.
4. Using a slide holder or clothespin, quickly **PASS** this slide through a flame two times. Pass the slide horizontally and so that only the middle of the slide comes in contact with the flame. The flame should touch the central section of the slide for one second during each pass (2 seconds total) .
5. While still hot, **ADD** a single drop (20 - 50 µL) of water to the center of each circle. If more than 50 µL of water is added, extend the incubation period in step 10. *NOTE: Be careful to stay within the boundaries of the circle, going over can cause the wax pencil to smear, contaminating results.*
6. **REMOVE** the lid of the agar plate. Using a sterile loop, **SCRAPE** a single colony of the bacteria that is consistent across all three plates.
7. Using the same loop, **MIX** the bacteria with the water in the circle on the ***left*** side of the slide. The solution may appear slightly cloudy but make sure that no clumps are present.
8. **SPREAD** the mixture within the circle.
9. Using a new loop, **REPEAT** steps 6-8 for the second strain of bacteria on the plate in the circle on the ***right*** side of the slide.
10. **INCUBATE** the slide at room temperature for 1 minute or until the water has dried completely.
11. **PASS** the slide through the flame two more times (like in step 4). Have the cells facing up so that they do not come into direct contact with the flame.
12. **PLACE** the slide aside, **REPEAT** steps 3-11 *twice* for the two other plates.
13. **TURN OFF** the Bunsen burner or extinguish the flame of the alcohol lamp.

STEP 6 NOTE:
Control plates can be provided by your instructor if you isolation streak was unsuccessful.

STOP **OPTIONAL STOPPING POINT:**
Slides can be stored in the dark at room temperature for multiple days.

Module II-B: Gram Staining



1. **LABEL** four transfer pipets CV (Crystal Violet), I (Iodine), EtOH (Ethanol), and S (Safranin).
2. Using a transfer pipet, **ADD** 2 drops of Gram's Crystal Violet to the center of each circle - on top of the film of bacterial cells.
3. **INCUBATE** at room temperature for 30 seconds.
4. **REMOVE** excess dye by either rinsing by slowly running distilled water down the slide or by repeatedly dipping the slide into a beaker of distilled water. **SHAKE** off any excess water.
5. Using a transfer pipet, **ADD** 2 drops of Gram's Iodine to the center of each circle.
6. **INCUBATE** at room temperature for 30 seconds.
7. **REMOVE** excess iodine by either rinsing by slowly running distilled water down the slide or by repeatedly dipping the slide into a beaker of distilled water. **SHAKE** off any excess water.
8. **HOLD** the slide at a 45 degree angle over a sink or beaker. Gently **FLUSH** the cells with 95% ethanol by creating a steady stream of drops that hit the center of the slide and then flow down the slide. Continue until the slide appears clear - between 5 to 10 seconds.
9. **REMOVE** excess ethanol by either rinsing by slowly running distilled water down the slide or by repeatedly dipping the slide into a beaker of distilled water. **SHAKE** off any excess water.
10. Using a transfer pipet, **ADD** 2 drops of Gram's Safranin to the center of each circle.
11. **INCUBATE** at room temperature for 30 seconds.
12. **REMOVE** excess Safranin by either rinsing by slowly running distilled water down the slide or by repeatedly dipping the slide into a beaker of distilled water. **SHAKE** off any excess water.
13. **DRY** the slide by gently blotting it with a paper towel or with filter paper. **DO NOT** rub dry and **DO NOT** blot directly on top of the cells. (To wick away water from the central cell spot, place paper on the side of any large droplets.)
14. **REPEAT** steps 2-13 for the two other prepared slides.

STOP

OPTIONAL STOPPING POINT:
Slides can be stored in the dark at room temperature for multiple days.

Module III: Cell Observation

1. **PLACE** a slide under a microscope and **FASTEN** it to the platform.
2. **LOCATE** the cells using the weakest magnification.
3. **SWITCH** to stronger magnifications. Remember to bring the stained cells into focus following each shift in magnification. If you are using an oil immersion microscope add a cover slip and a drop of microscope oil to the slide. ***NOTE: Microscope oil should only be used with the oil immersion lens. Using oil on a lower magnification will damage the lens.***
4. **OBSERVE** the color, shape, and arrangement of the different species of bacteria present on the slide. **RECORD** this information in your lab book or in Observation Table 2, below.

Slide	Circle	Gram Stain Result	Shape	Arrangement
1	left			
	right			
2	left			
	right			
3	left			
	right			

5. **REPEAT** steps 1-4 for the other two slides.
6. Reference Table B to **CONFIRM** the identity of the unidentified bacterial species that all three plates have in common.

TABLE B: BACTERIA IDENTIFICATION INFORMATION

Bacterial Genus and Species	Gram Stain Result	Shape	Arrangement
Escherichia coli	Negative	Bacilli	Single cells
Serratia marcescens	Negative	Bacilli	Chains
Bacillus megaterium	Positive	Bacilli	Chains
Staphylococcus epidermidis	Positive	Cocci	Clusters
Pseudomonas aeruginosa	Negative	Bacilli	Single cells
Bacillus subtilis	Positive	Bacilli	Single or chain
Staphylococcus aureus	Positive	Cocci	Clusters

Slide 1:_____ _____

Slide 2:_____ _____

Slide 3:_____ _____

Study Questions

1. Organelles are specialized subunits within a cell that have a specific function. Name three organelles that bacteria have and their functions. How do these organelles differ from most organelles found in eukaryotes?

2. Name at least two ways to classify bacteria. Why might there be several different ways to classify bacteria?

3. How are gram-positive and gram-negative bacteria different?

4. What is the purpose of each component of the gram stain (i.e. crystal violet, iodine, 95% alcohol, safranin)?

Instructor's Guide

NOTE TO THE INSTRUCTOR

This experiment is intended for 10 student groups. In this experiment, students will utilize Gram staining to characterize and identify bacteria, a diagnostic tool frequently used by medical professionals. While the students will be looking to identify *Staphylococcus aureus* as the culprit bacteria, *Micrococcus luteus* will actually be used as a non-pathogenic, classroom safe alternative. Note, while real patient samples are **NOT** being used and the bacteria used are **non-pathogenic**, students should always practice aseptic technique and use caution when working with bacteria.

APPROXIMATE TIME REQUIREMENTS

Preparation For:	What to do:	When:	Time Required:
Module I	Prepare LB agar plates	2-14 days before performing the experiment.	1 hour
	Prepare 30°C incubator	Anytime before Module I.	30 min.
	Prepare *micrococcus* culture	One day before Module I	10 min.
	Prepare patient samples and make source plates	One hour before performing the experiment.	1 hour
Module II	Dispense Grams crystal violet, iodine, ethanol, safranin and water.	Anytime before performing the experiment.	30 min.
	Prepare slides	Anytime before performing the experiment.	20 min.

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

IMPORTANT NOTE:
While the students will be looking to identify S. aureus as the culprit bacteria, M. luteus is actually being used as a non-pathogenic, classroom safe alternative.

PreLab Preparations Overview

1. Up to two weeks before Module I, **POUR** agar plates.

2. One day before Module I, **PREPARE** liquid *Micrococcus luteus* culture.

3. On the day of Module I:

   a. **LABEL** microcentrifuge tubes:
   - **LABEL** 10 microcentrifuge tubes "P1" for patient 1.
   - **LABEL** 10 microcentrifuge tubes "P2" for patient 2.
   - **LABEL** 10 microcentrifuge tubes "P3" for patient 3.
   - **LABEL** 10 microcentrifuge tubes "H_2O" for water.

   b. **ALIQUOT** 400 µL of each patient sample into the appropriately labeled tube (P1, P2, P3).

   c. **ALIQUOT** 500 µL of distilled water into ten microcentrifuge tubes.

FOR MODULE II:

4. **ALIQUOT** 5 mL of Gram's Crystal Violet into ten labeled flasks. **COVER** to prevent evaporation.

5. **ALIQUOT** 5 mL of Gram's Iodine into ten labeled flasks. **COVER** to prevent evaporation.

6. **ALIQUOT** 20 mL of 95% ethanol into ten labeled flasks. **COVER** to prevent evaporation.

7. **ALIQUOT** 5 mL of Gram's Safranin into ten labeled flasks. **COVER** to prevent evaporation.

8. For slide washing, either **PREPARE** ten beakers or eight ten bottles with water.

9. **DISTRIBUTE** solutions, slides, plastics, and equipment to each student group.

FOR THIS EXPERIMENT, EACH GROUP WILL NEED:

Module I-A

- Three plates of Nutrient Agar
- Three sterile loops
- Tube of P1 (400 µL)
- Tube of P2 (400 µL)
- Tube of P3 (400 µL)

Module II-A

- 3 Sample plates from Module I (or control plates)
- 6 Sterile loops
- 3 Slides
- Pencil
- Distilled water (500 µL)
- 1 Small transfer pipet
- Paper towels
- Bunsen burner or alcohol lamp

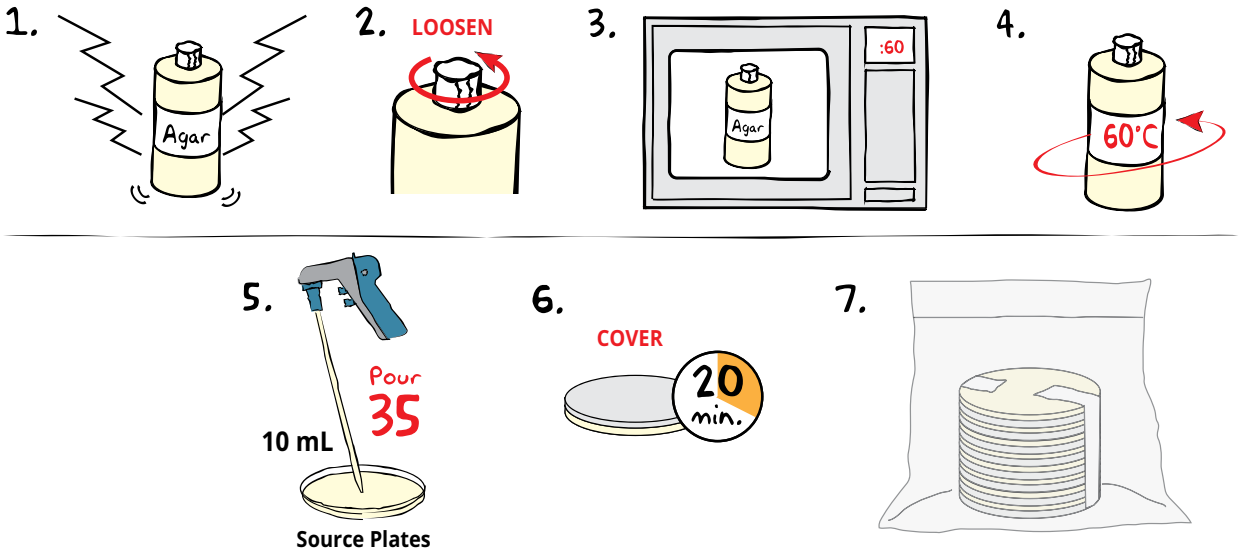
Module II-B

- 5 mL Gram's Crystal Violet
- 5 mL Gram's Iodine
- 20 mL 95% Ethanol
- 5 mL Gram's Safranin
- 4 Large transfer pipettes
- Bunsen burner or alcohol lamp
- Squeeze bottles filled with water and access to a sink OR beakers filled with distilled water
- Slide holder or clothespin
- Paper towels or blotting Paper

Module III

- Microscope
- Cover slip and mounting media
- Immersion oil, if desired

PreLab Preparations: Module I-A



POURING AGAR PLATES

1. **BREAK** solid ReadyPour Nutrient Agar into small chunks by vigorously squeezing and shaking the plastic bottle.

2. **LOOSEN**, but DO NOT REMOVE, the cap on the ReadyPour™ Nutrient Agar bottle. This allows the steam to vent during heating.

   CAUTION: Failure to loosen the cap prior to heating may cause the bottle to break or explode.

3. **MICROWAVE** the ReadyPour™ Nutrient Agar on high for 60 seconds to melt the agar. Carefully **REMOVE** the bottle from the microwave and **MIX** by swirling the bottle. Continue to **HEAT** the solution in 30-second intervals until the agar is completely dissolved (the amber-colored solution should be clear and free of small particles).

4. **COOL** the ReadyPour™ Nutrient Agar to 60°C with careful swirling to promote even dissipation of heat.

5. **POUR** 10 mL of the cooled ReadyPour™ Nutrient Agar into each of the thirty-five large petri dishes using a 10 mL pipet and pipet pump, if available. Alternatively, plates can be poured directly from the bottle, adding enough agar to make a thin layer that coats the bottom of the plates. *NOTE: Thirty plates are to be designated for students performing Module I-A: Streaking for Isolation. The remaining 5 plates are to be designated for the control plates (see page 20).*

6. **COVER** and wait at least twenty minutes for the plates to solidify. For optimal results, leave plates at room temperature overnight.

7. **STORE** plates at room temperature for no more than two days. Plates should be inverted and placed in a sealable plastic bag to ensure that they do not dry out. Inverted and bagged plates can also be stored in the refrigerator for up to two weeks.

   NOTE: When using plates out of the refrigerator, let warm up to room temperature before adding bacteria.

PreLab Preparations: Module I-A

PREPARING *MICROCOCCUS* LIQUID CULTURE

NOTE: The micrococcus culture must be started the DAY BEFORE performing the experiment. Remaining bacteria will be prepared immediately before performing the lab.

1. **ADD** 20 mL of Growth Medium to a 50 mL conical tube. **LABEL** the tube "*M. luteus* culture".
2. **ADD** 250 µL of Growth Medium to the vial labeled *M. luteus* LyphoCells™ to resuspend the bacteria. Let stand for several minutes to rehydrate.
3. **ADD** all of the resuspended *M. luteus* LyphoCells™ to the conical tube of Growth Medium labeled "*M. luteus* culture".
4. **INCUBATE** the culture at 30° C for 24 hours.

PREPARING THE PATIENT SAMPLES

NOTE: Complete this preparation right before you are about to perform this part of the experiment.
Be sure to save the remaining resuspended LyphoCells™ bacteria mixtures for preparing the control plates.

1. **LABEL** 10 snap-top tubes "P1", 10 snap-top tubes "P2", and 10 snap-top tubes "P3".
2. **COLLECT** your *M. luteus* culture from the previous day.
3. **LABEL** three 15 mL conical tubes: Patient 1, Patient 2, and Patient 3. **ALIQUOT** 5 mL of the *M. luteus* culture into each of the three labeled conical tubes.
4. **ADD** 250 µL Growth Medium to the vial labeled *E. coli* LyphoCells™ to resuspend the bacteria. Let stand for several minutes to rehydrate.
5. **ALIQUOT** 100 µL of the resuspended *E. coli* to the Patient 1 conical tube. **CAP** and **INVERT** to mix.

NOTE: Save remaining resuspended LyphoCells™ culture for preparing the control plates in the following section.

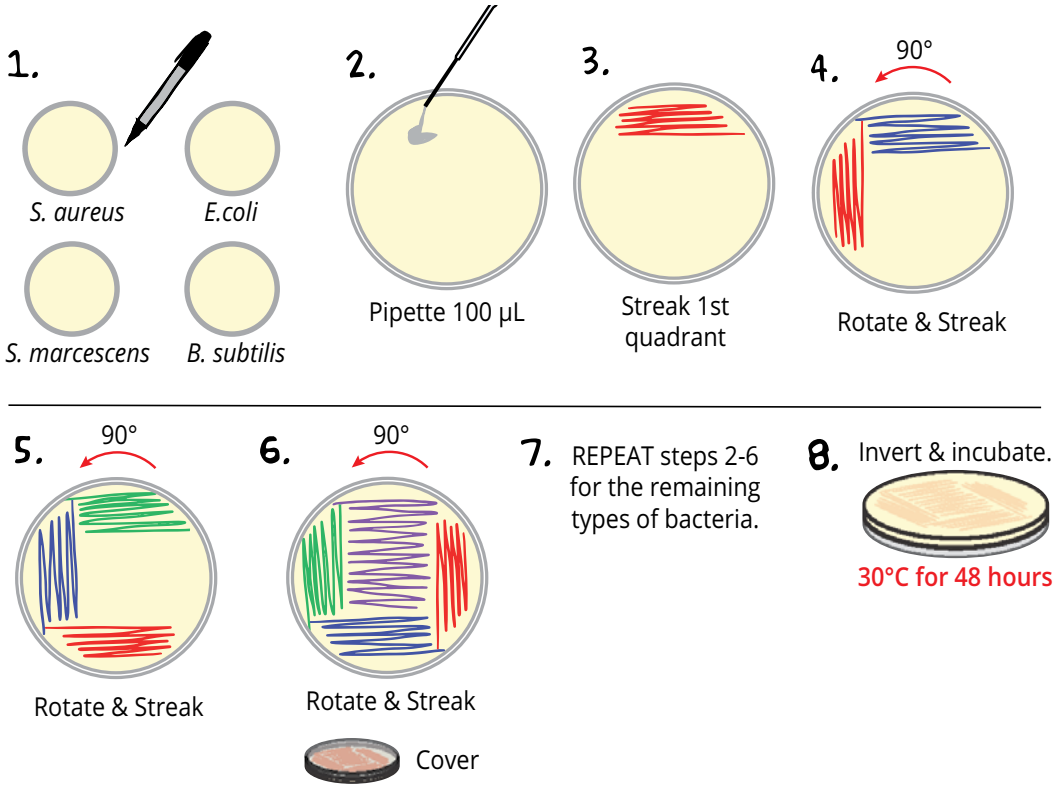
6. **ALIQUOT** 400 µL of the Patient 1 mixture into each of the "P1" labeled microcentrifuge tubes.
7. **REPEAT** steps 4-6 for the remaining patient samples using the *Serratia marcescens* LyphoCells™ for Patient 2 and the *Bacillus subtilis* LyphoCells™ for Patient 3.
8. Each group will receive three microcentrifuge tubes - one of each patient sample (P1, P2, and P3).

NOTE: Save remaining resuspended LyphoCells™ culture for preparing the control plates in the following section.

Patient 1	*Micrococcus Luteus*	*Escherichia coli*
Patient 2	*Micrococcus Luteus*	*Serratia marcescens*
Patient 3	*Micrococcus Luteus*	*Bacillus subtilus*

IMPORTANT NOTE:
While the students will be looking to identify S. aureus as the culprit bacteria, M. luteus is actually being used as a non-pathogenic, classroom safe alternative.

PreLab Preparations: Module I-A



PREPARING CONTROL PLATES

NOTE: Control plates can be used for Module I-B and Module II-A if students did not see growth on their plates. Control plates can be streaked using the remaining resuspended LyphoCells™ bacteria mixtures.

1. **OBTAIN** 4 nutrient agar plates and **LABEL** with bacteria species names: *S. aureus*, *E. coli*, *S. marcescens*, and *B. subtilis.*
2. **PIPETTE** 100 µL of the *M. luteus* culture onto the side of the corner of the labeled "*S. aureus*" nutrient agar plate*.
3. **STREAK** the loop back and forth through the sample to make a primary streak at the top of the plate. Try not to gouge the loop into the medium.
4. **ROTATE** the plate 90°. **STREAK** the loop through the primary streak once, then zig-zag across a clean part of the agar several times to create a secondary streak.
5. **ROTATE** the plate 90° again. **STREAK** the loop through the secondary streak once and then across a clean part of the agar several times.
6. **ROTATE** the plate 90° once more. **STREAK** the loop through the third streak and then zig-zag across the remaining clean agar. This should produce isolated colonies. **COVER** plates.
7. **REPEAT** steps 2-6 for additional control plates using the remaining rehydrated LyphoCells™ bacteria.
8. **INCUBATE** inverted at 30°C for 48 hours.

IMPORTANT NOTE:
While the students will be looking to identify S. aureus as the culprit bacteria, M. luteus is actually being used as a non-pathogenic, classroom safe alternative.

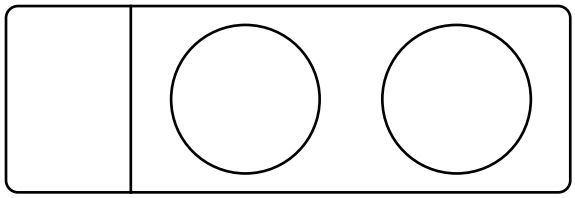
PreLab Preparations: Module II

FOR MODULE II-A

- **ALIQUOT** 500 µL of distilled water into ten microcentrifuge tubes.

Slide Preparation:

- Using the wax pencil, **DRAW** two circles on each of the 30 slides students will use for Module II. *NOTE: Ensure that circles are complete with NO GAPS in perimeter.*
- **REFERENCE** the diagram below for size and placement of the circles.



NOTE: Alternatively, students can share the wax pencil to draw circles on their own slides.

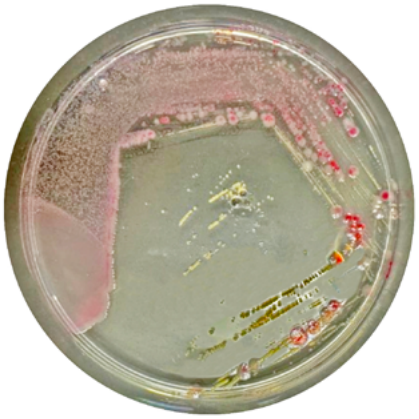
FOR MODULE II-B

1. **ALIQUOT** 5 mL of Gram's Crystal Violet into ten labeled flasks. **COVER** to prevent evaporation.
2. **ALIQUOT** 5 mL of Gram's Iodine into ten labeled flasks. **COVER** to prevent evaporation.
3. **ALIQUOT** 20 mL of 95% ethanol into ten labeled flasks. **COVER** to prevent evaporation.
4. **ALIQUOT** 5 mL of Gram's Safranin into ten labeled flasks. **COVER** to prevent evaporation.
5. For slide washing, either **PREPARE** ten beakers or ten squeeze bottles with water.
6. **DISTRIBUTE** solutions, slides, plastics, and equipment to each student group.

Experiment Results and Analysis

MODULE I-A

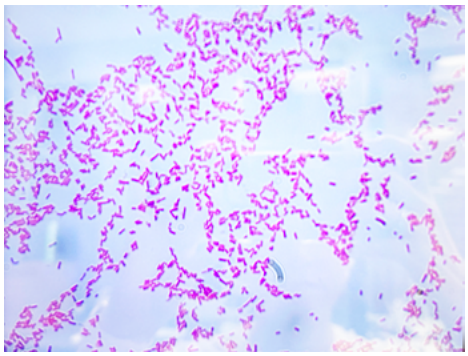
PATIENT 1:
Escherichia coli and
Staphylcoccus aureus



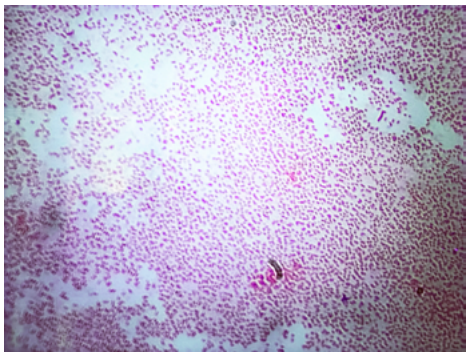
PATIENT 2:
Serratia marcescens and
Staphylcoccus aureus

PATIENT 3:
Bacillus subtilis and
Staphylcoccus aureus

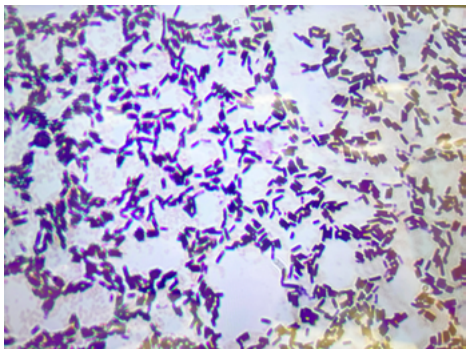
MODULE I-B



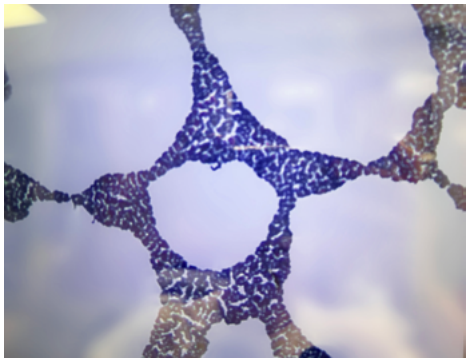
PATIENT 1:
Escherichia coli



PATIENT 2:
Serratia marcescens



PATIENT 3:
Bacillus subtilis



COMMON BACTERIA AMONGST ALL PATIENTS:
Staphylcoccus aureus

Answers to Study Questions

1. **Organelles are specialized subunits within a cell that have a specific function. Name three organelles that bacteria have and their functions. How do these organelles differ from most organelles found in eukaryotes?**

   Students can list several including nucleoid (organizes and maintains the cell's DNA), plasmids (also organizes and maintains the cell's DNA and enables DNA exchange), flagellum (movement), pilus (movement), ribosomes (translation of RNA into proteins), and cell wall (protection, structure, and support). These organelles are different from those found in eukaryotes because they do not have surrounding lipid membranes that make them distinct from the rest of the cell.

2. **Name at least two ways to classify bacteria. Why might there be several different ways to classify bacteria?**

   Bacteria can be classified by distinct cell features (their gram stain, cell shape, size, etc.), DNA sequences, growth requirements, appearance when grown on different agars, or by the presence or absence of certain proteins. Students may come up with several reasons for these different schemes. Bacteria are a diverse group where there are always exceptions to the rules. Consequently, having multiple classification options help to ensure that a species will be identifiable by at least a handful of traits. Bacteria are also classified for different reasons and in different contexts. For example, in a medical setting, a doctor will primarily want to quickly identify a well-studied species. In contrast, in a field setting, a researcher will want to be able to describe the relationship of a new species to other species using a more data-rich quality like DNA.

3. **How are gram-positive and gram-negative bacteria different?**

   Gram-positive bacteria have thick cell walls composed mostly of peptidoglycan. These bacteria appear purple-blue following gram staining. Gram-negative bacteria have thinner cells walls and only a thin or single layer of peptidoglycan. They do however have an additional outer membrane. These bacteria appear pink or red following gram staining. Gram-positive and gram-negative bacteria also differ in how they respond to different antibiotics.

4. **What is the purpose of each component of the gram stain (i.e. crystal violet, iodine, 95% alcohol, safranin)?**

   - **Crystal violet** dissociates into CV^+ and CV^- ions which permeate into the outer membrane of the cell, dying the cell purple.
   - **Iodine** traps the dye within the cell wall by binding to CV^+ ions, creating a large crystal violet iodine complex.
   - **Alcohol** helps to highlight the difference between gram-positive and gram-negative bacteria. It does this by tightening the links between certain peptidoglycan molecules in gram-positive bacteria which further entraps the crystal violet stain within the walls of these cells. While degrading the outer membrane and single peptidoglycan layer in gram-negative bacteria which allows the crystal violet stain to easily leave these cells.
   - **Safranin** is a secondary stain that dyes bacteria cells pink. It is used so that gram negative cells can be seen under the microscope.

Appendix A

Troubleshooting Guide

Problem	Cause	Answer
No individual colonies seen on plates	Cells were not properly quadrant streaked	Have students use another groups plates for gram staining or individual colonies from the source plates.
The bacteria on the slide are too dark	Too much bacteria on slide	Have students pick up only single colonies of each bacteria.
	The bacteria were kept in flame too long during heat fixing	Cells were burned by flame or the slide was not completely dry before heat fixing and the bacteria boiled.
The morphology of the cells does not match what it should be	The bacteria were kept in the flame too long	Have students quickly pass the bacteria through the flame.
The bacteria all appear pink when some should be purple	Cells have been over decolorized	Have students stop adding alcohol to the slide as soon as the solvent runs clear.
The bacteria all appear purple when some should be pink	Cells have been under decolorized	Have students add alcohol until the solvent runs clear.
There appears to be no bacteria on the slide	Cells were not properly heat fixed	The slide was not passed through the flame enough times or long enough.

Appendix B

Student Worksheet

FOR MODULE II-A

Each Student Group Should Receive:

Amt.	Component	Check (✓)
3	Patient Sample plates	❑
1	Tube containing 500 µL distilled water	❑
1	Small transfer pipet	❑
3	Slides	❑
1	Bunsen burner or alcohol lamp	❑

FOR MODULE II-B

Each Student Group Should Receive:

Amt.	Component	Check (✓)
1	Flask with 5 mL Crystal Violet	❑
1	Flask with 5 mL Iodine	❑
1	Flask with 20 mL 95% ethanol	❑
1	Flask with 5 mL Safranin	❑
4	Large transfer pipets	❑
1	Squeeze bottle or beaker filled with water (for rinsing)	❑
1	Paper towels or blotting paper	❑

FOR MODULE III

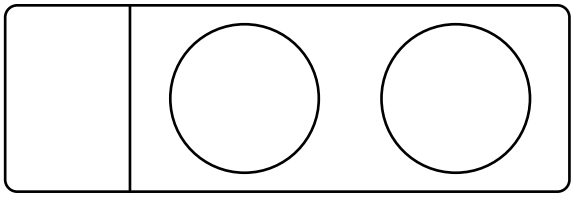
Each Student Group Should Receive:

Amt.	Component	Check (✓)
1	Microscope	❑
1	Observation Table 2 (page 13)	❑

PATIENT IDENTIFICATION

Students may use templates below to draw in to match slide or keep track of identifying characteristics.

PATIENT 1



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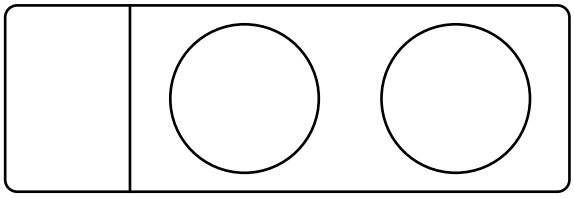
PATIENT 2



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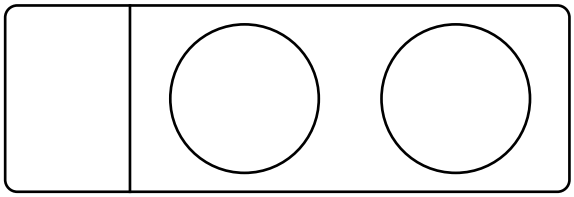
PATIENT 3



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Right Circle: