BIOLOGY 11
Cell Biology Lab: Gram-Staining of Bacteria

Overview:

Bacteria are divided into different groups based on a number of characteristics. One way to distinguish bacteria is by their ability to accept a specific type of stain called a Gram stain.

The Gram-staining method uses two stains, a dark purple one called crystal violet and a reddish-pink stain called safranin. Bacteria that are Gram positive retain the dark purple stain. Bacteria that lose the dark purple stain and show only the pink stain are called Gram negative. The difference in staining characteristics is due to differences in the bacterial cell wall.

In this lab you will be staining three species of bacteria:

* + - 1. *Escherichia coli*
			2. *Bacillus subtilis*
			3. *Serratia marcescens*

If you have any bacteria on your Petri plates growing from last time, you may stain those instead! Make sure that you stain at least three different species before you are finished.

Purpose:

To learn the technique of Gram staining and to classify the each of the three bacteria as either Gram positive or Gram negative.

Materials:

2 species of bacteria

crystal violet

Gram’s iodine

ethyl alcohol

safranin

paper towels

inoculating loop

microscope slide

Bunsen Burner

dying tray

disinfectant spray.

After slide has dried: Microscope with oil immersion lens.

Procedure:

See next pages for Parts A, B, and C.

**Part A: Slide Preparation**

1. The glass slide on which the preparation is to be made must be absolutely clean and free of grease. Wash it thoroughly with soap and water. Dry it over a Bunsen flame by passing it through three or four times. Do not try drying it with paper towels which produce fibers that will be visible on the slide.
2. Using an overhead pen, mark a circle about the size of a dime in the middle of the glass slide. You will put your bacteria on the slide inside this circle.
3. To make a slide from a liquid culture follow the instructions below:
4. Sterilize the inoculating loop by flaming it in the Bunsen burner. Cool the loop for 10 sec.
5. Hold the culture tube in your left hand and loosen the plug without removing it.
6. Remove the plug by grasping it between your fourth finger and thumb.
7. Flame the lip of the culture tube.
8. Insert the sterile loop into the culture and withdraw it
9. Place a “loop-full” of the liquid culture of bacteria on the slide. Use the loop to spread the liquid inside the area you marked with the overhead pen. Do NOT touch the overhead pen mark with the inoculation loop. Let the slide dry in the air. You may speed up the drying by holding the slide high above the Bunsen burner flame.
10. The next step is to “fix” the bacteria on the slide. In order to do this quickly pass the slide bacteria side up through the flame of the Bunsen burner three times. After each passage through the flame test the temperature of the slide on the back of your hand. The bottom of the slide should feel quite warm but not hot. The slide is now ready for staining.

**Part B: Staining the Bacteria with Gram’s Stains**

All bacteria react to a particular series of stains which was developed by a Danish bacteriologist, Christian Gram. The purple dye crystal violet reacts with the cell walls of certain (“Gram positive”) bacteria. Other bacteria (“Gram negative”) lose the colour of the crystal violet when the slide is treated with ethyl alcohol and would appear colourless in the slide preparations but are “counterstained” with a red stain safranin so they become visible. Because of the difficulty of classifying bacteria on the basis of structure, the Gram staining technique has been of great value in separating various types of bacteria.

1. Pour tap water into a beaker until it is about 3 cm. from the top. Place the slide across the top with the bacteria-surface up. (See the first figure).
2. Cover the film with 5 drops of crystal violet from the first bottle in the series. Leave the stain on for one minute.
3. Rinse off the stain by pulling the slide gently to one side until one end drops slowly into the water as shown in the second figure. Dip it up and down in the water. Remove the slide. Refill the beaker with clean water.
4. Place the slide on the top of the beaker again. Add 5 drops of Gram’s Iodine solution for one minute.
5. Rinse the slide in water, then gently blot (do not rub) the slide on paper toweling. Refill the beaker with clean water.
6. Decolourize the preparation by flooding it in 95% ethyl alcohol (EtOH), tipping the slide from side to side until no more stain washes off. The time for decolourization varies from 20 seconds to a minute. This is a very important step, as the Gram-negative organisms will lose the stain at this time. When no more purple streaks can be seen washing out of the smear, rinse the slide gently in water in your beaker.
7. Blot the slide gently and then counter-stain the preparation with 5 drops of safranin for about 30 seconds. This stain colours the gram negative bacteria so that they are visible.
8. Rinse the slide in clean water in your beaker and air dry it.

**Part C: Observing Bacteria using the Oil Immersion Lens**

Bacteria which have been fixed and stained as you have in this exercise are always examined without using a coverslip and usually using and oil immersion lens. Follow the steps below.

1. Lower the stage of the microscope to its lowest position, and rotate the nosepiece so that the low power lens is in position.
2. Place the slide on the stage and secure it with the calipers. Using the caliper adjustment knobs, move the smear to the center of the stage directly under the low power lens.
3. While watching from the side, move the stage to its highest position. (you watch from the side as a precaution to be sure the slide will not hit the objective lens!)
4. Locate the smear under low power. The bacteria may appear only as tiny coloured specks. Move the slide around until a group of specks is located near the center of the field of view. Focus using coarse adjustment on low power by moving the stage away from the lens.
5. Change to medium power. Center the specks in the field. Focus using coarse adjustment.
6. While watching from the side, turn the nosepiece to change to high power. Be careful! Do not let the lens touch the slide as you turn the nosepiece. You will scratch the lens, damaging it permanently.
7. Center the specks in the field. Focus using only fine adjustment.
8. While watching from the side, swing the high power lens to the half-way position between high and oil immersion.
9. Place a drop of immersion oil directly on top of the slide over the smear.
10. Carefully swing the oil immersion lens into place. Look at the lens and slide from the side to see that they do not touch. The oil should now “join” the immersion lens to the smear. The lens will touch the oil.
11. Focus only with the fine adjustment and do it very carefully.
12. If more than one smear has been placed on the slide, perform the steps above to allow you to see the bacteria on one smear. Next, place oil on the other smear and using the mechanical stage, move the slide to the next smear without refocusing the microscope. The other smear will be in focus or will need adjustment with the fine focus only.

Observations:

* Draw three biological diagrams of the three different bacteria species under oil immersion.
* Write the name of the bacteria at the top of the circle.
* Record the magnification of your drawing at the bottom right-hand of the circle by dividing your drawing size by the estimated actual size of the bacteria. Colour your drawing to illustrate which bacteria are Gram positive and which are Gram negative.

Discussion Questions:

1. Why do you think that you flame the slide with the smear facing away from the flame?
2. Why should water not be run directly on the smear?
3. Give two reasons for staining bacteria using Gram’s stains.
4. On the basis of your lab, does a bacterium’s reaction to Gram stain seem to be directly related to its shape? How could you test your hypothesis?
5. You are given an unknown bacterial culture to Gram stain. The bacteria are identical in size and shape but some are pink and some are purple. What could be a possible explanation for this?

Conclusion:

* Describe “what you did” and “what you found”.
	+ Be sure to answer the purpose in the “what you found” section of your conclusion.