

## Activity 5.1.4: Gram Staining

### Introduction

In the last activity, you isolated the bacteria that was responsible for Anna's illness and performed a gross examination of the resultant colonies. While gross examination of unknown bacteria is helpful in eliminating some possibilities, it is rarely sufficient alone to identify unknown bacteria due to the large amount of bacterial species and degree of variation between them. Therefore, further tests are required. All bacteria are divided into two main groups, depending on how they react to a specific set of dyes called the *Gram stain*. A microbiologist named Hans Christian Gram developed the staining protocol in the 1880s, and it remains one of the first steps in classifying or identifying bacteria. If the bacteria appear purple after being treated with the stain, they are classified as Gram positive. The bacteria are considered to be Gram negative if they appear pink.

A Gram stain is usually one of the first steps in identifying bacteria. In addition to the gram stain, the morphology, or structure, of the bacteria can also be observed. There are three basic morphologies of bacteria (based on the shape of a single cell): bacillus (rod), coccus (sphere), and spirillum (spiral). In this activity you will create bacterial smears on microscope slides and perform a Gram stain on three types of bacteria, including the bacteria isolated from Anna's sample. You will look at the stained samples under the microscope and identify the morphology of the bacteria as well as whether the bacteria are Gram positive or Gram negative. You will be working with live bacteria and dyes that permanently stain clothes, the floor, and counter tops. Read all the directions carefully before beginning and follow all safety precautions. If you have questions, ask your teacher.

### Equipment

- Computer with presentation software
- Laboratory journal
- PBS Course File
- Gram Stain presentation
- Activity 5.1.1 Medical History Resource Sheet
- Aseptic Technique Resource Sheet
- Microscope with oil immersion (100x) objective
- Immersion oil
- Commercially prepared and stained slides of bacteria
- "Anna's sample" plate from Activity 5.1.3
- Gram Stain and Bacterial Morphology Kit from Carolina Biological
- Alcohol lamp
- Matches
- 5 Microscope slides and cover slips
- 5 Individually wrapped sterile inoculation loops
- Colored pencils

- Test tube clamp or wooden spring-style clothes pin
- Safety goggles
- Latex or Nitrile exam gloves
- Laboratory apron
- Disinfectant in squirt bottle
- Disinfectant in 500 mL beaker

## Procedure

### Part I: Bacteria Morphology

1. Examine the structure of a bacterial cell. Use the Cells Alive *Bacterial Cell Structure* website found at: <http://www.cellsalive.com/cells/bactcell.htm#surface> as well as other reliable sites for information.
2. Draw and label a diagram of a bacterial cell in your laboratory journal. Use colored pencils to enhance your diagram. Include the following cellular parts on your diagram:
  - Nucleoid
  - Plasmid
  - Ribosomes
  - Cell wall
  - Plasma membrane (cell membrane)
  - Capsule
  - Flagella
  - Pili
  - Endotoxins
3. Create text boxes containing a description of the function for each labeled component on the drawing.
4. Research the difference in cell wall structure between a Gram positive and Gram negative bacteria. Take notes in your laboratory journal.
5. Access the Gram Stain presentation. As you work through the presentation, create a storyboard in your laboratory journal that depicts what is occurring at each step of the gram staining procedure for both Gram positive and Gram negative bacterial cells. Make sure to include colored pictures of the cells at each step.
6. Note that in addition to the Gram stain, the morphology of the bacteria can also be observed when looking under a microscope. There are three basic morphologies of bacteria (based on the shape of a single cell): bacillus (rod), coccus (sphere), and spirillum (spiral).
7. View the Gram positive and Gram negative bacteria prepared slides. Use the *How to Use a Microscope* resource sheet as your guide.
8. Use colored pencils to sketch what you see in your laboratory journal. Be sure to properly label your diagrams including the magnification.
9. View the prepared slide of *E. coli* and *Streptococci*. Use colored pencils to sketch what you see in your laboratory journal. Be sure to properly label your diagrams including the magnification.

10. Carefully examine the bacteria to determine whether the cell shapes are round (cocci), rod-shaped (bacilli), or spiral (spirillum) as well as if they are Gram positive or Gram negative. Record the shapes and Gram stain next to each sketch.

## **Part II: Gram Stain**

11. Before continuing, check with your teacher and watch a demonstration of the steps listed below or view the video that shows how the Gram stain is performed. This video is available from [http://highered.mheducation.com/sites/007337525x/student\\_view0/exercise9/gram\\_stain.html](http://highered.mheducation.com/sites/007337525x/student_view0/exercise9/gram_stain.html).
12. Put on gloves and safety goggles.
13. Note that you will perform a Gram stain for three different bacterial samples, including Anna's sample. Complete the entire Gram staining procedure for one sample before moving on to the next sample.
14. Place one drop of water onto the center of a slide.
15. Using aseptic technique and a disposable inoculation loop, remove a small amount of bacteria from the sample tube, or plate, as demonstrated by your teacher. You want to remove only a very small amount of bacteria; you should barely see anything on the end of the loop. Remember bacterial cells cannot be seen without a microscope and they are much smaller than human cells. So a small amount of culture on the tip of the loop will contain millions of cells.
16. Using a circular motion, mix the bacterial culture into the drop of water on the slide. You want to make a uniform smear in the center portion of the slide. Pretend the bacterial culture is a small amount of paint; you are mixing it with water to coat the middle of the slide. The smear should look like a thin, faintly cloudy film on the slide. It should not appear opaque or thick.
17. Place the used inoculation loop in the beaker containing the disinfectant. Be sure the portion of the loop that contacted the bacteria is completely submerged in the disinfectant.
18. Allow the water to completely evaporate from the slide. It is important that the slide air dries. After the water evaporates, you may not see anything on the slide, or you may see a slight haze. (If you see a very cloudy heavy coating, you added too much bacterial culture. Continue with the procedure, but use less culture the next time.)
19. Light the alcohol lamp.
20. Use a test tube clamp or clothes pin to hold the slide, as demonstrated by your teacher.
21. Make sure your hair and clothing are safely away from the flame.
22. Carefully pass the center portion of the slide through the flame 3 times, as demonstrated by your teacher. Remember not to hold the slide in the flame; you are not trying to cook the bacteria. The heat will cause the bacteria to stick to the slide so that you can stain them without washing them off the slide.
23. Move the area designated by your teacher as the staining area.

24. Carefully add the dye Crystal Violet to cover the center of the slide where you made the bacterial smear. Add only enough to cover the area. Do not waste the dye by overflowing the slide.
25. Allow the dye to remain on the slide for 1 minute.
26. Rinse the dye off using a squirt bottle filled with water. Be careful not to splash the dye on clothes or other materials. Crystal Violet is a permanent stain.
27. Do not wipe or dry the slide.
28. Carefully add the dye Gram's Iodine to cover the center of the slide. Add only enough to cover the area with the bacterial smear. Do not waste the dye by overflowing the slide.
29. Allow the dye to remain on the slide for 1 minute.
30. Rinse the dye off using a squirt bottle filled with water. Be careful not to splash the dye on clothes or other materials.

*This next step is the most critical part of the Gram stain; if the slide is rinsed for too long, the bacteria may be too decolorized and appear as false negatives. On the other hand, if the bacteria are not rinsed enough, they may retain stain and be false positives.*

31. Hold the slide at a 45° angle lengthwise (like a slide at the playground). You can hold the slide with your gloved hand or use a test tube holder (or a clothes pin). Using an eye dropper or squirt bottle, add drops of the Acid Alcohol at the "top" of the slide and allow the alcohol to run down the length of the slide. Continue this until no more color runs down the slide, usually about 15 or 30 seconds.
32. Use a squirt bottle to carefully rinse the slide with water.
33. Carefully add the dye Safranin to the center portion of the slide.
34. Allow the dye to remain on the slide for 30 seconds, and then carefully rinse it off with water.
35. Place the slide between two pieces of paper towel and press the towel onto the slide. Do not wipe the slide or you will wipe the bacteria off the slide. You want to blot the water off the slide, not remove the bacterial smear.
36. Wipe the bottom of the slide to remove any excess stain or water so you do not damage the microscope stage.
37. Wash the area where you were working with a disinfectant as directed by your teacher.
38. Place your gloves in the hazardous waste container.
39. Wash your hands.
40. Use proper microscope technique to view the bacteria; first, use the low power objective and then increase magnification until you are using the oil immersion objective.
41. Record in your laboratory journal the shape and Gram stain for each of the bacterial cultures as directed by your teacher.

42. Dispose of the slides as directed by your teacher. Do not place them in a trash can.
43. Refer to the Activity 5.1.1 Medical History document and fill in the test you just performed under the *Tests* section as well as the results you determined for Anna's sample under the *Results* section (i.e., the Gram stain and morphology of Anna's bacterial sample). You will fill in the remaining blanks in the subsequent activity.
44. Fill in the Gram staining results for *Bacillus megaterium* and *Micrococcus luteus* in the chart below. Match your Gram staining results for Anna's sample with the Gram stain results below in order to narrow down the possibilities of the species of bacteria responsible for Anna's infection.

Species	Gram Stain Results
<i>Enterobacter aerogenes</i>	Gram negative
<i>Enterobacter cloacae</i>	Gram negative
<i>Eschericia coli</i>	Gram negative
<i>Serratia liquefaciens</i>	Gram negative
<i>Serratia marcescens</i>	Gram negative
<i>Streptococcus pneumonia</i>	Gram positive
<i>Streptococcus pyrogenes</i>	Gram positive
<i>Bacillus megaterium</i>	
<i>Micrococcus luteus</i>	

45. Answer the Conclusion questions.

## Conclusion

1. Why are the results of a Gram stain taken from a patient important?
2. What bacterial species can be eliminated as possibilities for the bacteria responsible for Anna's infection? Explain your reasoning.

3. What is your preliminary idea(s) of the species of bacteria responsible for Anna's infection? Has your idea changed since the last activity? Explain your reasoning.