

## Lab Exercise 6: The Gram Stain

### Background

Christian Gram was a Danish physician in the 1800s who was trying to develop a staining procedure that would differentiate bacterial from eukaryotic cells in a stained tissue sample. Although he did not succeed in this endeavor, he did discover a monumental staining technique that distinguishes bacterial cell types based on cell wall structure. In the **Gram stain**, two kinds of cells, **Gram positive** and **Gram negative**, can be identified based on their ability to retain a primary stain throughout a staining process (*i.e.* Gram positive) or their inability to do so (*i.e.* Gram negative). Gram staining therefore divides the entire Domain of bacterial cells into two groups. Although Gram was unaware of it at the time, these differences in staining are caused by the amount of peptidoglycan in the cell wall. Gram positive cells appear purple because their thick layer of peptidoglycan will retain the primary stain, crystal violet, even when they are exposed to alcohol. Gram negative cells on the other hand have a very thin or absent peptidoglycan layer and so alcohol will completely remove all of the crystal violet from the cells (Figure 1).

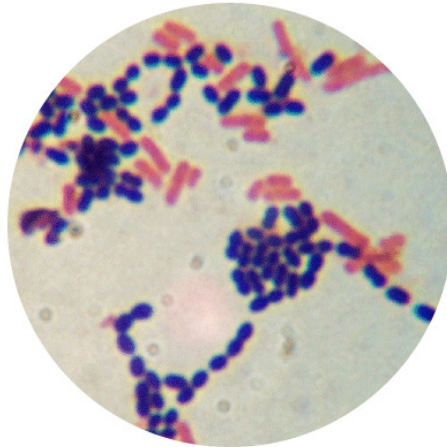


Figure 1: Micrograph of a Gram stain containing Gram positive cocci (purple) and Gram negative bacilli (pink) bacteria.

With the Gram stain, you are being introduced to a new type of staining technique, **differential staining**. Differential staining is a method of subdividing microbes into two groups based on a single characteristic. In this case, as stated above, the presence of a thick peptidoglycan layer is differentiated. All methods of differential staining employ two stains, one to color the cells that are “positive” for a specific characteristic and one to color the cells that are “negative” for it. In the case of the Gram stain, **crystal violet** will stain both Gram positive and Gram negative cells, because it is a basic (positively-charged stain). However, with the addition of **alcohol**, the crystal violet is removed from the Gram negative cells, making them colorless and impossible to identify. For this reason, as with all differential staining techniques, a second stain called a counterstain is used so that the Gram negative cells can be seen, in this case the basic stain **safranin**. The ability of certain cells to retain a stain through the process of decolorization is typically heightened in all differential staining techniques by the use of a **mordant**.

In the Gram stain, the mordant is **iodine**. The iodine complexes with crystal violet within the cell wall of Gram positive cells and keeps them from being decolorized. The basic steps of the Gram stain are as follows:

1. Stain the smear prep with crystal violet.
2. Apply the mordant iodine.
3. Decolorize with acetone:alcohol.
4. Counterstain with safranin.

The Gram stain is one of the most important stains to master in microbiology. It will be crucial in identifying your *Unknown Organisms* later in the semester as well. In addition, 200 years after its discovery, it is still used on a daily basis in medical and clinical microbiology labs for the identification of microbial pathogens.

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### Introduction

In today’s lab you’ll be performing Gram stains of several different organisms. You’ll prepare mixes of Gram positive and Gram negative bacteria to test your skill at the procedure and then have the opportunity to look at two cell types that cause a **Gram positive irregular** result: **spore formers** and **acid fast bacteria**. It’s a good idea to get used to what these two types of cells look like when Gram stained because it will make it easier for you to identify these organisms later on. The organism mixes that are outlined in the protocol below will allow you to identify any problem areas with your Gram stain technique because each of the pairings consists of organisms that are morphologically distinct. *Staphylococcus aureus* is a Gram positive coccus, while *Bacillus megaterium* is a Gram positive bacillus. Likewise, *Pseudomonas aeruginosa* is a Gram negative bacillus and *Branhamella catarrhalis* is a Gram negative coccus.

*B. megaterium* is also a spore-former, so look out for round areas with no stain within these Gram positive cells. *Mycobacterium smegmatis* is an acid fast bacterium which will stain Gram positive, but the coverage will be splotchy. Remember that these two characteristics cause that tell tale “**Gram positive irregular**” staining result- even when you’ve done the Gram stain perfectly.

Note that each of the cultures is listed as a 24 hour old culture. This is important because as a culture ages, individual cells age and their peptidoglycan layers become thinner. This can cause erroneous results which incorrectly cause Gram positive cells to appear Gram negative.

## Objectives

1. Understand the chemical and theoretical basis for differential staining procedures, including the Gram Stain.
2. Perform a successful Gram Stain to differentiate between gram-positive and gram-negative bacteria.

## Gram Stain Protocol

Team Supplies	Individual Supplies
24-hour old culture of <i>Staphylococcus aureus</i>	Gram staining kit (crystal violet, iodine, alcohol decolorizer and safranin)
24-hour old culture of <i>Bacillus megaterium</i>	Staining tray
24-hour old culture of <i>Pseudomonas aeruginosa</i>	Microscope slides
24-hour old culture of <i>Branhamella catarrhalis</i>	Water bottle
24-hour old culture of <i>Mycobacterium smegmatis</i>	Bibulous paper
	Inoculating loop

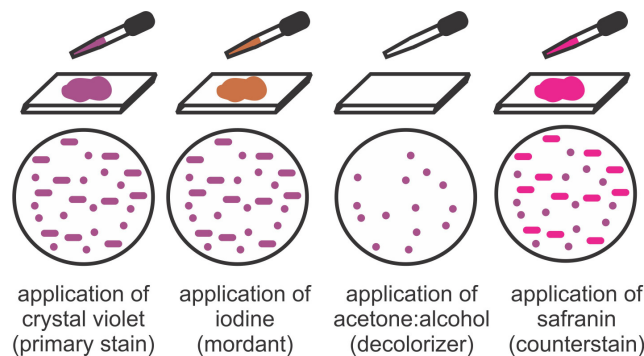
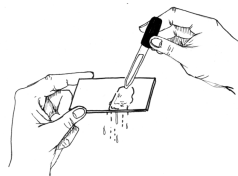


Figure 2: Diagram of the Gram stain procedure.

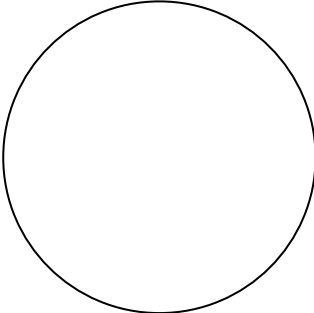
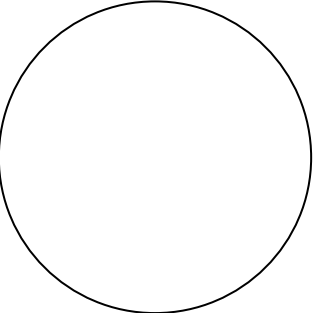
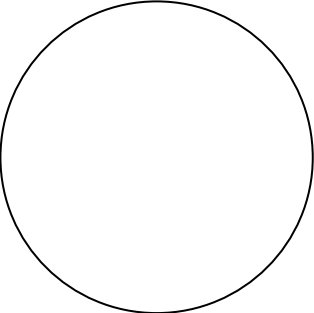
1. Prepare your lab bench.
2. Prepare your slides as directed in the Smear Preparation protocol with the following changes. Add a loopful of the first organism to the top of your slide. Sterilize your loop and obtain a loopful of organism 2. Place the loop of organism 2 directly on top of organism 1. If you are working with an organism from a liquid culture and a solid culture, add the liquid culture first and do not add water to the slide. Prepare slide of the following mixes, taking care not to contaminate the stock cultures while you are preparing them.
  - i. *S. aureus* & *P. aeruginosa*
  - ii. *B. megaterium* & *B. catarrhalis*
  - iii. *M. smegmatis* & *S. aureus*
3. As diagrammed in Figure 2, place your slides on the staining tray and proceed with the Gram stain:
  - iv. Flood the smear with crystal violet for 30 seconds.
  - v. Wash with water to remove excess dye.
  - vi. Flood the smear with Gram's iodine for 1 minute.
  - vii. Wash with water to remove excess dye.
  - viii. Decolorize with alcohol. **IMPORTANT STEP:** Hold slide at an angle. Get one full dropper of decolorizer and starting above the smear, slowly and steadily let the alcohol wash down over the smear for approximately 5 seconds. **IMMEDIATELY** wash with water to stop the decolorization process.



1. **Counterstain** by flooding smear with **safranin** for 1 minute.
2. Wash with water to remove excess dye.
3. Blot with bibulous paper.

### Data Collection and Analysis

1. Examine all slides microscopically. Draw the results in your lab manual, taking care to note morphology, arrangement, Gram reaction of each organism and total magnification.

Drawing	Drawing	Drawing
		
Total magnification _____ x	Total magnification _____ x	Total magnification _____ x

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### Discussion

1. Describe the purposes of each of the following reagents in a differential staining procedure and name the specific type used in the Gram stain: primary stain, counterstain, decolorizing agent and mordant.
2. How do Gram positive and Gram negative bacteria differ in cellular structure? How does this contribute to their differential staining properties in the Gram stain?
3. What is the most critical step in the Gram stain procedure? Why? If this procedure is not done correctly, how might it affect the results?
4. How does culture age affect the results of a Gram stain?