Gram Stain*

Objective/ purpose/ learning goals:

Learning Outcomes:

a) Students will demonstrate mastery of the Gram staining technique.
b) Students will be able to distinguish and categorize bacterial groups from Gram staining results.

Learning Objectives: After completing this experiment, you should understand:
a) The chemical and theoretical basis for differential staining procedures.
b) The chemical basis for the Gram stain.
c) The procedure for differentiating between two principle groups of bacteria: gram-positive and gram-negative.

Key terms: Gram-positive, Gram-negative, decolorizer, mordant, peptidoglycan, outer membrane

Introduction / theory/overview: The Gram stain, developed in 1884 by the Danish bacteriologist Dr. Hans Christian Gram, is a differential stain that is almost always the first step in the preliminary identification of a bacterial species. It classifies bacteria into two large groups (gram-positive and gram-negative) based on differences in cell wall chemical composition and physical properties.

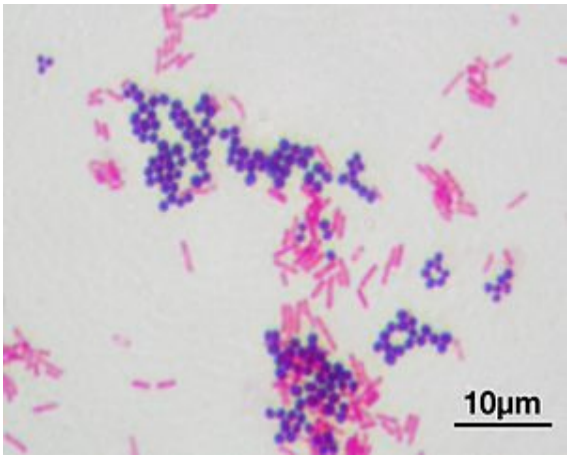
Gram-positive bacteria have a thick net-like cell wall made of peptidoglycan (50–90%), and as a result are stained purple by crystal violet, whereas gram-negative bacteria have a thinner layer of peptidoglycan (10%) plus an outer membrane composed of a phospholipid bilayer, integral membrane proteins and lipopolysaccharide, so do not retain the purple stain and are counter-stained pink by safranin.

There are four basic steps of the Gram stain:

- Applying a primary stain (**crystal violet**) to a heat-fixed smear of a bacterial culture. Heat fixation kills bacteria but is mostly used to affix the bacteria to the slide so that they do not rinse out during the staining procedure. Crystal violet readily enters bacterial cell walls because it is soluble.
- The addition of Gram's iodine. Gram's iodine alone readily enters bacterial cell walls because it is soluble. Gram's iodine is also known as a mordant (a substance which increases the cells'affinity for a stain). It does this by binding to crystal violet, forming an insoluble complex, that gets trapped in the cell wall and does not easily wash away.
- Rapid decolorization with 95% ethanol or acetone. This is the most critical step! The decolorizer serves a dual function: 1) protein-dehydrating agent, 2) lipid solvent.
  In gram-positive cell walls, the decolorizer dehydrates the cross-linking polypeptides found in the thick layer of peptidoglycan. This causes shrinking and narrowing in the diameter of the pores, which translates into a more stringent retention of the crystal violet – iodine (CV-I) complex. Therefore the tightly bound primary stain complex is difficult

to remove, resulting in the gram-positive cells remaining purple. In gram-negative cells, the alcohol dissolves the lipids in the outer membrane, increasing its porosity. Therefore the CV-I complex can be more easily removed from the thinner and less highly cross-linked peptidoglycan layer. After decolorization, gram-negative cells are colorless but gram-positive cells remain violet in color.

- Counterstaining with safranin.[10] Gram-negative cells will be stained reddish / pink from the safranin since they have lost the primary crystal violet stain from the decolorization. Gram-positive cells remain violet because this color is dominant over the reddish / pink color of safranin.



Gram-positive cocci (purple) and Gram-negative bacilli (pink).

https://commons.wikimedia.org/wiki/File:Gram_stain_01.jpg

***References for the Gram Stain came from:**

1. Cappuccino, J.G. & Sherman, N. Microbiology: A Laboratory Manual, 8th ed., Pearson Ed. Inc., 2008.

2. Leboffe, M.J. & Pierce, B.E. Microbiology: Laboratory Theory & Application, 2nd ed., Morton Publishing Company, 2012.

Materials, equipment and organisms (per student/ per group):

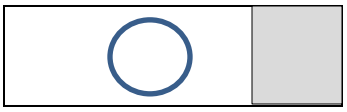
Each student will do gram stains on all of the provided bacterial species.

Live organisms: *Escherichia coli, Serratia marcescens, Klebsiella pneumoniae, Bacillus cereus, Staphylococcus aureus, Micrococcus luteus, Enterococcus fecalis*

Reagents and Materials Needed: microscope slides, marker, loop, slide warmer, Bunsen burner, crystal violet stain, Gram's Iodine, 95% ethanol decolorizer, safranin stain, tap water at sink, stain rack at sink, paper towel for blot drying, colored pencils.

Procedure:

1. Make smears and **heat fix before staining.**



2. Add **CRYSTAL VIOLET** and cover the smears completely. Stain for 1 min. & rinse with H_2O.
3. Add **GRAMS IODINE** (mordant) and rinse with water after 1 min.
4. *Decolorize with **95% ALCOHOL with one drop at a time**. Rinse with water.
   ***VERY CRITICAL STEP. Alcohol that runs off of the slide should be light purple and not completely clear.**
5. Rinse with water to kill the action of the decolorizer.
5. Add **SAFRANIN** and rinse with water after 1 minute.
6. Blot dry with paper towel (may place coverslip on each smear after blot drying).
7. **Wash your hands and remove stains, if any, before getting / handling the microscope.**
8. Observe your stained bacteria, starting with the lowest magnification. Focus @ 1000X magnification under oil and have your professor look at your slide.
9. Your stained bacteria should be (i) same color and shape (ii) mostly distinct and fairly evenly distributed (iii) not clumped or aggregated (iv) no uneven staining.

Safety and disposal: Lab apron and safety goggles should be worn for gram staining procedure. Stained slides can be saved in your slide box. Heat fixed slides should be disposed of in the glass disposal box.

Observations/ interpretation/ Drawings /Results:

Concept check/review/Take Home Lessons: The Gram stain tells you not only the morphology and arrangement of bacterial cells but also the cell wall structure [i.e., gram (+) or gram (-)]. You should know how the gram stain relates to the cell wall of the bacterium. You need to be able to recognize Gram-positive and Gram-negative rods, cocci, spirals and whether they occur in chains, clusters, single etc. by their appearance under the microscope. You should begin to associate particular organisms with their gram stain morphologies. You must know the reagents and the function of each in the staining procedure.

The Gram grade sheet goes along with this exercise. You are to make drawings in correct color of your best Gram positive and Gram-negative slide.