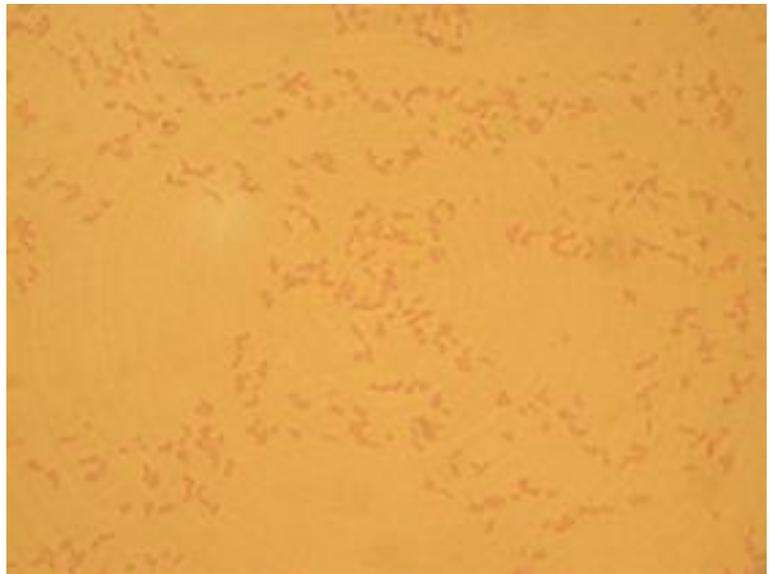


Gram Stain

The Gram stain is the most common differential stain used in microbiology. Differential stains use more than one dye. The unique cellular components of the bacteria will determine how they will react to the different dyes. The Gram stain procedure has been basically unchanged since it was first developed in 1884. Almost all bacteria can be divided into two groups, Gram negative or Gram positive. A few bacteria are gram variable. *Trichomonas*, *Strongyloides*, some fungi, and some protozoa cysts also have a Gram reaction. Very small bacteria or bacteria without a cell wall, such as *Treponema*, *Mycoplasma*, *Chlamydia*, or *Rickettsia* do not have a gram reaction. The characterization of any new bacteria must include their gram reaction.

Typically a differential stain has four components; the primary stain, a mordant that sets the stain, a decolorizing agent to remove the primary stain, and a counter stain. In the Gram stain, the primary stain is crystal violet. This gives the cell an intense purple color. The mordant, iodine, forms a complex with the crystal violet inside the cell wall. The cell is then washed with either Gram's decolorizer or 95% ethanol. Gram positive cells will retain the dye complex and remain purple. The dye rinses out in gram negative cells. The counter stain, safranin, is used to color the cells that lost the primary stain, other wise they would remain colorless and you wouldn't be able to see them.



The large iodine-crystal violet complex is retained within the cell walls of gram positive cells because of the molecular structure of the many layers of peptidoglycan in the cell wall. There are lots of cross-linked teichoic acids and the iodine-dye complex cannot physically get out. There are also fewer lipids in the membrane and the decolorizing agent cannot get to it as well. Gram negative cells have an outer membrane and only one layer of peptidoglycan, with more lipids. The crystal violet dye is easily washed out.

General Considerations

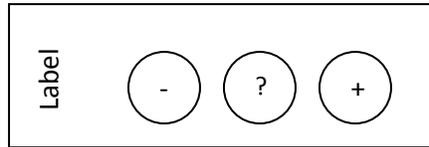
The accuracy of the Gram stain is dependent on the integrity of the bacterial cell wall. There are a variety of things that can influence the cell wall integrity; old cells (i.e. cultures over 24 hours old), the sample is from someone treated with antibiotics that target that cell wall such as penicillin, the cells have been roughly handled or you over heat fixed them. Under these conditions, gram positive cells will come out as gram-negative. If you de-colorize too long, Gram-positive cells will look like Gram-negative cells. Conversely, if you do not decolorize enough, Gram-negatives will look like Gram-positives. The only way you can trust your results it to always run a known Gram-positive and a known Gram-negative on the same slide. If they stain as predicted you can be pretty sure the result of your unknown sample is reliable.

The Gram staining takes practice to get right. Do not expect to get a good Gram stain on your first try. It is a good idea to hold your slide with a clothespin; your gloves will get pretty psychedelic as will everything you touch!

Gram Stain Procedure

1. Label your slide. Prepare your smears on a slide with a Gram negative on the left, your unknown in the middle, and a Gram positive on the right.

- Don't forget to methanol fix your slide!



2. Laying your slide on the staining rack, cover the smears with crystal violet for 1 minute.

- Use just enough stain to fully cover the smear but not so much that it runs or drips off the slide.

3. Tilt the slide and pour the crystal violet off and briefly rinse with water from your wash bottle.

- Remember not to spray directly on the smears or you will wash them off.

4. Flood the slide with iodine mordant for 1 minute.

5. Tilt the slide, pour off the excess iodine and gently decolorize with Gram's decolorizer until it just begins to run clear.

- This is the tricky part!

6. Tilt your slide on some paper towels to remove excess decolorizer.

7. Flood your slide with the safranin for 1 minute.

8. Tilt the slide to pour off the excess safranin and gently rinse with water until it runs clear.

9. Let the slide air dry

10. Observe under oil immersion.

- The Gram positive control should be purple and the Gram negative control should be pink.

11. Discard your used slide in the disinfectant bucket.