## Exercise 3.4 – Gram Stain

LEARNING OUTCOMES

1. List the steps for preparing a Gram stain.
2. State the purpose of the primary dye, mordant, decolorizer, and counterstain.

This very commonly used staining procedure was first developed by the Danish bacteriologist Hans Christian Gram in 1882 while working with tissue samples from the lungs of patients who had died from pneumonia. Since then, the Gram stain procedure has been widely used by microbiologists everywhere to obtain important information about the bacterial species they are working with. Knowing the Gram reaction of a clinical isolate can help the health care professional make a diagnosis and choose the appropriate antibiotic for treatment.

Gram stain results reflect differences in cell wall composition. These differences are reflected in the way the cells react with the stains used in the Gram stain procedure. Gram-positive bacteria have thick layers of the carbohydrate peptidoglycan in their cell walls, while the cell walls of Gram-negative bacteria are relatively thin. However, unlike Gram-positive bacteria, Gram-negative cells possess an outer membrane in addition to the plasma membrane. This outer membrane contains lipopolysaccharides (LPS) which act as endotoxins when Gram-negative cells are destroyed by the host's immune system. Endotoxins can heighten the inflammatory response in a patient and cause elevated fever. Figure 3.6 shows the major differences between the Gram positive and Gram-negative cell walls.

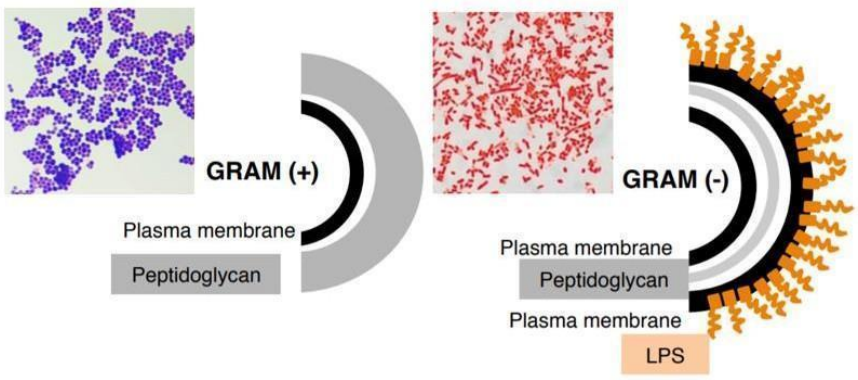


*Figure 3.6. The thick peptidoglycan cell walls of Gram-positive bacteria retain crystal violet throughout the staining procedure. Gram-negative bacteria have much thinner cell walls and additional outer membranes. These cells are readily decolorized despite application of a mordant and must be counterstained with safranin to be observed microscopically.*

Although most bacteria are either Gram-positive or Gram-negative, it is important to remember that not all bacteria can be stained with this procedure. For example, *Mycoplasma* bacteria, which have no cell wall, stain poorly with the Gram stain. Figure 3.7 shows a microscopic image of two cell types stained by the Gram method, where Gram-positive cells appear purple in color and Gram-negative cells appear pink (note that cell wall thickness, rather the shape of cells, determines Gram reaction).

## Steps of the Gram Stain

The Gram Stain is a differential stain because it separates bacteria into two groups based on differences in their cell wall structure. The protocol involves more steps than a simple stain, but is still performed on air-dried, heat-fixed smear preps. The smear prep is critical. If the smear is too thick the bacteria will not stain evenly, nor will they decolorize evenly. This can be a major source of error in evaluating the Gram reaction of a culture.

The four steps of the Gram stain procedure are outlined in Table 3.3 and Figure 3.7. It is important to understand the purpose of every step as well as the color of cells after the application of each chemical reagent.

Table 3.3. Steps of the Gram stain.

| STEP | REAGENT | PURPOSE | GRAM (+) | GRAM (-) |
|---|---|---|---|---|
| **Primary dye** | Crystal violet | Stains peptidoglycan | Purple | Purple |
| **Mordant** | Gram's iodine | Fixes crystal violet into peptidoglycan | Purple | Purple |
| **Decolorizer** | Alcohol or Acetone-Alcohol | Removes crystal violet from Gram (-) cells | Purple | Colorless |
| **Counterstain** | Safranin | Stains the colorless Gram (-) cells | Purple | Pink |

**STEP 1 – Primary Dye:** The first, or primary, stain is crystal violet. Crystal violet is a basic dye that attaches to peptidoglycan of both Gram-positive and Gram-negative bacteria. The primary dye is applied for one minute and then rinsed off with water. Since both Gram-positive and Gram-negative cells have peptidoglycan, application of the primary dye stains all cells purple.

**STEP 2 – Mordant:** A mordant is a chemical that stabilizes or fixes a stain with its target. In this case, Gram's iodine is applied as a mordant, enhancing the action of the primary dye by forming strong complexes between crystal violet and peptidoglycan. Following application of the mordant and a water rinse, all cells remain purple.

**STEP 3: Decolorizer** – The decolorization step is the most critical step of the procedure because it differentiates Gram-positive and Gram-negative cells. If not done carefully, over-decolorization or under-decolorization can lead to incorrect or ambiguous results. An alcohol or acetone/alcohol solution is applied to the smear to dissolve the outer membrane and remove the crystal violet from Gram-negative cells. Gram-positive cells retain the crystal violet/iodine complex due to the many layers of peptidoglycan and lack of an outer membrane. The action of decolorizer is stopped by a water rinse. After decolorization, Gram-positive cells are still purple but Gram-negative cells are colorless. Stopping here would make it very difficult to observe Gram-negative cells microscopically, so a final step is needed to make these cells visible.

**STEP 4: Counterstain** – The second stain, or counterstain, in the Gram procedure is safranin. Safranin is a basic dye that stains cells pink and imparts color to the Gram-negative cells that lost the primary dye following decolorization. Safranin may also attach to Gram-positive cells, but since crystal violet is so strongly complexed with the cell wall it obscures any additional pink staining. Following the application of safranin and a final water rinse, Gram-positive cells are purple and Gram-negative cells are pink (Figure 3.8). Note that after all four steps of the procedure, Gram-positive cells remained purple.

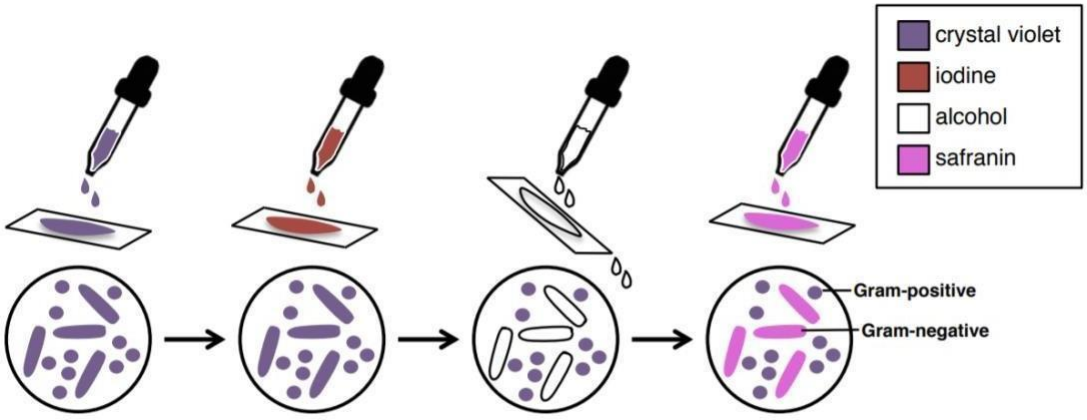


*Figure 3.7: Steps of the Gram stain. Each step is followed by a water rinse.*

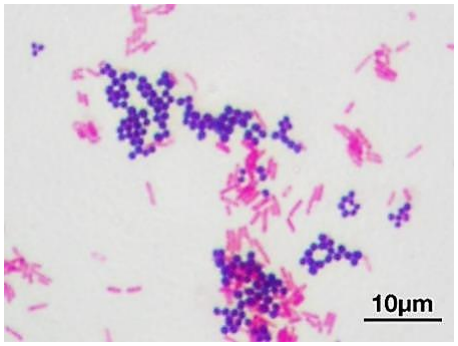


*Figure 3.8: Microscopic image of a Gram stain of mixed Gram-positive cocci (Staphylococcus aureus, purple) and Gram-negative bacilli (Escherichia coli, pink). Total magnification 1000X.*

Gram stains are best performed on fresh cultures—older cells may have damaged cell walls which may not produce an accurate Gram reaction. Some species of bacteria are Gram-variable, appearing as a mix of both Gram-positive and Gram-negative reactions. The decolorization step is also critical for accurate results. This step uses an alcohol/acetone mixture that disrupts the outer membrane and thin layer of peptidoglycan of Gram-negative cells. When under-decolorization occurs, the decolorizer is not left on long enough and Gram-negative bacteria retain too much of the crystal violet, causing them to appear purple instead of pink. Likewise, over-decolorization causes Gram-positive cells to lose crystal violet and appear pink  after counterstaining with safranin.

Another common mistake is in the preparation of the heat-fixed bacterial smear. The main purpose of heat-fixing is to adhere the bacterial cells to the microscope slide (it also denatures the proteins, killing the cells as well). If you forget to do this step, or do it inadequately, then the cells will be washed off in all the subsequent steps of your staining process and there will be no bacteria on the slide to observe! Variable colors are also observed if bacterial cells are not spread evenly in a drop of water when preparing a smear from solid media. This could result in uneven decolorization during the Gram staining procedure and both purple and pink cells in a slide made from one type of bacteria. For example, thick areas of a smear made from Gram- negative bacteria might be under-decolorized, causing cells in those areas only to appear purple, while remaining cells are pink as expected (Figure 3.9).

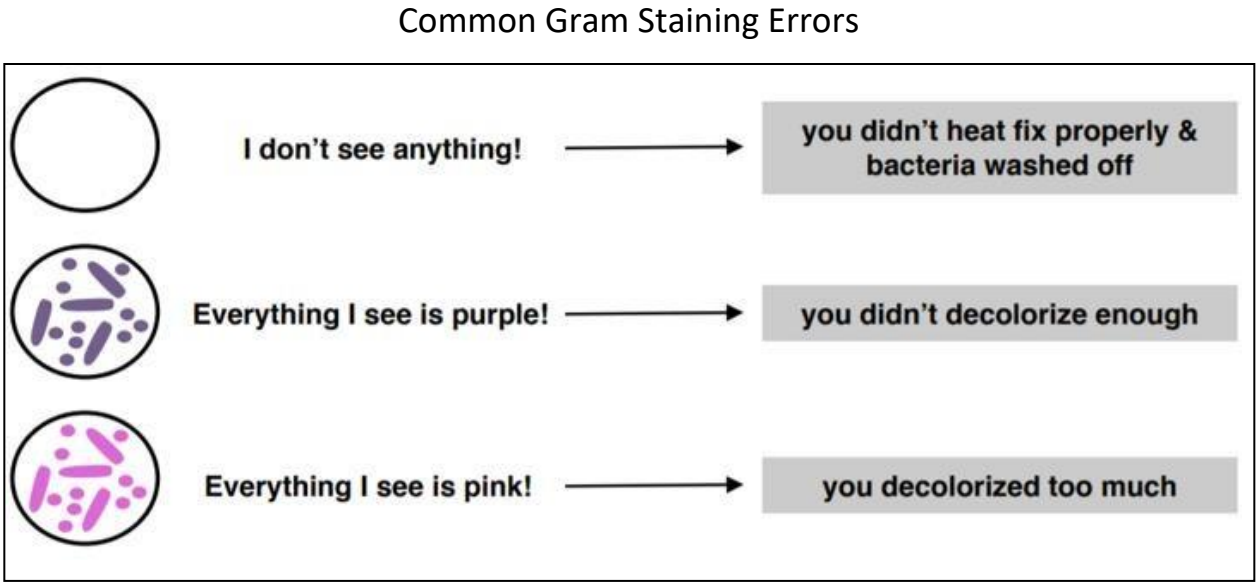


*Figure 3.9: Common errors in Gram staining.*

## Exercise 3.4 – Gram Stain

OBJECTIVE

Stain heat-fixed smears with an acidic dye to determine cell size, shape, arrangement, and cell wall type.

MATERIALS

- SLIDES: *Staphylococcus aureus, Escherichia coli,* and *Unknown* heat-fixed smears
- SOLUTIONS: Gram stain kit (crystal violet, iodine, alcohol decolorizer, safranin)
- EQUIPMENT: Stain pan, rack, wash bottle, bibulous paper, clothespin/slide holder

PROCEDURE – STUDENTS WORK IN PAIRS

*IMPORTANT: If your staining pan becomes full, empty it into the sink at the center of your bench.* ***Never carry a full pan across the room****!*

1. Place the heat-fixed smears on the rack over the staining pan.
2. Cover smears entirely with **crystal violet** and let stand for one minute.
3. Using the clothespin or slide holder, rinse both sides of each slide with water; do not blot.
4. Cover smears entirely with **iodine** and let stand for one minute.
5. Using the clothespin or slide holder, rinse both sides of each slide with water; do not blot.
6. Lifting one slide at a time, apply **alcoho**l until the color just starts to run (10-20 seconds) and immediately rinse the slide with water to stop action of the decolorizer; do not blot.
7. Cover smears entirely with **safranin** and let stand for one minute.
8. Using the clothespin or slide holder, rinse both sides of each slide with water.
9. Blot slides gently in the bibulous paper booklet and put the slides aside.
10. Dispose of the pan water in the bench sink (do not carry the full pan to the room sink!).
11. View the stained slides microscopically under oil immersion and complete the lab report.