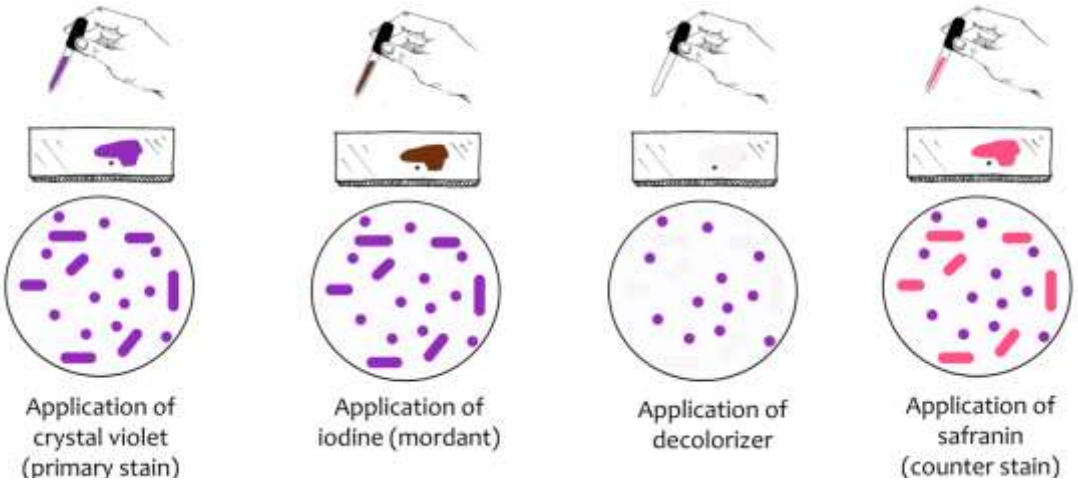
Differential Stains (Gram Stain)

Differential Stains use two or more stains and allow the cells to be categorized into various groups or types. Both the techniques allow the observation of cell morphology, or shape, but differential staining usually provides more information about the characteristics of the cell wall (Thickness).
The Gram staining method is named after the Danish bacteriologist Hans Christian Gram (1853-1938). It is a differential staining method as it differentiates bacterial species into two large groups (Gram positive and Gram-negative) based on the chemical and physical properties of their cell walls.

Gram Staining Technique:

1. **Crystal violet acts as the primary stain**. Crystal violet may also be used as a simple stain because it dyes the cell wall of any bacteria.
2. **Gram's iodine acts as a mordant** (Helps to fix the primary dye to the cell wall).
3. **Decolorizer (95% ethanol)** is used next to remove the primary stain (crystal violet) from Gram Negative bacteria (those with LPS imbedded in their cell walls).
4. **Finally, Secondary stain or Counterstain (Safranin)** is applied to stain those cells (Gram Negative) that has lost the primary stain as a result of decolorization.



Applications of Gram staining:

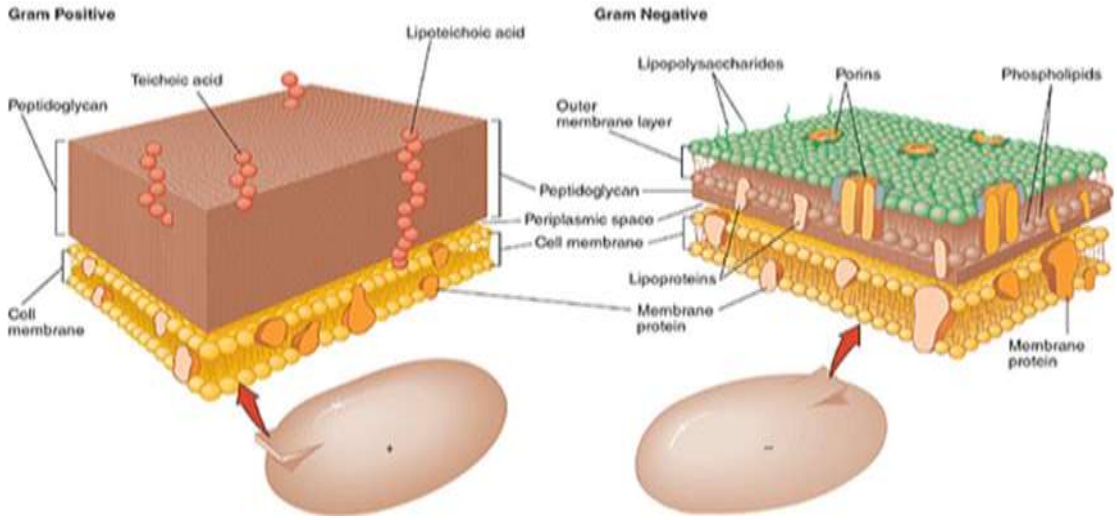
1. Differentiation of bacteria into Gram positive and Gram negative is the first step towards the classification of bacteria.
2. Observation of bacteria in clinical specimens provides a vital clue in the diagnosis of infectious diseases.
3. Useful in estimation of total count of bacteria.
4. Empirical choice of antibiotics can be made based on Gram stain's report.
5. Choice of culture media for inoculation can be made empirically based on Gram's stain report.

Mechanism of Gram reaction:

Various theories have been proposed to explain why some bacteria retain the dye and some don't. Theories such as differences in cytoplasmic pH (2 in Gram+ and 3 in Gram- bacteria), and presence of Magnesium ribonucleate in Gram+ and its absence in Gram- bacteria have not received widespread acceptance. The thickness of Gram+ cell wall and the presence of more lipids in Gram- cell walls have been more acceptable reasons for Gram stain reactions.

In Gram-positive Cells: peptidoglycan (Polymer consisting of sugars and amino acids that forms a mesh-like layer outside the plasma membrane) makes up as much as 90% of the thick, compact cell wall, which is the outermost cell wall structure of Gram+ cells.

Gram-negative Cells: The cell walls are more chemically complex, thinner and less compact. Peptidoglycan makes up only 5-20% of the cell wall, and is not the outermost layer, but lies between the plasma membrane and an outer membrane. This outer membrane (lipopolysaccharide membrane is removed by alcohol and thin peptidoglycan layer allows the dye to escape) is similar to the plasma membrane, but is less permeable and composed of lipopolysaccharides (LPS), a harmful substance classified as an endotoxin.



Factors affecting staining:

1. Smear preparation

a. Thin smear: If the smear is too thick, the cells can appear Gram-positive in very thick area.

b. Heat fix appropriately: flaming too much will cause all cells to appear Gram-negative.

c. Young culture: Old culture smears can cause cells to appear Gram-negative (weak walls).

2. Staining

a. If the mordant is no added Gram-positive bacteria will turn to Gram- negative bacteria.

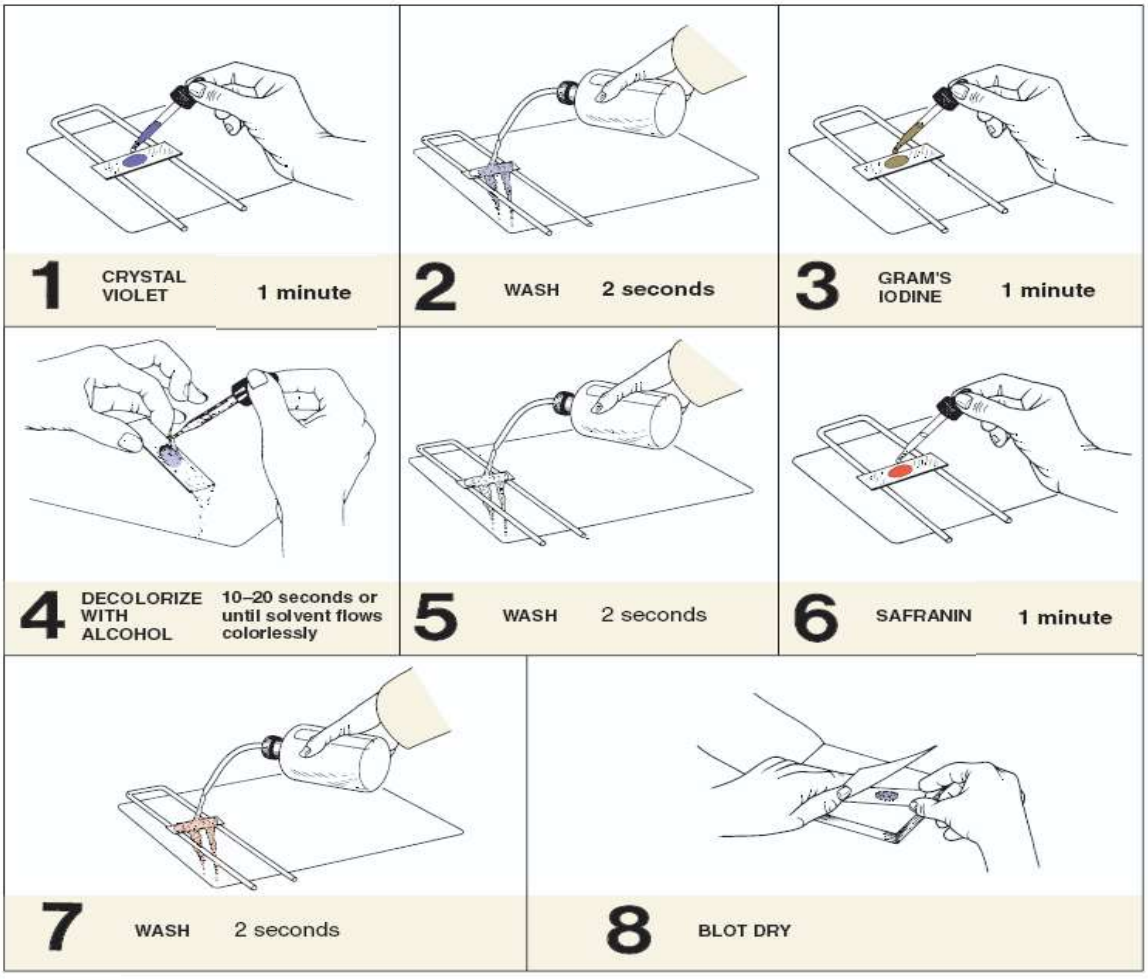
b. If the decolorizing agent stays too long some Gram-positive bacteria will change to Gram-negative bacteria.

c. Concentration and freshness of the reagents that affect the quality of the stain.

d. Washing and drying between the steps should be consistent, excess water will dilute some reagents.

Procedure:

1. Prepare and heat fix smear and place slide on a staining rack.
2. Cover the smear with crystal violet for 30-60 seconds.
3. Wash with distilled water to remove excess dye.
4. Cover the smear with Gram's iodine for 60 seconds.
5. Wash off with distilled water.
6. Decolorize with Gram decoloriser for 10-15 seconds.
7. Wash thoroughly with distilled water.
8. Cover the smear with safranin for 30-60 seconds.
9. Wash with distilled water and air dry.
10. Examine under immersion oil.



The gram-staining procedure