Gram Staining (differential staining)

Principle : Gram Staining is a differential staining technique used for classification of microorganisms according to their staining property. It is named after Dr. Christian Gram (1884), the discoverer. It divides bacterial cell into two major groups, Gram +ve and Gram-ve.

Differential staining requires the use of at least 3 chemical reagents that are applied sequentially to a heat-fixed smear. The first reagent is called primary stain. Its function is to impart its colour to all cells. In order to establish a colour contrast the second reagent is used which is a decolourising agent. Based on the chemical composition of the cellular components, the decolorizing agent may or may not remove the primary stain from the entire cell or from certain cell structurs. The third and final reagent, the counter stain, has a contrasting colour to that of the primary stain. Following decolourization, if the primary stain is not washed out, the counter stain be absorbed and the cell or its components will retain the colour of the primary stain. If the primary stain is removed the decolorizing cellular components will accept the counter stain and assume the contrasting colour. In this way the cell types or their structures can be distinguished from each other on the basis of the stain that is retained.

Sometimes mordants are also used to improve the fidelity of the staining process.

A mordant serves as a substance that increases the cells affinity for a stain. It does this by binding to primary stain thus forming an insoluble complex. The resultant primary stain-mordant complex serves to intensify the colour of the stain.

The Gram stain reaction is based on the difference in chemical composition of the bacterial cell wall. Gram +ve cells have a thick peptidoglycan layer whereas the peptidoglycan layer in Gram –ve organisms is much thinner and surrounded by outer lipid-containing layers. Peptidoglycan is mainly a polysaccharide composed of 2 chemical subunits found only in bacterial cell wall.

Gram +ve: Thick peptidoglycan, Low lipid content
Gram -ve: Thin peptidoglycan, High lipid content (outer layer)

When the smear is flooded with crystal violet and iodine both Gram +ve and Gram-ve bacteria take up the stain (CVI complex) and look violet. However, when washed with alcohol, dehydration takes place and in the case of Gram +ve bacteria small pores are formed as the lipid content is low. In the case of Gram –ve bacteria large pores are formed because alcohol washes away the large amount of lipids present there. (alcohol serves as a solvent for lipids). Thus the CVI complex is retained in the case of Gram +ve but washed off in the case of Gram –ve organisms. Safranin stains both, rendering the Gram –ve look pink while the Gram +ve deep purple.

Procedure: 1. Make a heat-fixed smear of bacteria on a grease-free slide.
2. Cover the slide with crystal violet solution (primary stain) [0.5% aq sol] and allow to act for 30-60 sec.

3. Drain off stain. Wash under gentle stream of tap water.
4. Cover with Gram's Iodine (mordant) solution and allow it to act for 30-60sec.
5. Wash of the iodine soln. with absolute alcohol until colour ceases to come out of the prep.
6. Wash with tap water (optional)
7. Cover with safranin (counter stain) for 1-2 minutes.
8. Wash with water and air-dry.
9. Examine under microscope.

Observation : (under 40 X objective of microscope)

Name of bacteria	Color,taken.	Morphology (size,shape & arrangement)	Gram-character.
E.coli.	Red in color.	Short rod in shape, some were in singly form, some were in pairs, few were in clusters.	Gram-negative
Bacillus subtilis	Violet in color.	Large rod in shape, some were in singly form, some were in pairs, very few were in clusters.	Gram-positive
Staphylococcus aureus	Violet in color.	Spherical in shape,most of them were in clusters, some were in singly form, very few were in pairs.	Gram-positive