Objective: Classification of bacteria based on gram staining

Mechanism: Gram positive and negative bacteria differ in cell wall composition. In Gram positive organism, the cell wall is composed of peptidoglycan layer entirely whereas in gram negative bacteria, the cell wall is composed of thin layer of peptidoglycan and thick layer of lipopolysaccarides. Etahnol wash dissolve the lipopolysaccaride layer in case of gram negative bacteria whereas ethanol dehydrates in gram positive bacteria leads to shrinkage of the cell wall. Due to change in cell wall composition, Crystal violet-gram's iodide complex is retained in case of gram positive bacteria and give purple color whereas Crystal violet-gram's iodide complex leached out from the gram negative bacteria after washing with ethanol and give pink color coming from counter stain i.e safranin.

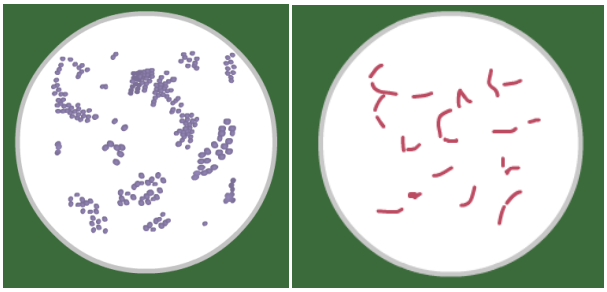
Materials required:

1. Glass slides
2. Inoculating loop
3. Laminar air flow chamber
4. Microscope
5. Immersion oil
6. Distilled water
7. Bacterial culture
8. Crystal Violet
9. Grams Iodine
10. Ethanol
11. Safranin

Procedure:

1. Take fresh glass slide for making the microbial smear and make a circle at the center of the slide
2. Take sterile loop and take bacterial broth with loop for making the smear on the slide. Alternatively, you can take bacterial colony and make smear with the help of sterile saline
3. The smear should be thin and transparent and air dried
4. Fix the smear on glass slide by passing through the flame quickly by keeping smear on upside
5. Now moved to gram staining
    - First, smear was flooded with crystal violet and kept it for 1 min
    - Remove crystal violet and wash slide with dist. water
    - Smear was flooded with gram's Iodine and kept it for 1 min
    - Remove gram's Iodine and wash slide with dist. water (slide appear purple color)
    - Smear was flooded with decolorizing agent (ethanol or acetone) for 15 sec (don't keep it for long time)
    - Immediately rinse slide with dist. water
    - Smear was flooded with safranin (counter stain) and kept for 45 sec
    - Remove safranin and wash slide with dist. water
    - Dry the slides with blot paper followed by air dry.

Results:



Gram positive organisms look purple color and gram negative organisms look pink color

Functions of the stains:

1. **Crystal violet (CV):** Crystal violet dissociates into CV+ and Cl- ions. CV+ ions able enter into the cell wall and cell membrane and react with negatively charged cell wall components. CV can able to enter into both gram positive and negative bacteria
2. **Gram's iodine (GI):** Gram's iodine act as fixing agent or mordant. It reacts with crystal violet in cell wall and form insoluble GI-CV complex and make the cell wall purple color.
3. **Decolorizing agent/ethanol:** Ethanol treatment washes the outer lipid membrane in gram negative bacteria and increases the cell wall porosity by exposing the peptidoglycan layer. The GI-CV insoluble complex will leach out from the cell wall due to increased porosity in gram negative organisms. However, ethanol also leads to dehydration which in turn leads to shrinkage of cell wall in gram positive organisms which retain the GI-CI complex.
4. **Safranin (Counter stain):** Safranin stains gram negative organism due to increased porosity and easily can enter inside (also stain has positive charge and react with negatively charged cell wall) whereas cannot stain in gram positive organisms due to dehydration and shrinkage of cell wall.