► Gram staining procedure:

1. flood air-dried, heat-fixed smear of cells for 1 minute with **crystal violet** staining reagent, please note that the quality of the smear (too heavy or too light cell concentration) will affect the Gram Stain results.

2. Wash slide in a gentle and indirect stream of tap water for 2 seconds.

3. Flood slide with mordant: **Grams' iodine**. Wait 1 minute.

4. Wash slide in a gentle and indirect stream of tap water for 2 seconds.

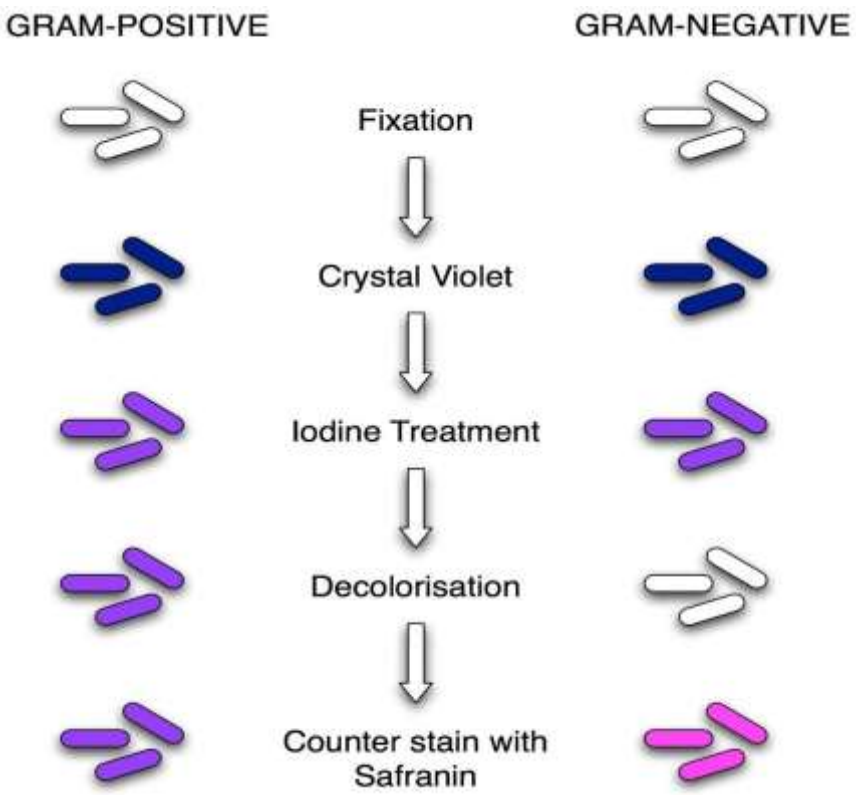
5. Flood slide with **decolorizing agent (Acetone-alcohol decolorizer)**. Wait 10-15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear.

6. Flood slide with counterstain, **Safranin**. Wait 30 seconds to 1 minute.

7. Wash slide in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper.

8. Observe the results of the staining procedure under microscope.

9. Gram-negative bacteria will stain pink/red and Gram-positive bacteria will stain blue/purple.



Procedure of Gram staining: note color change after each step

B. Acid fast stain or acid bacilli test (AFB)

The main aim of this staining is to differentiate bacteria into acid fast group and non-acid fast groups. This method is used for those microorganisms which are not stained by simple or Gram staining methods, particularly the most medically important AFB *Mycobacterium tuberculosis* which are resistant and can only be visualized by acid-fast staining.

When the smear is stained with **carbol fuchsin**, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat, carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm. Then after all cell

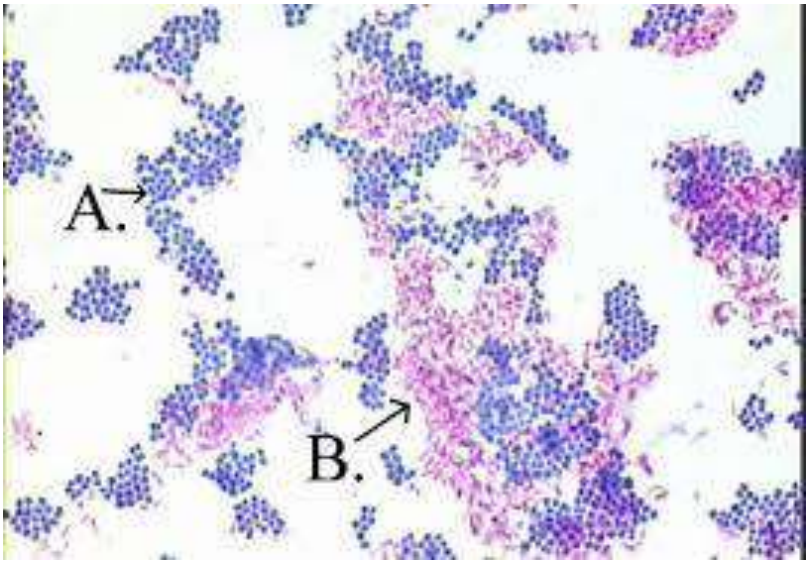
appears red. Then the smear is decolorized with decolorizing agent (3% HCl in 95% alcohol) but acid fast cells resistant due to the presence of large amount of lipoidal material in their cell wall which prevents the penetration of decolorizing solution. The non-acid fast organism lacks the lipoidal material in their cell wall due to which they are easily decolorized, leaving the cells colorless. Then the smear is stained with counterstain, methylene blue. Only decolorized cells absorb the counter stain and take its color and appears blue while acid-fast cells retain the red color.

► Procedure of Acid-Fast Stain

1. Prepare bacterial smear on clean slide, using sterile technique.
2. Cover the smear with carbol fuchsin stain.
3. Heat the stain until vapour just begins to rise (i.e. about 60 °C). Do not overheat. Allow the heated stain to remain on the slide for 5 minute.
4. Wash off the stain with clean water.
5. Cover the smear with 3% v/v acid alcohol for 5 minutes or until the smear is sufficiently decolorized, i.e. **pale pink**.
6. Wash well with clean water.
7. Cover the smear with methylene blue stain for 1-2 min., using the longer tome when the smear is thin.
8. Wash off the stain with clean water.
9. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry.
10. Examination the smear microscopically, using the 100 X oil immersion objective.

Application of	Reagent	Cell colour	
		Acid fast	Non-acid fast
Primary dye	Carbol fuchsin	Red	Red
Decolorizer	Acid alcohol	Red	Colorless
Counter stain	Methylene blue	Red	Blue

Summary of Acid-Fast Stain



Interpretation of Acid-Fast stain

Acid fast: Bright red to intensive purple (B), Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded. Non-acid fast: Blue color (A)