NPPC protocol on Gram Staining of bacteria (modified from Gephardt et al., 1981)

Principle:

Gram staining is a diagnostic test that gives an early indication of potential bacteria through visualization of the bacteria. The Gram stain helps to differentiate the organism, whether it is gram-positive or gram-negative. Gram-positive bacteria appear purple in colour and gram-negative bacteria appear pink. Differences of Gram-positive and Gram-negative bacteria arise from cell wall composition; Gram-positive bacteria have a thick peptidoglycan layer with teichoic acids, making them resistant to decolorization.

The Gram staining procedure involves four basic steps:

1. The bacteria are first stained with the basic dye **crystal violet**. Both Gram-positive and Gram-negative bacteria become directly stained and appear purple after this step.

2. The bacteria are then treated with **Gram's iodine solution**. This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex. Both Gram-positive and Gram-negative bacteria remain purple after this step.

3. **Gram's decolorizer**, a mixture of **ethyl alcohol and acetone**, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while Gram-negative are decolorized.

4. Finally, the counterstain **safranin** (also a basic dye) is applied. Since the Gram-positive bacteria are already stained purple, they are not affected by the counterstain. Gram-negative bacteria, which are now colourless, become directly stained by the safranin. Thus, Gram-positive appear purple, and Gram-negative appear pink.

Interpretation

Gram-positive bacteria (thick layer of peptidoglycan) - **stains violet /purple**

Gram-negative bacteria (thin layer of peptidoglycan and high lipid content) –**stains red/pink**

Materials

- Culture containing bacteria (18 to 24 hours old)
- Bunsen burner
- Compound microscope
- Slides and coverslip
- Inoculation needle or Inoculating loop
- Immersion oil
- Gram staining kit (Reagents)

Reagents needed for Gram staining include:

- Crystal violet (primary stain)
- Gram's iodine solution (the mordant)
- Acetone/ethanol (50:50 v: v) (the decolorizer)
- Safranin solution (the counterstain/secondary stain)
- Water

The procedure of Gram Staining

1. **Preparing Bacterial smear**

1. Take a clean microscopic slide
2. Place a drop of sterilized distilled water on the microscopic glass slide with the help of an inoculation loop.
3. Sterilize the inoculating loop by flaming and transfer a small portion of the bacterial colony from the bacterial culture plate and emulsify it with a drop of water on the glass slide. (If culture is on a petri dish or slant, place a drop of water in the middle of the glass slide and using a sterile loop, transfer a small amount of colony and suspend with the water drop).
4. After emulsifying spread the smear to a size of about 1cm sq., to form a thin film.
5. After spreading allow it to air dry for 2-3 minutes.

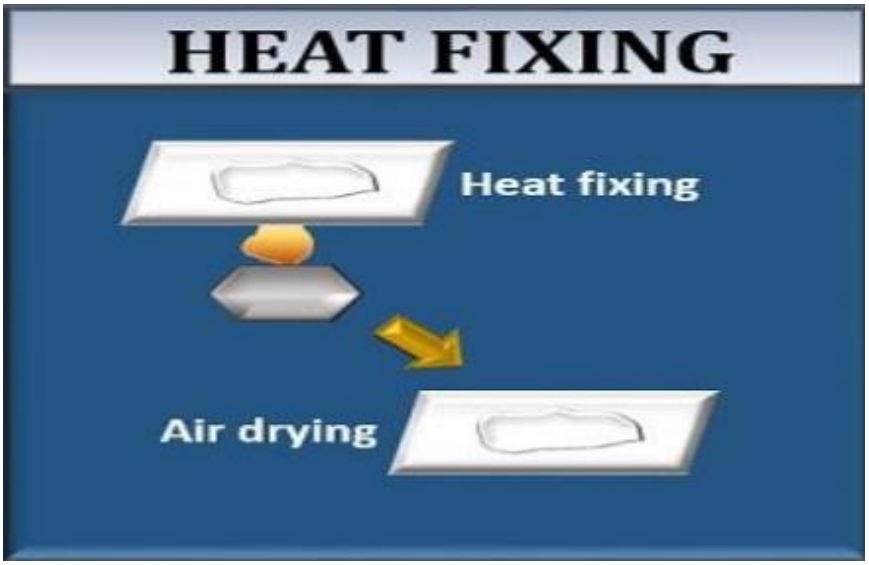
2. Heat Fixing

After smear preparation, move the prepared slides over the Bunsen burners flame at least three times. Then, allow the slide to air dry.

• Heat fixing helps in the fixation of a specimen to the glass slide.

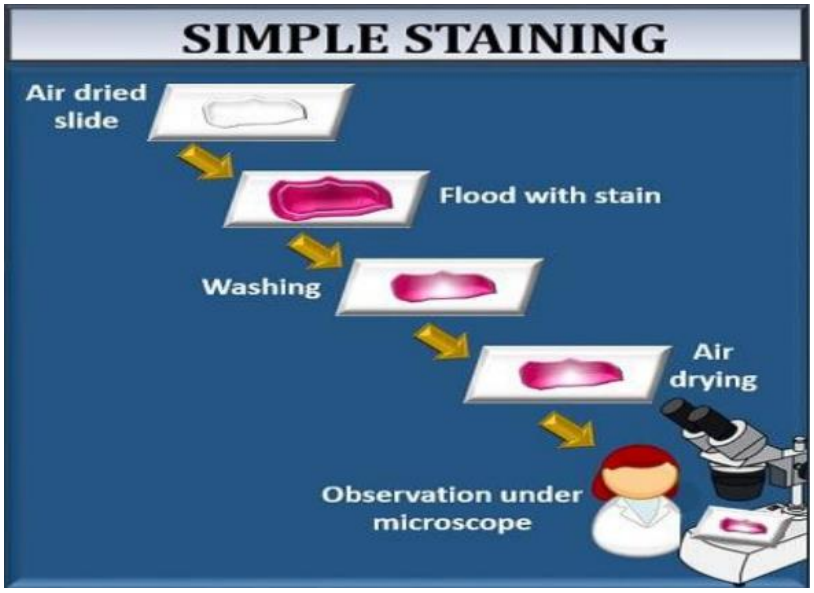
• Heat fixing helps the stain to penetrate the smear.

(Slide must be moved up and down or circularly over the flame to prevent from overheating)



3. Staining the Bacterial smear

1. Add crystal violet staining solution over the smear and allow it to stand for 1 minute.
2. Wash slide in a gentle and indirect stream of distilled water for 2 seconds.
3. Flood slide with the mordant: Gram's iodine. wait for 1 minute.
4. Wash slide in a gentle and indirect stream of distilled water for 2 seconds.
5. Flood slide with a decolorizing agent (Acetone-alcohol decolourizer). Wait 10-15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear.
6. Flood slide with a counterstain, safranin. Wait 30 seconds to 1 minute.
7. Wash slide in a gentile and indirect stream of distilled water until no colour appears in the effluent and then blot dry with absorbent paper.
8. Observe the results of the staining procedure under oil immersion (100x) using a bright field microscope.



Reagent preparation if the Gram staining kits are not available from solid stock or powder

1. **Crystal Violet Preparation**
    - For preparing solution A (Crystal Violet stock solution), add 20 gm of 85% crystal violet dye in 100 ml ethanol (95%) and dissolve by mixing thoroughly.
    - For preparing solution B (Oxalate Stock solution), add 1 gm ammonium oxalate in 100 ml distilled water and dissolve by mixing thoroughly.
    - For preparing the working solution, add 1 ml of crystal violet stock solution in 10 ml distilled water and add 40 ml of oxalate stock solution.
    - Let the solution sit for 24 hours at room temperature
    - Store the solution in a dark bottle for use.
2. **Gram's Iodine Preparation**
    - Dissolve 1 gm of Iodine, and 2 gm of potassium iodide in 300 ml distilled water.
    - Mix properly till the iodine dissolve and keep the solution in a dark bottle.
3. **Decolorizing Solution**
    - Mix 50 ml of acetone with 50 ml of 95% ethanol
4. **Safranin**
    - Mix 2.5 gm of safranin-O in 100 ml of 95% ethanol
    - Mix 10 ml of the above solution with 90 ml of distilled water to prepare a working solution
5. **Carbol-fuchsin**
    - Dissolve 3 gm of basic fuchsin in 100 ml of 95% ethanol
    - Mix 5 ml liquid phenol with 95 ml of distilled water to prepare 5% phenol solution
    - Mix 10 ml of basic fuchsin solution with 100 ml of 5% phenol solution
    - Let the solution sit for 24 hours at room temperature
    - Store in a dark bottle

References

Smith, A. C., and Hussey, M. A., 2005. Gram Stain Protocols. American Society for microbiology.

Gephart, P., Costilow, R, N., Krieg, N.R., Murray, R.G.E., Nester, E.W., Phillips, G.B., and Wood, A.W. B 1981. Manual of Methods for General Bacteriology, ASM Press, Washington D.C. Gram. C.1884. Ueber die isolirte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. Fortschritte der Medcin, (2):185-189.