

EXERCISE # 6**PROCEDURE USED TO CLASSIFY BACTERIA INTO TWO LARGE GROUPS (GRAM POSITIVE AND GRAM NEGATIVE)****LIST OF REAGENTS AND INSTRUMENTS****EQUIPMENT**

Bunsen burner, alcohol-cleaned microscope slide, water

REAGENTS

Crystal violet, Gram's iodine solution, acetone/ethanol (50:50 v:v) or 95% ethanol, 0.1% basic fuchsin(red stain) / Safranin (Pink) solution

PROCEDURES**1. PREPARE A SLIDE SMEAR:**

A. Transfer a drop of the suspended culture to be examined on a slide with an inoculation loop. The bacterial culture should not be more than 24-36 hours old to get optimum results. If the culture is to be taken from a Petri dish or a slant culture tube, first add a drop or a few loop-full of water on the slide and aseptically transfer a minute amount of a colony from the Petri dish. Note that only a very small amount of culture is needed; a visual detection of the culture on an inoculation loop already indicates that too much is taken.

If staining a clinical specimen, smear a very thin layer onto the slide, using a wooden stick. Do not use a cotton swab, if at all possible, as the cotton fibres may appear as artefacts. The smear should be thin enough to dry completely within a few seconds. Stain does not penetrate thickly applied specimens, making interpretation very difficult.

B. Spread the culture with an inoculation loop to an even thin film over a circle of 1.5 cm in diameter, approximately the size of a dime. Thus, a typical slide can simultaneously accommodate 3 to 4 small smears if more than one culture is to be examined.

C. Air-dry the culture and fix it over a gentle flame, while moving the slide in a circular fashion to avoid localized overheating. The applied heat helps the cell adhesion on the glass slide to make possible the subsequent rinsing of the smear with water without a significant loss of the culture. Heat can also be applied to facilitate drying the smear. However, ring patterns can form if heating is not uniform, e.g. taking the slide in and out of the flame.

2. GRAM STAINING:

- A. Add **crystal violet stain** (it will stain everything that present there) over the fixed culture. Let stand for 60 seconds; for thinly prepared slides, it is usually acceptable to pour the stain on and off immediately. Pour off the stain and gently rinse the excess stain with a stream of water from a faucet or a plastic water bottle. Note that the objective of this step is to wash off the stain, not the fixed culture.
- B. Add the **gram-iodine solution** (It will penetrate the wall and stick in there. If bacterium is gram positive, iodine will form a complex with crystal violet and form crystals. We will remain with purple cells when we decolourize. While if bacterium is gram negative, the lipid layers of bacteria will not allow iodine to penetrate and it will wash off after next step) on the smear, enough to cover the fixed culture. Let stand for 60 seconds. Pour off the iodine solution and rinse the slide with running water. Shake off the excess water from the surface.
- C. Add **a few drops of decolourizer (alcohol)** so the solution trickles down the slide. Rinse it off with water immediately to stop decolourizing (Maximum time is 12 seconds. Be accurate otherwise everything will change). The exact time to stop is when the solvent is no longer coloured as it flows over the slide. Further delay will cause excess decolourization in the gram-positive cells, and the purpose of staining will be defeated.
- D. Counterstain with **safranin or basic fuchsin solution (Counter stain)** for 45 seconds. Wash off the solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shake to remove most of the water and air-dried.

3. QUALITY CONTROL:

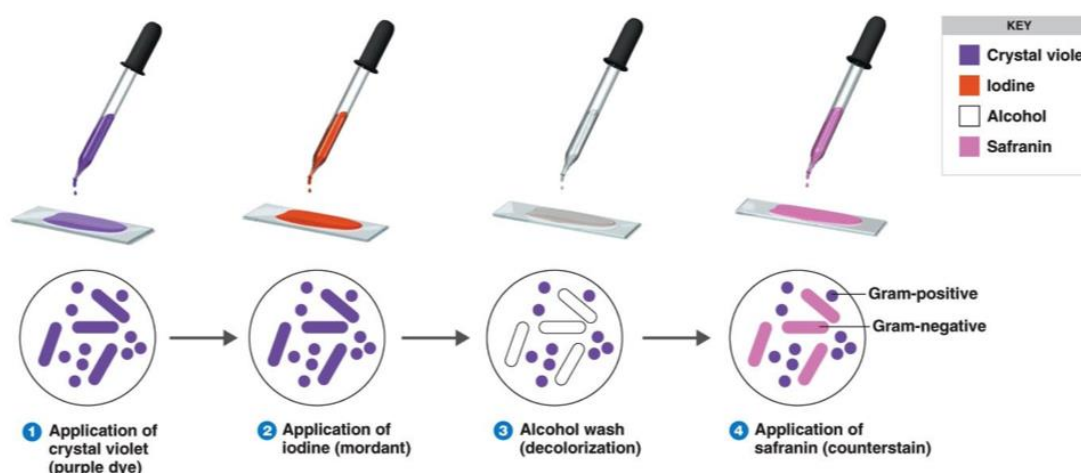
It is a simple matter to prepare a control slide by breaking a clean wooden applicator stick and picking a small amount of material from the interproximal space of one's teeth. This should be smeared into a drop of clean tap water on a clean glass slide. The slide may be stained as above. This material will consistently display a few neutrophils and a mixture of Gram (+) and (-) organisms. Neutrophil nuclei should be pink.

4. EXAMINE THE FINISHED SLIDE UNDER A MICROSCOPE.

A caveat in the examination of the Gram smears is the distortion in morphology that can be caused by antimicrobial therapy. This is especially likely to occur in urine

specimens. Filamentous and pleomorphic forms may be observed among the Gram (-) rod species. Gram reaction of the organism may also change after antimicrobial therapy, Gram (+) bacterial may become gram variable. Look at areas that are one cell thick only; observation of thick areas will give variable and often incorrect results. White blood cells and macrophages should stain Gram-negative, whereas squamous epithelial cells are Gram-positive.

Microscopically examination of gram staining slides under 100X objective of microscope. Observe several fields on the slide for bacterial organism. Describe the gram reaction of any organism seen. If Gram positive bacteria cell will remain purple because of the complex of crystal violet and iodine solution because it will not wash off when we will apply decolorizer. If gram negative bacteria it should be white or clear when we apply decolorizer. Safranin or basic fuchsin (Counter stain) will stain it pink to red because there is no complex of iodine and crystal violet.



QUESTIONS

1. What is the purpose of Gram Staining?
2. What will happen if we allow decolorizer to stay long?
3. What is counter staining? Why we do it?
4. What should be the age of bacteria used for Gram staining?
5. Why Gram positive stain Pink or Red?