NEATH PORT TALBOT COLLEGE
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School of Maths & Science
Science Practical

Gram staining

◆ Aim

To become familiar with:
- The chemical and theoretical basis for differential staining procedures.
- The chemical basis of the Gram stain.
- Performance of the procedure for differentiating between the two principal groups of bacteria: gram-positive and gram-negative.

◆ Introduction

Differential staining requires the use of at least three chemical reagents that are applied sequentially to a heat-fixed smear: The first reagent is called the primary stain. Its function is to stain all cells. In order to establish a colour contrast, the second reagent used is the decolourising agent. Based on the chemical composition of cellular components, the decolourising agent may or may not remove the primary stain from the entire cell or only from certain cell structures. The final reagent, the counter-stain, has a contrasting colour to that of the primary stain. Following decolourisation, if the primary stain is not washed out, the counter-stain cannot be absorbed and the cell or its components will retain the colour of the primary stain. If the primary stain is removed, the decolourised cellular components will accept and assume the contrasting colour of the counter-stain. In this way, cell types or their structures can be distinguished from each other on the basis of the stain that is retained. The most important differential stain used in bacteriology is the Gram stain, named after Dr. Christian Gram. It divides bacterial cells into two major groups, gram-positive and gram-negative, which makes it an essential tool for classification and differentiation of micro organisms.

◆ Safety

Control Measures

- The wearing of **safety glasses** and a **laboratory coats at all times** will be sufficient to take account of most hazards and significant risks.
- You are reminded of the need of good laboratory practice in order to maintain a safe working environment.

Hazards

Biological Hazard — E. coli, Bacillus subtilis

Irritant — Crystal Violet, Grams Iodine, Safranin, Propanone

Flammable — Propanone

The Gram stain uses four different reagents. Descriptions of these reagents and their mechanisms of action follow.

Primary Stain
Crystal Violet. This violet stain is used first and stains all cells purple.

Mordant
Gram's Iodine. This reagent serves as a mordant, a substance that forms an insoluble complex by binding to the primary stain. The resultant crystal violet—iodine (CV-I) complex serves to intensify the colour of the stain, and all the cells will appear purple-black at this point. In gram-positive cells only, this CV-I complex binds to the magnesium-ribonucleic acid component of the cell wall.

Decolourising Agent
Propanone. This reagent serves a dual function as a lipid solvent and as a protein-dehydrating agent. Its action is determined by the lipid concentration of the microbial cell walls. In gram-positive cells, the low lipid concentration is important to retention of the Mg-RNA-CV-I complex. Therefore, the small amount of lipid content is readily dissolved by the action of the propanone, causing formation of minute cell wall pores. These are then closed by propanone's dehydrating effect. As a consequence, the tightly bound primary stain is difficult to remove, and the cells remain purple. In gram-negative cells, the high lipid concentration found in outer layers of the cell wall is dissolved by the propanone, creating large pores in the cell wall that do not close appreciably on dehydration of cell wall proteins. This facilitates release of the unbound CV-I complex, leaving these cells colourless or unstained.

Counter-stain
Safranin. This is the final reagent, used to stain red those cells that have been previously decolourised. Since only gram-negative cells undergo colourisation, they may now absorb the counter-stain. Gram-positive cells retain the purple colour of the primary stain. The preparation of adequately stained smears requires that you bear in mind the following precautions:

The most critical phase of the procedure is the decolourisation step, which is based on the ease with which the CV-I complex is released from the cell. Remember that over-decolourisation will result in loss of the primary stain, causing gram-positive organisms to appear gram-negative. Under-decolourisation, however, will not completely remove the CV-I complex, causing gram-negative organisms to appear gram-positive. Strict adherence to all instructions will help remedy part of the difficulty, but individual experience and practice are the keys to correct decolourisation.
It is imperative that slides be thoroughly washed under running tap water between applications of the reagents. This removes excess reagent and prepares the slide for application of the subsequent reagent.

The best Gram stained preparations are made with fresh cultures, that is, not older than 24 hours. As cultures age, especially in the case of gram-positive cells, the organisms tend to lose their ability to retain the primary stain and may appear to be gram-variable; that is, some cells will appear purple, while others will appear red.

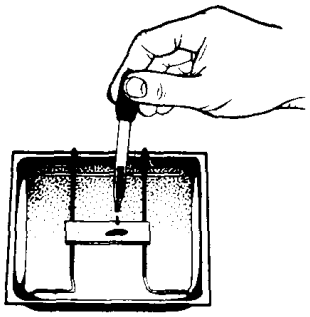
◆ Procedure

1. Obtain three clean glass slides.

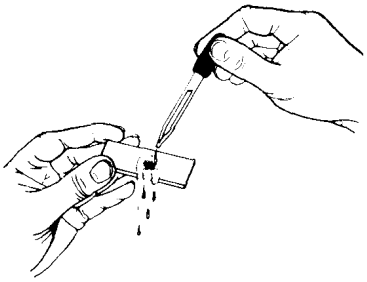
2. Using sterile technique, prepare a smear of each of the two organisms and on the remaining slide prepare a smear consisting of a mixture of Bacillus subtilis and E. coli. Do this by placing a drop of water on the slide and then transferring each organism separately to the drop of water on the slide with a sterile, cooled loop. Mix and spread both organisms by means of a circular motion of the inoculating loop.

3. Allow smears to air dry and then heat fix.

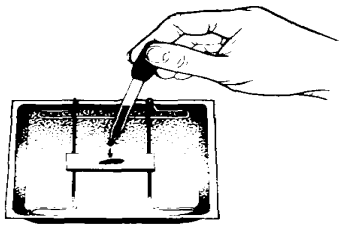
4. Flood smears with crystal violet and let stand for 1 minute.



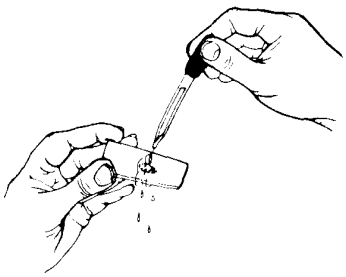
5. Wash with tap water.



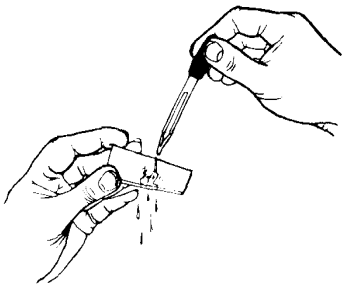
6. Flood smears with the Gram's iodine mordant and let stand for 1 minute.



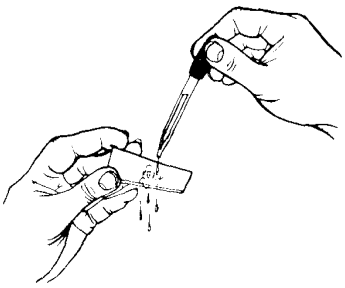
7. Wash with tap water.



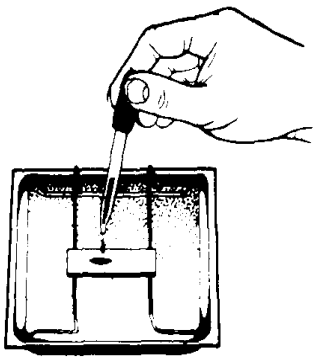
8. Decolourise with propanone.
   Caution:
   Do not over-decolourise.
   Add reagent drop by drop until crystal violet fails to wash from smear.



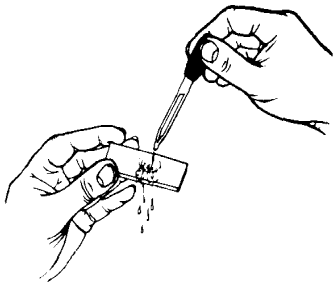
9. Wash with tap water.



10. Counter stain with safranin for 45 seconds.



11. Wash with tap water.



12. Blot dry with lens tissue, cover with a coverslip and examine under the microscope.