Gram Staining Protocol
for Bacterial Differentiation

Introduction

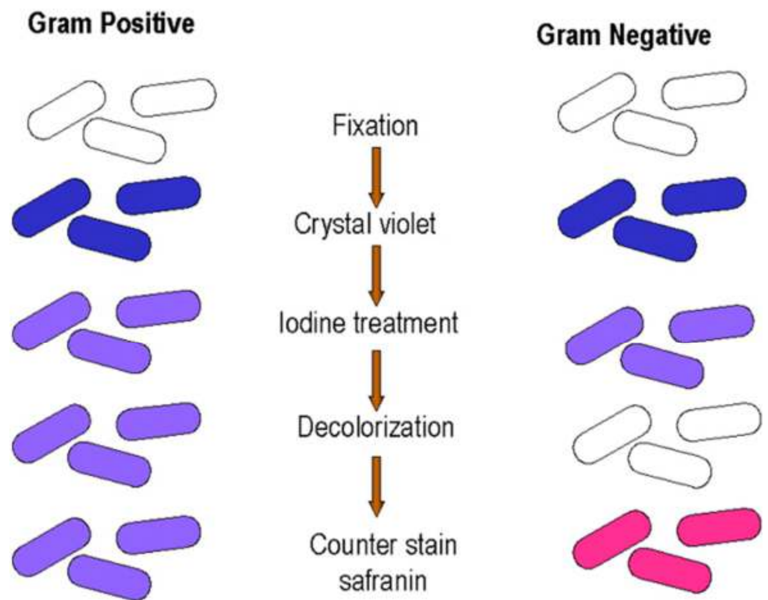
The Gram staining method is named after Hans Christian Gram, the Danish bacteriologist who originally devised it in 1844, and is one of the most important staining techniques in microbiology. It is almost always the first test performed for the identification of bacteria. The primary stain of this method is Crystal Violet, which can be sometimes substituted with equally effective Methylene Blue. The microorganisms that retain the crystal violet-iodine complex appear purple brown under microscopic examination. Stained microorganisms are classified as gram positive, while the unstained are classified as gram negative.

While there is a wide range of staining methods available, the procedures for those are similar to that of Gram's stain. By using appropriate dyes, different parts of the cell structure such as capsules, flagella, granules or spores can be stained. Staining techniques visualize components that are too difficult to see under an ordinary light microscope either because of lack of color contrast between background and object being examined or because of the limited power of the light microscope. In addition, these techniques are useful in the detection or absence of cell components. This simple differentiation technique ranks among the most important diagnostic tools in biological science.

Gram's Method uses retained crystal violet dye during solvent treatment to amplify the difference in the microbial cell wall. The cell walls for gram-positive microorganisms have a higher lipid content than gram-negative cells. First, crystal violet ions penetrate the cell wall of both types of cells. Then, iodine is added to form a complex that makes the dye difficult to remove, in a step referred to as "fixing" the dye. Following iodine, the cells are treated with decolorizer, a mixture of ethanol and acetone, which dissolves the lipid layer from the gram-negative cells, and dehydrating the thicker gram-positive cell wall. As a result, the stain leaches from gram-negative cells and is sealed in gram-positive cells. With expedient removal of the decolorizer, cells will remain stained. The addition of a safranin counterstain to dye the gram-negative cells with a pink color for easier observation under a microscope. Thus, gram-positive cells will be stained purple and gram-negative cells will be stained pink.

Materials

- Crystal Violet (GoldBio Catalog # C-328)
- Methylene Blue (GoldBio Catalog # M-680)
- Potassium Iodide (GoldBio Catalog # P-440)
- Ethanol, 95%
- Acetone
- Ammonium oxalate
- Iodine
- Sodium bicarbonate
- Safranin O
- Bunsen Burner
- Microscope
- Slide
- Cloth pin
- dH_2O



Credit: Dr. Alivin Fox, University of South Carolina School of Medicine. Bacteriology- Chapter 2, Culture and identification of infectious agents.

Preparation of solutions

Gram Crystal Violet Solution:

- Dissolve 20 g of crystal violet in 100 ml of ethanol to make a crystal violet stock solution.
- Similarly, dissolve 1 g of ammonium oxalate in 100 ml of water to make an oxalate stock solution.
- The working solution is obtained by mixing 1 ml of the crystal violet stock solution with 10 ml of water and 40 ml of the oxalate stock solution. Store the working solution in a drop bottle.

Methylene Blue Solution:

- Dissolve 1 g of methylene blue, 90% dye content, in 100 ml of ethanol, this is solution A.
- Mix 0.03 g of KOH in 300 ml of water, this is solution B.
- Mixing solutions A and B yields the working solution.

Gram Iodine Solution:

- Dissolve 1 g of iodine, 2 g of potassium iodide and 3 g of sodium bicarbonate in 300 ml of water.

Gram Decolorizer Solution:

- Mix equal volumes of 95% ethanol and acetone.

Gram Safranin Solution:

- Dissolve 2.5 g of Safranin O in 100 ml of 95% ethanol to make a stock solution.
- Working solution is obtained by diluting one part of the stock solution with five parts of water.

Method

Prepare a Slide Smear

1. Transfer a drop of the suspended culture to be examined on a slide with an inoculation loop. If the culture is to be taken from a petri dish or a slant culture tube, first add a drop of water on the slide and aseptically transfer a minute amount of a colony from the petri dish.

   Note: 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.

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

3. Hold the slide with a cloth pin. Air-dry the culture and fix it or over a gentle flame while moving the slide in a circular fashion to avoid localized overheating.

   Note: 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 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).

Gram Staining

1. Add about 5 drops of crystal violet stain over the fixed culture. Let stand for 60 seconds. Note that a clothes pin is used to hold the slide during the staining procedure to avoid staining one’s hand.

2. Pour off the stain and gently rinse the excess stain with a stream of dH_2O.

   Note: The objective of this step is to wash off the stain, not the fixed culture.

3. Add about 5 drops of the iodine solution on the smear, enough to cover the fixed culture. Let stand for 30 seconds.

4. Pour off the iodine solution and rinse the slides with running water. Shake off excess water from the surface.

5. Add a few drops of decolorizer so the solution trickles down the slide. Rinse it off with water after 5 seconds. Stop when the solvent is no longer colored as it flows over the slide.

    Note: Leaving the decolorizer on for longer than 5 seconds will cause excess decolorization in the gram-positive cells, and proper staining will not occur.

6. Counterstain with 5 drops of the Safranin solution for 20 seconds.

7. Wash off the red Safranin solution with water. Blot with bibulous paper to remove any excess water. Alternatively, the slide may be shaken to remove most of the water and air-dried.

8. Liberally wash off any spilled stain immediately with water to avoid leaving permanent marks on the sink, lab bench or glassware.

9. Examine the finished slide under a microscope.

Associated Products

- Crystal Violet (GoldBio Catalog # C-328)
- Methylene Blue (GoldBio Catalog # M-680)
- Potassium Iodide (GoldBio Catalog # P-440)

References

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