Department of biology

Microbiology Lab

((Gram Stain))

Lab/5

2 stage

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Gram Stain:

The Gram staining is one of the most crucial staining techniques in microbiology. It gets its name from the Danish bacteriologist Hans Christian Gram, who first introduced it in 1882, mainly to identify organisms causing pneumonia.

Often, the first test performed, gram staining, involves the use of crystal violet or methylene blue as the primary color. The term for organisms that retain the primary color and appear purple-brown under a microscope is Gram-positive organisms. The organisms that do not take up primary stain appear red under a microscope and are Gram-negative organisms.

How Does Gram Staining Work?

Gram staining involves three processes: staining with a water-soluble dye called crystal violet, decolorization, and counterstaining, usually with safanin. Due to differences in the thickness of a peptidoglycan layer in the cell membrane between Gram positive and Gram negative bacteria, Gram positive bacteria (with a thicker peptidoglycan layer) retain crystal violet stain during the decolorization process, while Gram negative bacteria lose the crystal violet stain and are instead stained by the safranin in the final staining process.

The process involves three steps:

1- Cells are stained with crystal violet dye. Next, a Gram's iodine solution (iodine and potassium iodide) is added to form a complex between the crystal violet and iodine. This complex is a larger molecule than the original crystal violet stain and iodine and is insoluble in water.

2- A decolorizer such as ethyl alcohol or acetone is added to the sample, which dehydrates the peptidoglycan layer, shrinking and tightening it. The large crystal violet-iodine complex is not able to penetrate this tightened peptidoglycan layer, and is thus trapped in the cell in Gram positive bacteria. Conversely, the the outer membrane of Gram negative bacteria is degraded and the thinner peptidoglycan layer of Gram negative cells is unable to retain the crystal violet-iodine complex and the color is lost.

3- A counterstain, such as the weakly water soluble safranin, is added to the sample, staining it red. Since the safranin is lighter than crystal violet, it does not disrupt the purple coloration in Gram positive cells. However, the decolorized Gram negative cells are stained red.

Staining Protocol:

- Crystal violet (primary stain)
- Iodine solution/Gram's Iodine (mordant that fixes crystal violet to cell wall)
- Decolorizer (e.g. ethanol)
- Safranin (secondary stain)
- Water (preferably in a squirt bottle)

1-Make a slide of cell sample to be stained. Heat fix the sample to the slide by carefully passing the slide with a drop or small piece of sample on it through a Bunsen burner three times.

2-Add the primary stain (crystal violet) to the sample/slide and incubate for 1 minute. Rinse slide with a gentle stream of water for a maximum of 5 seconds to remove unbound crystal violet.

3-Add Gram's iodine for 1 minute- this is a mordant, or an agent that fixes the crystal violet to the bacterial cell wall.

4-Rinse sample/slide with acetone or alcohol for ~3 seconds and rinse with a gentle stream of water. The alcohol will decolorize the sample if it is Gram negative, removing the crystal violet. However, if the alcohol remains on the sample for too long, it may also decolorize Gram positive cells.

5-Add the secondary stain, safranin, to the slide and incubate for 1 minute. Wash with a gentle stream of water for a maximum of 5 seconds. If the bacteria is Gram positive, it will retain the primary stain (crystal violet) and not take the secondary stain (safranin), causing it to look violet/purple under a microscope. If the bacteria is Gram negative, it will lose the primary stain and take the secondary stain, causing it to appear red when viewed under a microscope.