The Gram Stain: Theory and Procedure

The Gram stain is a staining procedure that makes bacterial cells more visible under a microscope and differentiates cell types based on composition of cell walls. Explore the Gram stain procedure and learn how and why it works.

The Classic Gram Stain

Every student and professional who works with bacteria has to, at some point, learn how to do a Gram stain. The Gram stain is a staining procedure used to not only make bacterial cells more visible under the microscope, but also to differentiate cell types based on the cell wall composition. In this lesson, we will go step by step through the Gram stain procedure, explaining exactly what to do and how each step contributes to coloring and differentiating bacteria. For more in-depth background information and several key example species, see the lesson 'The Gram Stain: Background & Example Organisms.'

The Gram Stain Procedure

The best way to appreciate how the Gram stain is able to differentiate bacteria based on their cell walls is to dive right into the process itself. Before we begin, remember Gram-positive cells will appear purple and Gram-negative cells will appear red after proper staining. Before we begin the process, we have to prepare your bacterial specimen.

The bacteria should be less than 24 hours old to ensure accurate results. The bacteria need to be spread in a thin layer across a microscope slide. A thin layer will ensure even and complete staining. Once applied to the slide, the bacteria need to be heat fixed by waving the bottom of the slide over an open flame. The slide should get warm, but not hot. Too much heat can distort the cells or even shatter the glass slide. A gentle warming will kill the bacteria and attach them to the slide so they can't be easily rinsed off. Now it's time to start staining.

Step 1: Cover the surface of the slide with crystal violet for one minute. **Crystal violet** is the primary stain. It will stain the bacteria a deep purple color. The crystal violet will penetrate the cell walls of all bacteria, making them all appear purple under the microscope.

Step 2: Gently rinse the stain off the slide. This is most easily accomplished by tilting the slide under a lightly running tap and rinsing with regular water until the water runs clear. The

excess crystal violet will drain into the sink, while the stain that penetrated into the cell walls will remain.

Step 3: Cover the slide with Gram's iodine for one minute. Gram's iodine serves as a mordant. A **mordant** is a substance that combines with stain or dye to enhance the staining ability. The iodine forms a tight complex with the crystal violet, forming compounds with a larger size. This larger size helps the crystal violet resist rinsing out of the cell walls during the later rinsing steps.

Step 4: Gently rinse the Gram's iodine off the slide. The same tap water rinse works well here too. At this point, if you looked at the slide under the microscope, all the cells will still appear to be purple from the crystal violet stain.

Step 5: Decolorize the slide with 95% ethanol. This is the most important step in the procedure and the easiest to mess up, which would invalidate your results. You want to tilt the slide slightly and very slowly trickle the ethanol decolorizer over the slide. You will notice that the decolorizer picks up the purple stain as it runs across your cells. You must stop decolorizing as soon as the decolorizer looks clear running off the slide.

What is actually happening is the ethanol is dissolving the fats, also called lipids, in the outer membrane on the Gram-negative cells. These are the cells with the thin peptidoglycan layer. Without the outer membrane, the cell wall becomes porous, allowing all of the crystal violet to be rinsed out. The thick peptidoglycan of the Gram-positive cells traps the crystal violet between the layers. If you looked at the cells under the microscope now, the Gram-positive cells would still appear purple. The Gram-negative cells, having lost their stain, would appear transparent.

Take care here, this step is the easiest to mess up. Decolorize too much and you can strip the crystal violet from the Gram-positive cells too, making them appear transparent. Decolorize too little and the Gram-negative cells will retain enough crystal violet to appear purple.

Step 6: Gently rinse with water as before.

Step 7: Apply safranin stain for one minute. **Safranin** is the counterstain, turning cells a red color. It will stain both Gram-positive and Gram-negative cells, but the dark purple of the crystal violet will overpower the lighter red of the safranin in the Gram-positive cell walls. Gram-positive bacteria will still appear purple. The previously transparent Gram-negative cells will take up the safranin and appear red.

Step 8: Gently rinse with water as before.

Step 9: Gently blot dry the slide. At this point, ordinary paper towels or special tablets of blotting paper are used to gently blot off the excess stain and water. Never rub dry the slide. This will wipe off all your hard work.

That's it. Now you can put your slide on the microscope and view your results! If you did it all correctly, you should have a mix of purple, Gram-positive cells and red, Gram-negative cells. It is not uncommon for first (or second, third, and fourth) attempts to be inconclusive or improperly stained. This is a delicate technique that requires a lot of practice to get down. So don't get discouraged. Just keep staining!