



Core Microbiology Skills

How to Perform a Gram Stain

In this video, we're following the CDC safety protocols for the use of PPE and equipment. However, the use of goggles and gloves for performing these tests are not a universal safety requirement, so each laboratory must determine its own protocols based on its risk assessment. In this video, we're also following the CDC safety protocols for the disposal of sharps and biohazard waste.

The Gram stain is a laboratory technique commonly used to differentiate bacterial species into two large groups: Gram-positive and Gram-negative bacteria. The technique is based on the chemical and physical properties of their respective cell walls.

In the staining technique, cells on a microscope slide are heat-fixed and stained with a basic dye utilizing four reagents: crystal violet (the primary stain), iodine solution (the mordant), decolorizer (which is usually a 50/50 mixture of acetone and alcohol), and safranin or carbol fuchsin (which is the counterstain). Cells that retain the basic dye (crystal violet) after the procedure are called Gram-positive, and those that do not are called Gram-negative. Gram-positive cells are purple in color, while Gram-negative cells are pink in color.

After this program, you will be able to list the reagents used in the Gram stain procedure, correlate the results seen in the bacterial cells with the effects of the various reagents during the Gram stain procedure, and finally, you'll be able to list the steps in the Gram stain procedure.

For this procedure, you'll need the following: personal protective equipment, a clean water source, a slide rack, and a timer. For reagents, you'll need crystal violet, Gram's iodine, a decolorizer, which is usually a 50/50 acetone/alcohol mixture, and safranin or carbol fuchsin. Your sample should be a thin, heat-fixed bacterial smear. In this video, you'll observe the Gram stain procedure based on ASM protocols. However, the timing and reagents may vary, so you should follow the manufacturer's instructions.

This is the typical setup for the Gram stain that is seen in most clinical and public health laboratories. The Gram stain reagents are placed beside the sink, and a slide rack is placed over the sink to hold the slide. A rubber hose may be attached to the faucet for ease of rinsing the slides. The procedure starts with a thin, fixed smear on a glass microscope slide. The first reagent to use is the crystal violet, which is an alkaline dye. Slowly pour the crystal violet reagent over the slide in an even manner. During this step, the dye, crystal violet, is binding to the cell wall of the bacteria. The smear will be a purple color. Immediately after pouring the crystal violet over the slide, set the timer for 15 seconds. After the time is up, using the hose attached to the faucet, gently rinse the slide with a steady stream of water to remove any excess crystal violet.

The next step in the procedure is to add the mordant, Gram's iodine. The mordant binds with the crystal violet to form an insoluble complex in the bacterial cell. If you looked at the cells now, they would all be purple. As soon as the iodine is poured over the slide, set the timer for 15 seconds. After the time is up, rinse the slide gently with water to remove excess mordant. The next step in the procedure is adding the decolorizer. There are other types of decolorizers that can be used, so check

with your lab's protocol. During this step, due to the presence of a low concentration of peptidoglycan in the cell walls of some bacteria, the crystal violet-iodine complex is leached out, and the bacterial cells are rendered colorless. Hold the slide at an angle and pour or drip the decolorizer down the slide until the runoff is clear; this may take only a few seconds. Immediately rinse the slide with water to stop decolorization.

Please note: the timing of this step may vary depending on the type of decolorizer and the thickness of the smear. This is a critical step: you must take care not to over or under decolorize the smear. The cells that are Gram-positive, those with high peptidoglycan content, remain purple. And those that are Gram-negative, with a low peptidoglycan content, are colorless. At this point, the slide may not have any visible color.

Due to the fact that some bacteria are colorless after the decolorizing step, a counterstain such as safranin or carbol fuchsin is then poured over the slide to stain the colorless bacteria. Set the timer for 30 seconds. During this step, the safranin or carbol fuchsin is taken up by the colorless cells and is seen as having a pink color under the microscope. Those bacteria where the crystal violet-iodine complex was not leached from the cell during the decolorizing step will remain purple. After 30 seconds, rinse the slide gently with water to remove excess counterstain and allow the slide to air dry.

After the slide has air dried, it can be examined under the microscope. Looking under the microscope, you will see organisms that are Gram-positive purple or Gram-negative pink. Here, you can see *Staphylococcus aureus*, which is Gram-positive. And here, you see *E. coli*, which is a Gram-negative organism.

To summarize the steps for the Gram stain, pour the crystal violet onto the fixed smear. Set the timer for 15 seconds. Rinse with water. Pour the iodine onto the slide. Set the timer for 15 seconds and rinse with water. Hold the slide at an angle and pour the decolorizer onto the smear until the runoff is clear. Rinse with water. Pour the counterstain onto the slide. Set the timer for 30 seconds and rinse with water.

Link to video job aid: <https://reach.cdc.gov/jobaid/how-perform-gram-stain>.