

Gram Stain Protocols

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Information

History

The Gram stain was first used in 1884 by Hans Christian Gram (Gram, 1884). Gram was searching for a method that would allow visualization of cocci in tissue sections of lungs of those who had died of pneumonia. Already available was a staining method designed by Robert Koch for visualizing tubercle bacilli. Gram devised his method that used Crystal Violet (Gentian Violet) as the primary stain, an iodine solution as a mordant followed by treatment with ethanol as a decolorizer. This staining procedure left the nuclei of eukaryotic cells in tissue samples unstained while the cocci found in the lungs of those who had succumbed to pneumonia were stained blue/violet. Gram found that his stain worked for visualizing a series of bacteria associated with disease such as the "cocci of suppurative arthritis following scarlet fever". He found however that Typhoid bacilli were easily decolorized after the treatment with crystal violet and iodine, when ethanol was added. We now know that those organisms that stained blue/violet with Gram's stain are **gram-positive** bacteria and include *Streptococcus pneumoniae* (found in the lungs of those with pneumonia) and *Streptococcus pyogenes* (from patients with Scarlet fever) while those that were decolorized are **gram-negative** bacteria such as the *Salmonella Typhi* that is associated with Typhoid fever.

Purpose

The Gram stain is fundamental to the phenotypic characterization of bacteria. The staining procedure differentiates organisms of the domain Bacteria according to cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple. Gram-negative cells have a thin peptidoglycan layer and stain red to pink.

Theory

The Gram stain, the most widely used staining procedure in bacteriology, is a complex and differential staining procedure. Through a series of staining and decolorization steps, organisms in the Domain Bacteria are differentiated according to cell wall composition. Gram-positive bacteria

have cell walls that contain thick layers of peptidoglycan (90% of cell wall). These stain purple. Gram-negative bacteria have walls with thin layers of peptidoglycan (10% of wall), and high lipid content. These stain pink. This staining procedure is not used for Archaea or Eukaryotes as both lack peptidoglycan. The performance of the Gram Stain on any sample requires four basic steps that include applying a primary stain (crystal violet) to a heat-fixed smear, followed by the addition of a mordant (Gram's Iodine), rapid decolorization with alcohol, acetone, or a mixture of alcohol and acetone and lastly, counterstaining with safranin.

Details of the chemical mechanism of the Gram stain were determined in 1983 (Davies et al., 1983 and Beveridge and Davies, 1983). In aqueous solutions crystal violet dissociates into CV^+ and Cl^- ions that penetrate through the wall and membrane of both gram-positive and gram-negative cells. The CV^+ interacts with negatively charged components of bacterial cells, staining the cells purple. When added, iodine (I^- or I_3^-) interacts with CV^+ to form large CVI complexes within the cytoplasm and outer layers of the cell. The decolorizing agent, (ethanol or an ethanol and acetone solution), interacts with the lipids of the membranes of both gram-positive and gram-negative Bacteria. The outer membrane of the gram-negative cell is lost from the cell, leaving the peptidoglycan layer exposed. Gram-negative cells have thin layers of peptidoglycan, one to three layers deep with a slightly different structure than the peptidoglycan of gram-positive cells (Dmitriev, 2004). With ethanol treatment, gram-negative cell walls become leaky and allow the large CV-I complexes to be washed from the cell. The highly cross-linked and multi-layered peptidoglycan of the gram-positive cell is dehydrated by the addition of ethanol. The multi-layered nature of the peptidoglycan along with the dehydration from the ethanol treatment traps the large CV-I complexes within the cell. After decolorization, the gram-positive cell remains purple in color, whereas the gram-negative cell loses the purple color and is only revealed when the counterstain, the positively charged dye safranin, is added. At the completion of the Gram stain the gram-positive cell is purple and the gram-negative cell is pink to red.

Some bacteria, after staining with the Gram Stain yield a pattern called gram-variable where a mix of pink and purple cells are seen. The genera *Actinomyces*, *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, and *Propionibacterium* have cell walls particularly sensitive to breakage during cell division, resulting in gram-negative staining of these gram-positive cells. In cultures of *Bacillus*, *Butyrivibrio*, and *Clostridium* a decrease in peptidoglycan thickness during growth coincides with an increasing number of cells that stain gram-negative (Beveridge, 1990). In addition, in all bacteria stained using the Gram stain, the age of the culture may influence the results of the stain.

Some bacteria do not stain as expected with the Gram stain. For example, members of the genus *Acinetobacter* are gram-negative cocci that are resistant to the decolorization step of the Gram stain. *Acinetobacter* spp. often appear gram-positive after a well prepared Gram stain (Visca et al. 2001). For *Mycobacterium* spp., the waxy nature of the coat renders the bacteria not readily stainable with dyes used in

the Gram stain, though the bacteria are considered to be gram positive (Saviola and Bishai, 2000). *Gardnella* has an unusual gram-positive cell wall structure that causes bacteria of this genus to stain gram-negative or gram-variable (Sadhu et al 1989).

Misinterpretation of the Gram stain has led to misdiagnosis or delayed diagnosis of infectious disease (Visca et al., 2001, Noviello et al., 2004)

RECIPE

(Gephardt et al., 1981) This is Hucker's modification of the Gram Stain method. Gram originally used Gentian Violet as the primary stain in the Gram stain. Crystal violet is generally used today. In Hucker's method ammonium oxalate is added to prevent precipitation of the dye (McClelland, 2001) and uses an alcoholic solution of the counterstain. Burke's modification of the Gram Stain adds sodium bicarbonate to the crystal violet solution. Sodium bicarbonate prevents the acidification of the solution as iodine oxidizes (McClelland, 2001) and uses an aqueous solution of Safranin for the counterstain (Gephardt et al., 1981).

The reagents listed below can be made or purchased commercially from biological supply houses

1. Primary Stain: Crystal Violet Staining Reagent.

Solution A for crystal violet staining reagent

Crystal violet (certified 90% dye content), 2g
Ethanol, 95% (vol/vol), 20 ml

Solution B for crystal violet staining reagent

Ammonium oxalate, 0.8 g
Distilled water, 80 ml

Mix A and B to obtain **crystal violet staining reagent**. Store for 24 h and filter through paper prior to use.

2. Mordant: Gram's Iodine

Iodine, 1.0 g
Potassium iodide, 2.0 g
Distilled water, 300 ml

Grind the iodine and potassium iodide in a mortar and add water slowly with continuous grinding until the iodine is dissolved. Store in amber bottles.

3. Decolorizing Agent

Ethanol, 95% (vol/vol)

*Alternate Decolorizing Agent

Some professionals prefer an acetone decolorizer while others use a 1:1 acetone and ethanol mixture. Commercially, a variety of mixtures are available, most using 25 – 50% acetone with the ethanol. A few include a small quantity of isopropyl alcohol and/or methanol in the formulation.

Acetone, 50 ml
Ethanol (95%), 50 ml

4. Counterstain: Safranin

Stock solution:

2.5g Safranin O
100 ml 95% Ethanol

Working Solution:

10 ml Stock Solution
90 ml Distilled water

PROTOCOL (Gephardt et al, 1981, Feedback from ASMCUE participants, ASMCUE , 2005)

1. Flood air-dried, heat-fixed smear of cells for 1 minute with crystal violet staining reagent. **Please note** that the quality of the smear (too heavy or too light cell concentration) will affect the Gram Stain results.
2. Wash slide in a gentle and indirect stream of tap water for 2 seconds.
3. Flood slide with the mordant: Gram's iodine. Wait 1 minute.
4. Wash slide in a gentle and indirect stream of tap water for 2 seconds.
5. Flood slide with decolorizing agent. Wait 15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear (see Comments and Tips section).
6. Flood slide with counterstain, safranin. Wait 30 seconds to 1 minute.
7. Wash slide in a gentle and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper.
8. Observe the results of the staining procedure under oil immersion using a Brightfield microscope. At the completion of the Gram Stain, gram-negative bacteria will stain pink/red and gram-positive bacteria will stain blue/purple.

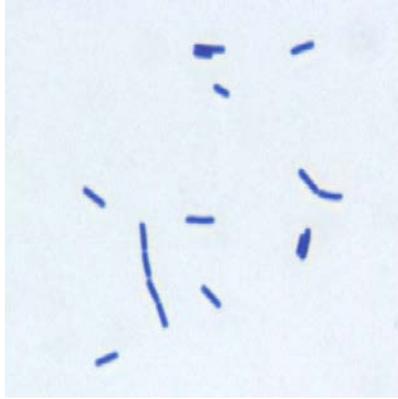


FIG. 1



FIG. 2

In a smear that has been stained using the Gram Stain protocol, the shape, arrangement and gram reaction of a bacterial culture will be revealed. FIG. 1. shows gram-positive (blue/purple) rods and FIG. 2. shows gram-negative (pink/red) rods.

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

MSDS links:

Acetone information:

<http://www.jtbaker.com/msds/englishhtml/A0446.htm>

Ammonium oxalate information:

<http://www.jtbaker.com/msds/englishhtml/A6072.htm>

Crystal violet Information:

<http://www.jtbaker.com/msds/englishhtml/C5720.htm>

Ethanol information:

<http://www.jtbaker.com/msds/englishhtml/D0210.htm>

Iodine Information:

<http://www.jtbaker.com/msds/englishhtml/I2680.htm>

Potassium iodide information:

<http://www.jtbaker.com/msds/englishhtml/P5906.htm>

Safranin Information:

<http://www.jtbaker.com/msds/englishhtml/S0240.htm>

COMMENTS AND TIPS

Comments and tips come from discussions at ASM Conference for Undergraduate Educators 2005.

The thickness of the smear used in the Gram stain will affect the result of the stain. **The step that is most crucial in effecting the outcome of the stain is the decolorizing step.** Over-decolorizing will lead to an erroneous result where gram-positive cells may stain pink to red indicating a gram-negative result, and under-decolorizing will lead to an erroneous result where gram-negative cells may appear blue to purple indicating a gram-positive result. The degree of decolorizing required is determined by the thickness of the smear (number of cells on the slide). The Gram stain was discussed in detail at the American Society for Microbiology Conference for Undergraduate Educators in 2005 when this site was reviewed. The group recommends that cells be prepared with a thin smear with no areas of clumping or inconsistency. When staining the thin smear a short decolorizing time should be used. Some individuals will flood the slide for 15 seconds or less with decolorizing agent, while others recommend adding decolorizing agent drop wise for 5- 15 seconds or until the color of the decolorizing agent running from the slide no longer shows any color.

It is recommended that young, actively growing cultures be used for gram staining. An intact cell wall is required for an accurate gram stain. Older cultures may have breaks in the cell wall and often give gram-variable results where a mixture of pink/red cells are seen among blue/purple cells.

Using a gram stain control is recommended. On the same slide as the test culture include a sample of cells with a known gram stain reaction to serve as a control for success in the gram stain technique.

Gram-stained bacteria should be viewed with a brightfield microscope at 1000X magnification with oil immersion. If the smear of cells is crowded it will be difficult to note cell shape and arrangement.

When viewing slides use brightfield microscopy and adjust the brightness sufficiently to reveal the color of the specimen.

Freshly made staining reagents are recommended. With older staining reagents, filter stains before use.

In the Gram Stain technique, two positively charged dyes are used: crystal violet and safranin. The use of the designation "gram-positive"

should not be confused with the concept of staining cells with a simple stain that has a positive charge.

KOH string test may be used as a confirmatory test for the Gram Stain (Powers, 1995, Arthi et al., 2003): The formation of a string (DNA) in 3% KOH indicates that the isolate is a gram-negative organism.

Procedure:

Place a drop of 3% KOH onto a glass slide.

Emulsify in KOH a loopful of the culture from a BA incubated for 18-24 hours.

Continue to mix the suspension for 60 sec and by slowly lifting the loop, observe for the formation of a string.

Interpretation:

Gram-negative cells form a string within 60 seconds.

Gram-positive cells are not affected.

Various formulations of decolorizing agents may be used (acetone, acetone/ethanol, ethanol). Acetone is the most rapid decolorizer followed by acetone/ethanol and then ethanol. Ethanol is recommended for student use to prevent over-decolorization of samples (McClelland, 2001)

When the counterstain is added this positively charged dye will replace the crystal violet dye in the gram-positive bacteria as well as stain the gram-negative bacteria, although the presence of the mordant slows this process considerably it is important not to overexpose cells to counterstain (McClelland, 2001)

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