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 Archeae 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 yeild 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

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 gentile 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.

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 A

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-

General Bacteriology, ASM Press, Washington D.C. **Gram. C.**1884. Ueber die isolirte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. Fortschritte der Medcin, (2):185-189. Available at: (pdf)
9. **Noviello S, Gallo R, Kelly M, Limberger RJ, DeAngelis K, Cain L, et al.** Laboratory-acquired brucellosis. Emerg Infect Dis [serial on the Internet]. 2004 Oct [cited August 6,2005]. Available from http://www.cdc.gov/ncidod/EID/vol10no10/04-0076.htm
10. **McClelland, R.**, 2001. Gram's stain the key to microbiology. Medical

Jeff Pommerville
Glendale Community College, Scottsdale, AZ

Mary Rees
Moorpark College, CA

Steven Roschke
Good Samaritan College of Nursing, Cincinnati, OH

Jackie Reynolds
Richland College, Dallas, TX

Erica Suchman
Colorado State University, Ft. Collins, CO

Dan Williams
Truckee Meadows Community College, Reno, NV