THE GRAM STAIN

Principle and Purpose

The Gram stain is perhaps the most useful test conducted in the clinical microbiology laboratory. It was first developed by the Danish scientist Hans Christian Gram in an effort to make bacteria more visible in stained sections of lung tissue. In Gram's original staining procedure (https://www.asm.org/ccLibraryFiles/FILENAME/0000000235/1884p215.pdf), published in 1884, some bacteria stained ". . . intense blue (often almost black) . . .", whereas others did not stain at all. At the time, the basis for this differential staining was unknown. Today, we clearly understand the reason for this difference in staining patterns and often exploit it in helping to identify environmentally and clinically relevant microbes.

As opposed to a simple stain, which is used to assess cell morphology, the Gram stain is not only used to distinguish the shape of prokaryotic cells, but also to differentiate between two types of bacteria (Fig. 1). The difference between these two groups of bacteria, termed Gram positive and Gram negative, lays in the structural and chemical composition of the cell wall (Fig. 2). Gram-positive bacteria, which are stained dark blue or purple by the modern Gram stain procedure, generally have a single plasma membrane surrounded by a thick peptidoglycan layer. By contrast, Gram-negative bacteria, which stain pink or red, generally possess a thin layer of peptidoglycan between the

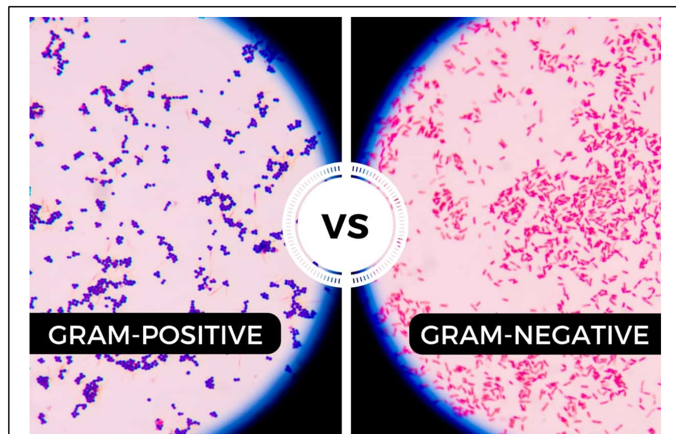

Figure 1. Comparison of staining patterns of Gram-positive and Gram-negative bacteria. (https://thisonevsthatone.com/gram-positive-vs-gram-negative/)

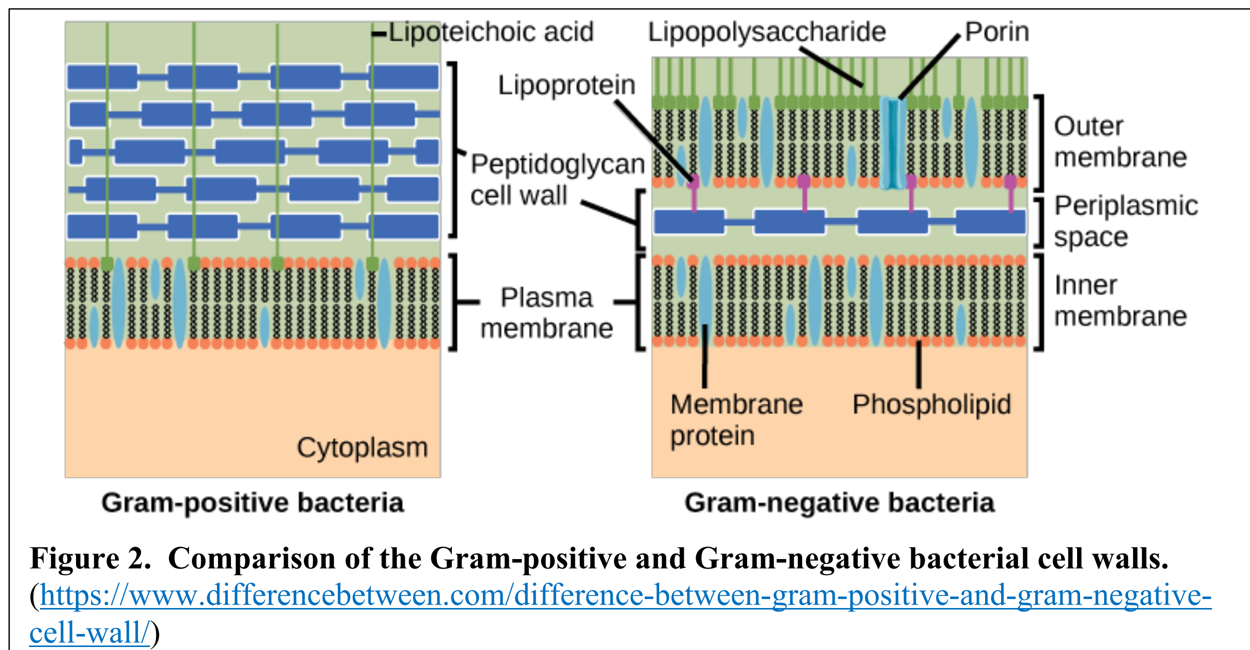

Figure 2. Comparison of the Gram-positive and Gram-negative bacterial cell walls. (https://www.differencebetween.com/difference-between-gram-positive-and-gram-negative-cell-wall/)

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plasma membrane and a somewhat thicker outer membrane. How the cell wall contributes to these staining differences is described below.

Gram vs. gram: Students will note throughout this exercise and in all documents written by the author of these laboratory exercises, the differential staining method originally developed Hans Christian Gram is designated as the "***G***ram stain", NOT the "***g***ram stain". Please note the emphasis. Typically, when a technique or useful item is attributed to an individual, the individual's name should be properly presented with the first letter of the name capitalized. Some examples include the following: 'Sanger sequencing' is named after Frederick Sanger for developing a method to decipher DNA sequences; the 'Petri dish' is named after the German bacteriologist, Julius Richard Petri, for development of a lidded dish for culturing cells; and the 'Erlenmeyer flask' is named after its inventor, Emil Erlenmeyer.

Following this practice for the present staining procedure, the "g" in gram should always be capitalized, i.e., "G", thereby resulting in 'Gram stain'. Moreover, whereas "BIG G" refers to a name, "little g" gram denotes a unit of weight in the metric system. Hence, the author of these exercises strongly opposes the use of the term 'gram stain' in textbooks and other materials to designate the method being explored in this exercise. Students in this course are expected to appease the author by acquiescing to his fondness for scientific tradition.

The first step of the modern Gram stain involves using crystal violet, a basic dye (Fig. 3). This is the primary stain which is initially applied to a heat-fixed smear of bacteria. This is followed by treating the smear with an iodine solution. The latter acts as a mordant, i.e., in this case, a substance that heightens the interaction of the crystal violet with the bacterial cell. This causes the dye to bind more tightly bound to the cell. The next step is the most critical one in the Gram stain procedure and is effectively the differentiating step. If not performed properly, the staining results will be incorrect. The smear is decolorized, i.e., the stain may be removed, by washing with ethanol or isopropanol-acetone. In the case of Gram-positive bacteria, the dye-iodine complex is retained by the cell because the decolorizing agent shrinks any pores in the thick peptidoglycan layer, thereby halting the flow of the complex out of the cell. In contrast, the decolorizing agent dissolves a significant portion of the lipid-based outer membrane of Gram-negative bacteria making it more porous resulting in the release of the crystal violet-iodine complex. In essence, these cells become colorless just like those originally noted by Gram in

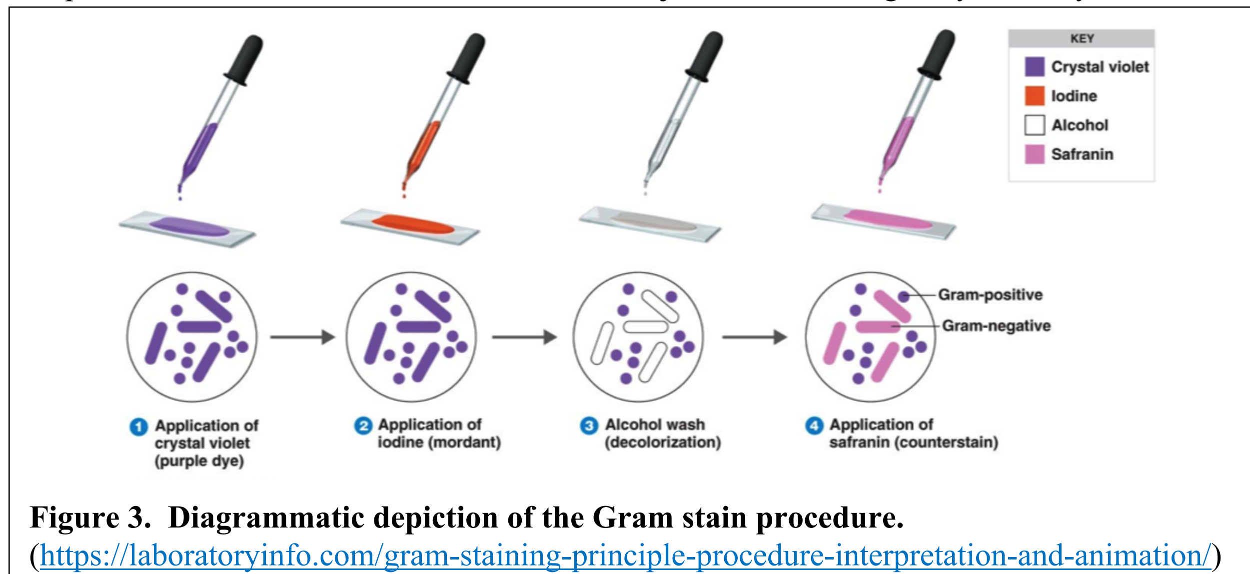

Figure 3. Diagrammatic depiction of the Gram stain procedure.
(https://laboratoryinfo.com/gram-staining-principle-procedure-interpretation-and-animation/)

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1884. To visualize these cells, the contemporary Gram stain procedure applies the counterstain safranin to the smear causing cells to appear red or pink.

Gram Stain Video: A brief video that reviews the key concepts of the Gram stain can be found at the following URL: http://highered.mheducation.com/olcweb/cgi/pluginpop.cgi?it=swf::530::530::/sites/dl/free/0073525502/930300/Gram_Stain.swf::Gram%20Stain

It is important to note that the expected results of the modern Gram stain are best generated from young, actively growing (18-24 hour-old) cultures. Gram-positive bacteria from older and less vigorously growing cultures may stain red. Moreover, some bacteria are variable in their Gram-staining reactions and others are resistant to staining at all. Fortunately, most prokaryotes are readily amenable to the Gram stain procedure. Nonetheless, it is generally considered good practice to use young cultures of known Gram-positive and Gram-negative bacteria as controls when staining an unknown bacterium.

Another note of interest is that variations of the Gram stain procedure have been developed over the years. Some employ slight modifications of the staining reagents and processing times, whereas others use methanol fixation instead of heat. Regardless of the method used, the key remains the decolorization step.

The present exercise will introduce students to one of the more common Gram staining techniques. This skill will be critical for conducting some future exercises in this course. In addition, students should gain a valuable skill as well as an appreciation of variability of microbes in terms of shape and structure.

Learning Objectives

Upon completion of this exercise, a student should be able to:

- Explain the chemical basis for the differentiation of bacteria by the Gram stain;
- Properly stain and observe microbes using the Gram stain technique; and
- Differentiate among Gram-positive and Gram-negative bacteria.

Materials Required

The following materials are necessary to successfully conduct this exercise:

Organisms

- The following organisms should be provided as 18-24 hour-old TSA slant cultures:
  - *Staphylococcus aureus* (ATCC 25923) [abbreviated as *S. aureus*]
  - *Escherichia coli* (ATCC 25922) [abbreviated as *E. coli*]
- Mixed TSB culture of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922)

Reagents

- Crystal violet solution
- Gram's iodine solution
- Decolorization solution
- Safranin solution

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Procedures

Special Note: This exercise depends upon an individual's ability to discern colors. If you are color blind, please PRIVATELY consult your laboratory instructor.

Students shall review and use the BIOL 3702L Standard Practices regarding the labeling, incubation, and disposal of materials.

Part A: Making Bacterial Smears

Perform the following procedure to produce three slides of heat-fixed, bacterial smears. Each slide will have a smear of *S. aureus*, a smear of *E. coli*, and a mixed smear of *S. aureus* and *E. coli* between the other two. These smears will be subjected to the Gram staining method described in Part B.

Note: When making these slides, use caution not to have one or more smears overlap with another.

1) Obtain three glass slides and label them appropriately at one end. (If the end of the slide is frosted, then mark the slide in this area.)

   For each of these slides, follow the steps described below for Part A to produce three slides upon which each contains three separate smears of organisms – a Gram-positive coccus (*S. aureus* on the left side of the slide), a Gram-negative bacillus (*E. coli* on the right side of the slide), and a mixture of these bacteria in the middle of the slide. Although the steps below describe the procedure for a single slide, the three slides, each containing the three smears, should be processed simultaneously.
2) Place a *tiny* drop of water (about a quarter the size of a finger nail) on both the right and left sides leaving the middle portion of the slide clear. Use a loop or a disposable plastic bulb pipette to obtain water from the labeled water bottle on the bench top.
3) Sterilize the microbiological loop and, using aseptic technique, obtain a very small mass of cells on the loop from the surface growth on the TSA agar slant culture of *S. aureus.* The cellular mass obtained should be barely visible on your loop.
4) Transfer the *S. aureus* cells to the water droplet on the left side of the glass slide and make a bacterial smear by spreading the water suspension of cells using the loop such that the liquid is about the size of a quarter.

Note: The cell mass should not produce a suspension the consistency of paste nor should the cells be clumped.

5) Sterilize the microbiological loop.
6) Using aseptic technique, obtain a very small mass of cells on the loop from the surface growth on the TSA agar slant culture of *E. coli.* The cellular mass obtained should be barely visible on your loop.
7) Transfer the *E. coli* cells to the water droplet on the right side of the glass slide and make a bacterial smear by spreading the water suspension of cells using the loop such that the liquid is about the size of a quarter.

Note: The cell mass should not produce a suspension the consistency of paste nor should the cells be clumped.

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8) Sterilize the microbiological loop.
9) Ensure that the bacterial cells in the mixed *S. aureus*/*E. coli* broth culture are fully mixed by rolling the tube between both palms ten times or more to suspend any sediment of cells that may have formed. Roll the tube quickly, but not so harshly that the broth splashes onto the tube cap or such that the tube rolls out of the hands causing leakage or breakage.
10) Using a sterilized microbiological loop, transfer 2-6 loopfuls of mixed culture broth from the bacterial culture to be the glass slide. Be sure to sterilize the loop between each transfer (the glass slide is not sterile!).
11) Using the microbiological loop, make a bacterial smear by spreading the broth such that the liquid is about the size of a quarter.
12) Sterilize the microbiological loop and place it aside.
13) The resulting slide now has three wet bacterial smears – a Gram-positive coccus (*S. aureus* on the left side of the slide), a Gram-negative bacillus (*E. coli* on the right side of the slide), and a mixture of these bacteria in the middle of the slide.

    The wet smear must now be dried. The slide may be allowed to air dry or set the slide on a hot plate operating on the lowest setting to facilitate drying.

Note: If a hot plate is used, do not allow the smear to remain on it longer than necessary.

14) The dry slide now needs to be heat fixed. There are two methods to do so. One uses the flame of a gas burner (14a) and the other uses a hot plate (14b). Each is described below.
    a) [PREFERRED METHOD] Using slide holders, tongs, etc., pass the slide containing the bacterial smear through the flame of a gas burner three times. Each pass should be of moderate speed. The slide should not linger in the flame, nor should it pass through so quickly that it is not warmed. Once heated, allow the slide to cool on the bench top.

Note: Exercise caution when using this method due to the extreme heat, the possibility of burns, and the presence of an open flame.

    b) [ALTERNATIVE METHOD] Set the slide containing the bacterial smear on a hot plate operating on the highest (hottest) setting. Use slide holders, tongs, etc., to place the slide on the hot plate surface for 3 seconds. Remove the slide immediately and allow it to cool on the bench top.

Note: Exercise caution when using this method due to the extreme heat produced by the hot plate and the possibility of burns.

Note: At this point, if the above procedure was performed properly, the slide is heat fixed and the bacteria are no longer viable. The slide can be placed in a slide container for storage/transport and stained at a later time.

Part B: The Gram Stain Procedure

Perform the following staining procedure on one of the slides containing the heat-fixed, bacterial smears generated in Part A above. Stain the cells on this slide and observe the results under the microscope using the oil immersion objective. If the appropriate results are not observed, then the remaining slides that can be used to repeat this procedure.

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1) Place a slide containing the heat-fixed bacterial smears on the slide support rails sitting across the sink.
2) Obtain the vial of crystal violet and flood the entire slide with this dye. Allow the stain to remain for 20-30 seconds.
3) Lifting the slide at an angle, use the water bottle on the bench to rinse the slide of excess crystal violet for five (5) seconds.
4) Obtain the vial of Gram's iodine and flood the entire slide with this mordant one (1) minute.
5) Lifting the slide at an angle, use the water bottle on the bench to rinse the slide of excess Gram's iodine for five (5) seconds.
6) Lifting the slide at an angle, decolorize the smears with the decolorizing agent adding this reagent one drop by drop until the color no longer runs off the slide.

Note: This is a very critical step in the Gram stain procedure. Do not over decolorize the smears, nor do not under decolorize.

7) Immediately, lifting the slide at an angle, use the water bottle on the bench to rinse the slide of excess decolorizing agent for five (5) seconds.
8) Obtain the vial of safranin and flood the entire slide with this counter stain one (1) minute.
9) Lifting the slide at an angle, use the water bottle on the bench to rinse the slide of excess safranin for five (5) seconds.
10) Gently blot (do not rub or scrape) the slide dry with bibulous paper or paper towel.

Note: The bibulous paper/paper towel can be discarded in the regular trash receptacle.

11) Examine the slide under the microscope using both the high dry and immersion oil objective lenses.

    Record any observations on the data report sheet attached to this document.

Note: Should a student wish to keep their work product, any slides can be stored and transported in the slide container

Practical Hints for a Successful Gram Stain

- Do not make the bacterial smears too thick. The cells may not have proper access to the staining reagents causing a 'mixed Gram stain' reaction.
- Stop the decolorization step once the reagent flows colorlessly from the slide. Be sure not to over decolorize (no more than 30-40 seconds for a typical bacterial smear already stained with crystal violet).
- Do not blot the slide by rubbing, scraping, or pressing too hard on the slide. The cells can be scratched off the cells or, worse, the slide will break (and possibly produce an injury).
- The Gram's iodine reagent should not be pale yellow. If so, then it has lost its potency and should be replaced.
- Do not excessively heat fix smears. This may damage the cells and affect the results of the Gram stain.

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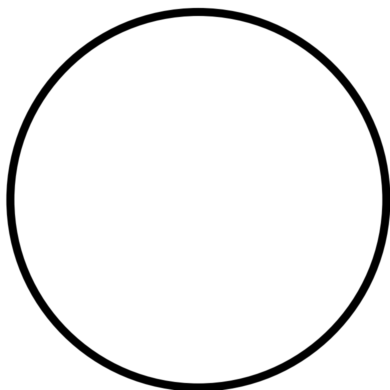
Student Name: ________________________________

DRAWINGS OF GRAM STAIN OF *STAPHYLOCOCCUS AUREUS*



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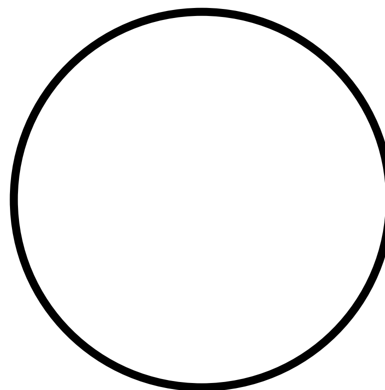

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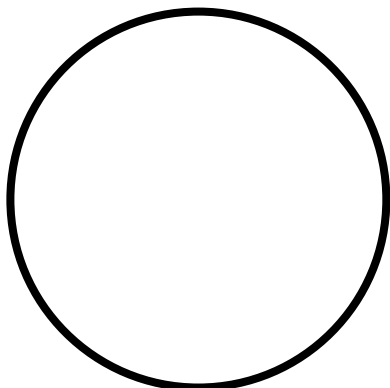
DRAWINGS OF GRAM STAIN OF *ESCHERICHIA COLI*



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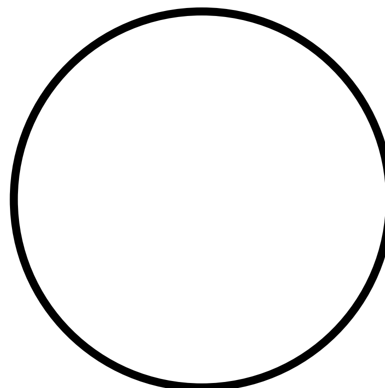

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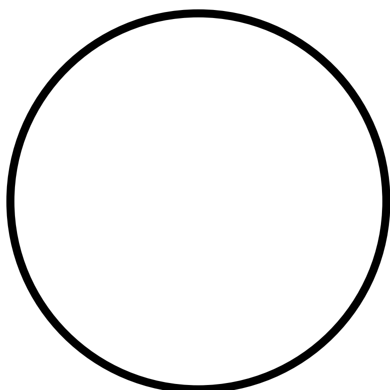
DRAWINGS OF GRAM STAIN OF *E. COLI* AND *S. AUREUS* MIXTURE



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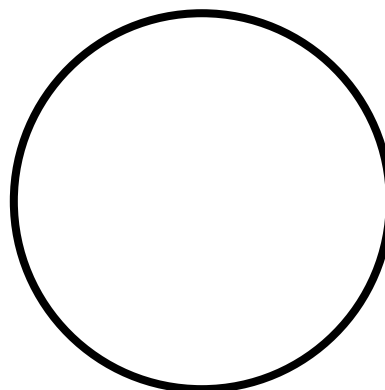

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Discussion Questions:

1. Briefly describe the difference between the simple and differential stain.

2. For the Gram stain, briefly state the purpose of the following reagents:

   a. Crystal Violet

   b. Gram's Iodine

   c. Decolorizing agent

   d. Safranin

3. What is the molecular basis for the Gram staining differences between Gram-positive and Gram-negative bacteria?

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4. Speculate on how the eukaryotic cells of the yeast *Saccharomyces cerevisiae* and the ciliate *Paramecium* would appear following a Gram stain. [**Note:** This answer shall likely require some outside research. If so, properly cite the sources.]

5. The cell walls of members of the Archaea differ significantly from Eubacteria. How might the microbe *Methanobacterium*, an archeon, appear after being subjected to the Gram stain? Why? [**Note:** This answer shall likely require some outside research. If so, properly cite the sources.]

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