

LAB 6: GRAM STAIN AND CAPSULE STAIN

A. THE GRAM STAIN

DISCUSSION

The Gram stain is the most widely used staining procedure in bacteriology. It is called a **differential stain** since it differentiates between Gram-positive and Gram-negative bacteria. Bacteria that stain **purple** with the Gram staining procedure are termed **Gram-positive**; those that stain **pink** are said to be **Gram-negative**. The terms positive and negative have nothing to do with electrical charge, but simply designate two distinct morphological groups of bacteria.

Gram-positive and Gram-negative bacteria stain differently because of fundamental differences in the structure of their cell walls. The bacterial cell wall serves to give the organism its size and shape as well as to prevent osmotic lysis. The material in the bacterial cell wall which confers rigidity is peptidoglycan.

In electron micrographs, the **Gram-positive cell wall** appears as a broad, dense wall 20-80 nm thick and consisting of numerous interconnecting layers of peptidoglycan (see Figs. [1A](#) and [1B](#)). Chemically, 60 to 90% of the Gram-positive cell wall is peptidoglycan. Interwoven in the cell wall of Gram-positive are teichoic acids. Teichoic acids, which extend through and beyond the rest of the cell wall, are composed of polymers of glycerol, phosphates, and the sugar alcohol ribitol. Some have a lipid attached (lipoteichoic acid). The outer surface of the peptidoglycan is studded with proteins that differ with the strain and species of the bacterium.

The **Gram-negative cell wall**, on the other hand, contains only 2-3 layers of peptidoglycan and is surrounded by an outer membrane composed of phospholipids, lipopolysaccharide, lipoprotein, and proteins (see Figs. [2A](#) and [2B](#)). Only 10% - 20% of the Gram-negative cell wall is peptidoglycan. The phospholipids are located mainly in the inner layer of the outer membrane, as are the lipoproteins that connect the outer membrane to the peptidoglycan. The lipopolysaccharides, located in the outer layer of the outer membrane, consist of a lipid portion called lipid A embedded in the membrane and a polysaccharide portion extending outward from the bacterial surface. The outer membrane also contains a number of proteins that differ with the strain and species of the bacterium.

For further information on the Gram-negative and Gram-positive cell wall, see the following Learning Objects in your Lecture Guide:

- [The Prokaryotic Cell Wall; Unit 1, Section IIB2](#)
- [The Gram-Positive Cell Wall; Unit 1, Section IIB2a](#)
- [The Gram-Negative Cell Wall; Unit 1, Section IIB2b](#)

The Gram staining procedure involves four basic steps:

1. The bacteria are first stained with the basic dye **crystal violet**. Both Gram-positive and Gram-negative bacteria become directly stained and appear purple after this step.
2. The bacteria are then treated with **Gram's iodine solution**. This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex. Both Gram-positive and Gram-negative bacteria remain purple after this step.
3. **Gram's decolorizer**, a mixture of **ethyl alcohol and acetone**, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while Gram-negative are decolorized.
4. Finally, the counterstain **safranin** (also a basic dye) is applied. Since the Gram-positive bacteria are already stained purple, they are not affected by the counterstain. Gram-negative bacteria, which are now colorless, become directly stained by the safranin. Thus, [Gram-positive appear purple](#), and [Gram-negative appear pink](#).

[Flash animation illustrating the interaction of the Gram's stain reagents at a molecular level](#)

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With the current theory behind Gram staining, it is thought that in Gram-positive bacteria, the crystal violet and iodine combine to form a larger molecule that precipitates out within the cell. The alcohol/acetone mixture then causes dehydration of the multilayered peptidoglycan, thus decreasing the space between the molecules and causing the cell wall to trap the crystal violet-iodine complex within the cell. In the case of Gram-negative bacteria, the alcohol/acetone mixture, being a lipid solvent, dissolves the outer membrane of the cell wall and may also damage the cytoplasmic membrane to which the peptidoglycan is attached. The few layers of peptidoglycan are

unable to retain the crystal violet-iodine complex and the cell is decolorized.

It is important to note that Gram-positivity (the ability to retain the purple crystal violet-iodine complex) is not an all-or-nothing phenomenon but a matter of degree. There are **several factors that could result in a Gram-positive organism staining Gram-negatively**:

1. **The method and techniques used.** Overheating during heat fixation, over decolorization with alcohol, and even too much washing with water between steps may result in Gram-positive bacteria losing the crystal violet-iodine complex.
2. **The age of the culture.** Cultures more than 24 hours old may lose their ability to retain the crystal violet-iodine complex.
3. **The organism itself.** Some Gram-positive bacteria are more able to retain the crystal violet-iodine complex than others.

Therefore, one must use very precise techniques in Gram staining and interpret the results with discretion.

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ORGANISMS

Trypticase Soy agar plate cultures of *Escherichia coli* (a small, Gram-negative bacillus) and *Staphylococcus epidermidis* (a Gram-positive coccus with a staphylococcus arrangement).

PROCEDURE (to be done individually)

1. *Escherichia coli*

a. Heat-fix a smear of *Escherichia coli* as follows:

1. Using the dropper bottle of **deionized water found in your staining rack**, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide ([see Fig. 1](#)). Alternately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

2. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it 2 - 3 times to the drop of water** until the water **becomes visibly cloudy** ([see Fig. 2](#)). A good smear with the correct amount of bacteria is essential to Gram staining.

- **Too many bacteria on the slide could result in under-decolorization; too few could lead to over-decolorization.**

3. **Incinerate the remaining bacteria on the inoculating loop.** If too much culture is added to the water, you will not see stained individual bacteria and you may not have a reliable Gram stain.

4. After the inoculating loop cools, **spread the suspension over approximately half of the slide** to form a thin film. A correctly prepared smear with the right amount of bacteria should look similar to [Fig. 3](#).

5. Allow this thin suspension to **completely air dry** ([see Fig. 4](#)). **The smear must be completely dry before the slide is heat fixed!**

6. To heat-fix the bacteria to the slide, **pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear near the opening of the microincinerator for 10 seconds** ([see Fig. 5](#)) as demonstrated by your instructor. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

b. Stain with Hucker's **crystal violet** for **one minute** ([see Fig. 6](#)). **Gently wash with water** ([see Fig. 7](#)). Shake off the excess water but **do not blot dry between steps**.

c. Stain with **Gram's iodine solution** for **one minute** ([see Fig. 8](#)) and **gently wash with water**.

d. Decolorize by picking up the slide and **letting the Gram's decolorizer run down the slide until the purple just stops flowing at the bottom of the slide** ([see Fig. 9](#)).

- **Make sure the entire smear is evenly decolorized and that you are not under-**

decolorizing or over-decolorizing.

- **Wash immediately with water.**

e. Stain with **safranin** for **one minute** ([see Fig. 10](#)). **When you wash off the excess safranin, be very careful to wash gently and briefly** as it is possible to wash out some of the safranin in the bacterium.

f. **Blot dry** ([see Fig. 11](#)) and observe using oil immersion microscopy.

2. *Staphylococcus epidermidis*

a. Heat-fix a smear of *Staphylococcus epidermidis* as follows:

1. Using the dropper bottle of **deionized water found in your staining rack**, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide ([see Fig. 1](#)). Alternately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

2. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it 2 - 3 times to the drop of water** until the water **becomes visibly cloudy** ([see Fig. 2](#)).

- **Too many bacteria on the slide could result in under-decolorization; too few could lead to over-decolorization.**

3. **Incinerate the remaining bacteria on the inoculating loop.** If too much culture is added to the water, you will not see stained individual bacteria and you may not have a reliable Gram stain.

4. After the inoculating loop cools, **spread the suspension over approximately half of the slide** to form a thin film ([see Fig. 3](#)).

5. Allow this thin suspension to **completely air dry** ([see Fig. 4](#)). **The smear must be completely dry before the slide is heat fixed!**

6. To heat-fix the bacteria to the slide, **pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear near the opening of the microincinerator for 10 seconds** ([see Fig. 5](#)) as demonstrated by your instructor. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

b. Stain with Hucker's **crystal violet** for **one minute** ([see Fig. 6](#)). **Gently wash with water** ([see Fig. 7](#)). Shake off the excess water but **do not blot dry between steps**.

c. Stain with **Gram's iodine solution** for **one minute** ([see Fig. 8](#)) and **gently wash with water**.

d. Decolorize by picking up the slide and **letting the Gram's decolorizer run down the slide until the purple just stops flowing at the bottom of the slide** ([see Fig. 9](#)).

- **Make sure the entire smear is evenly decolorized and that you are not under-decolorizing or over-decolorizing.**
- **Wash immediately with water.**

e. Stain with **safranin** for **one minute** ([see Fig. 10](#)). **When you wash off the excess safranin, be very careful to wash gently and briefly** as it is possible to wash out some of the safranin in the bacterium.

f. **Blot dry** and observe using oil immersion microscopy.

3. **Make sure you carefully pour the used dye in your staining tray into the waste dye collection container, not down the sink.**

[Animation of The Gram Stain Procedure.](#)

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[Instructions for Focusing a Microscope from Lab 1.](#)

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B. THE CAPSULE STAIN

DISCUSSION

Many bacteria secrete a slimy, viscous covering called a [capsule or glycocalyx](#). This is usually composed of polysaccharide, polypeptide, or both.

The ability to produce a capsule is an inherited property of the organism, but the capsule is not an absolutely essential cellular component. Capsules are often produced only under specific growth conditions. Even though not essential for life, capsules probably help bacteria to survive in nature. Capsules help many pathogenic and normal flora bacteria to initially **resist phagocytosis** by the host's phagocytic cells. In soil and water, capsules help prevent bacteria from being engulfed by protozoans. Capsules also help many bacteria to **adhere to surfaces** and thus resist flushing. It also enables many bacteria to form biofilms. A **biofilm** consists layers of bacterial populations adhering to host cells and embedded in a common capsular mass.

For further information on the bacterial capsules, see the following Learning Objects in your Lecture Guide:

- [The Glycocalyx \(Capsule\) and S-Layer; Unit 1, Section IIB4a](#)
- [The Ability to Resist Phagocytic Engulfment; Unit 3, Section B5b](#)

ORGANISM

Skim Milk broth culture of *Enterobacter aerogenes*. The skim milk supplies essential nutrients for capsule production and also provides a slightly stainable background.

PROCEDURE (to be done individually)

1. **Stir up the Skim Milk broth culture** with your loop and place **2-3 loops** of *Enterobacter aerogenes* on a microscope slide.
2. Using your inoculating loop, **spread the sample out to cover about one inch of the slide**.
3. Let it completely air dry. **Do not heat fix**. Capsules stick well to glass, and heat may destroy the capsule.
4. Stain with **crystal violet** for **one minute**.
5. **Wash off the excess dye with 20% copper sulfate solution**.
6. **Shake off the excess copper sulfate solution and immediately blot dry**.
7. **Observe using oil immersion microscopy**. The organism and the milk dried on the slide will pick up the purple dye while the [capsule](#) will remain colorless.
8. **Make sure you carefully pour the used dye in your staining tray into the waste dye collection container, not down the sink**.
9. Observe the demonstration [capsule stain of Streptococcus lactis](#), an encapsulated bacterium that is normal flora in milk.



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RESULTS

A. The Gram Stain



Make drawings of each bacterium on your Gram stain preparation.

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<u>Gram stain of <i>Escherichia coli</i></u>	<u>Gram stain of <i>Staphylococcus epidermidis</i></u>
Color =	Color =
Gram reaction =	Gram reaction =
Shape =	Shape =
	Arrangement =

B. The Capsule Stain

Make a drawing of your capsule stain preparation of *Enterobacter aerogenes* and the demonstration capsule stain of *Streptococcus pneumoniae*.

	
<u>Capsule stain of <i>Enterobacter aerogenes</i></u>	<u>Capsule stain of <i>Streptococcus lactis</i></u>

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PERFORMANCE OBJECTIVES LABORATORY 6

After completing this lab, the student will be able to perform the following objectives:

A. THE GRAM STAIN

DISCUSSION

1. State why the Gram stain is said to be a differential stain.
2. Describe the differences between a Gram-positive and a Gram-negative cell wall.
3. Describe a theory as to why Gram-positive bacteria retain the crystal violet-iodine complex while Gram-negatives become decolorized.
4. Describe three conditions that may result in a Gram-positive organism staining Gram-negatively.

PROCEDURE

1. State the procedure for the Gram stain.
2. Perform a Gram stain when given all the necessary materials.

RESULTS

1. Determine if a bacterium is Gram-positive or Gram-negative when microscopically viewing a Gram stain preparation and state the shape and arrangement of the organism.

B. THE CAPSULE STAIN

DISCUSSION

1. State the chemical nature and major functions of bacterial capsules.

RESULTS

1. Recognize capsules as the structures observed when microscopically viewing a capsule stain preparation.

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SELF-QUIZ

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Updated: March, 2012
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