

Smear Preparation

10

EXERCISE

The success for most staining procedures depends upon the preparation of a good **smear**. There are several goals in preparing a smear. The first goal is to cause the cells to adhere to the microscope slide so that they are not washed off during subsequent staining and washing procedures. Second, it is important to insure that shrinkage of cells does not occur during staining, otherwise distortion and artifacts can result. A third goal is to prepare thin smears because the thickness of the smear will determine if you can visualize individual cells, their arrangement, or details regarding microstructures associated with cells. Thick smears of cells with large clumps obscure details about individual cells and, furthermore, the smear can entrap stain keeping it from being removed by washing or destaining, leading to erroneous results. The procedure for making a smear is illustrated in figure 10.1.

The first step in preparing a bacteriological smear differs according to the source of the organisms. If the bacteria are growing in a liquid medium (broths, milk, saliva, urine, etc.), one starts by placing two or more loopfuls of the liquid medium directly on the slide.

From solid media such as nutrient agar, blood agar, or some part of the body, one starts by placing one or two loopfuls of water on the slide and then using an inoculating loop to disperse the organisms in the water. Bacteria growing on solid media tend to cling to each other and must be dispersed sufficiently by dilution in water; unless this is done, the smear will be too thick. *The most difficult concept for students to understand about making slides from solid media is that it takes only a very small amount of material to make a good smear.* When your instructor demonstrates this step, pay very careful attention to the amount of material that is placed on the slide.

The organisms to be used for your first slides may be from several different sources. If the plates from Exercise 6 were saved, some slides may be made from them. If they were discarded, the first slides may be made for Exercise 11, which pertains

to simple staining. Your instructor will indicate which cultures to use.

FROM LIQUID MEDIA

(Broths, saliva, milk, etc.)

If you are preparing a bacterial smear from liquid media, follow this routine, which is depicted on the left side of figure 10.1.

MATERIALS

- microscope slides
- Bunsen burner
- wire loop
- Sharpie marking pen
- slide holder (clothespin)

1. Wash a slide with soap or Bon Ami and hot water, removing all dirt and grease. Handle the clean slide by its edges.
2. Write the initials of the organism or organisms on the left-hand side of the slide with a marking pen.
3. To provide a target on which to place the organisms, make a $\frac{1}{2}$ " circle on the *bottom* side of the slide, centrally located, with a marking pen. Later on, when you become more skilled, you may wish to omit the use of this "target circle."
4. Shake the culture vigorously and transfer two loopfuls of organisms to the center of the slide over the target circle. Follow the routine for inoculations shown in figure 10.2. *Be sure to flame the loop after it has touched the slide.*

CAUTION: Be sure to cool the loop completely before inserting it into a medium. A loop that is too hot will spatter the medium and move bacteria into the air.

5. Spread the organisms over the area of the target circle.
6. Allow the slide to dry by normal evaporation of the water. Don't apply heat.

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7. After the smear has become completely dry, place the slide in a clothespin and pass it several times through the flame of a Bunsen burner. Avoid prolonged heating of the slide as it can shatter from excessive exposure to heat. The underside of the slide should feel warm to the touch.

Note that in this step one has the option of using or not using a clothespin to hold the slide. *Use the option preferred by your instructor.*

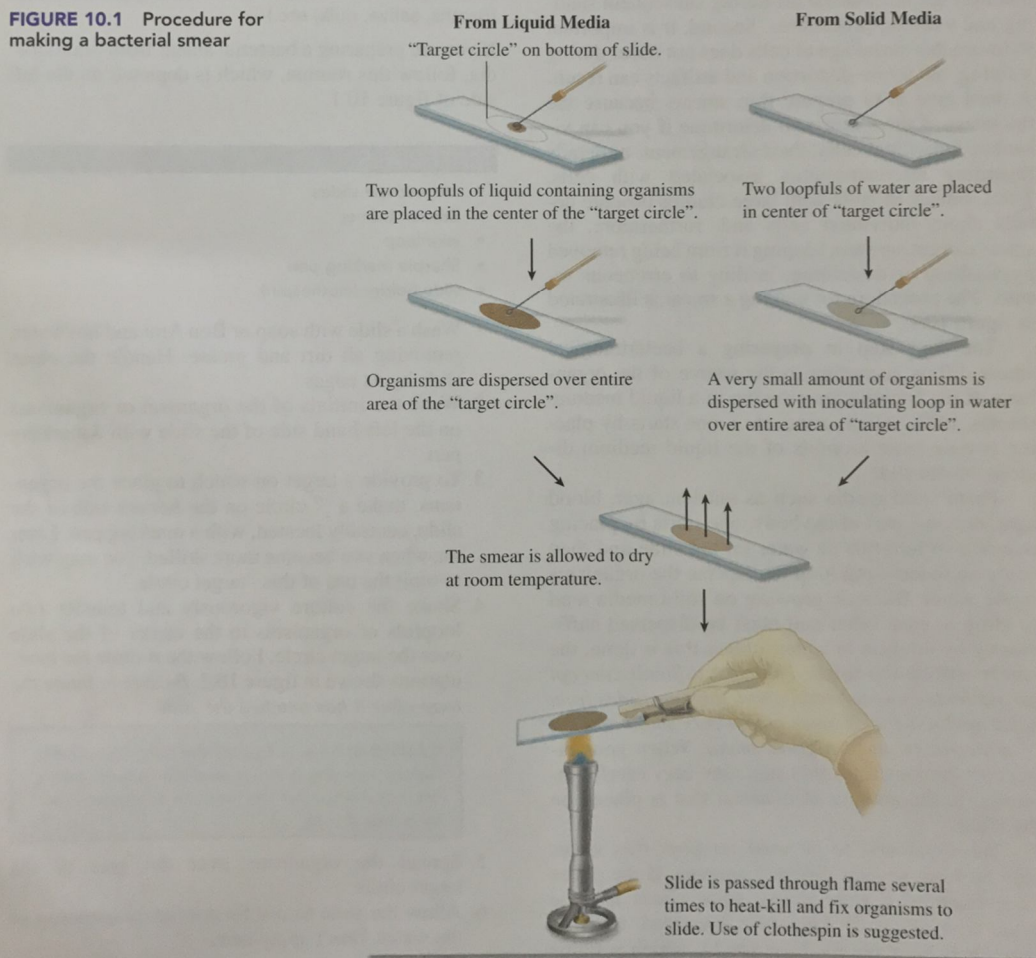
FROM SOLID MEDIA

When preparing a bacterial smear from solid media, such as nutrient agar or a part of the body, follow this routine, which is depicted on the right side of figure 10.1.

MATERIALS

- microscope slides
- inoculating needle and loop

FIGURE 10.1 Procedure for making a bacterial smear



- Sharpie marking pen
- slide holder (clothespin)
- Bunsen burner

1. Wash a slide with soap or Bon Ami and hot water, removing all dirt and grease. Handle the clean slide by its edges.

2. Write the initials of the organism or organisms on the left-hand side of the slide with a marking pen.
3. Mark a "target circle" on the bottom side of the slide with a marking pen. (See comments in step 3 on page 87.)

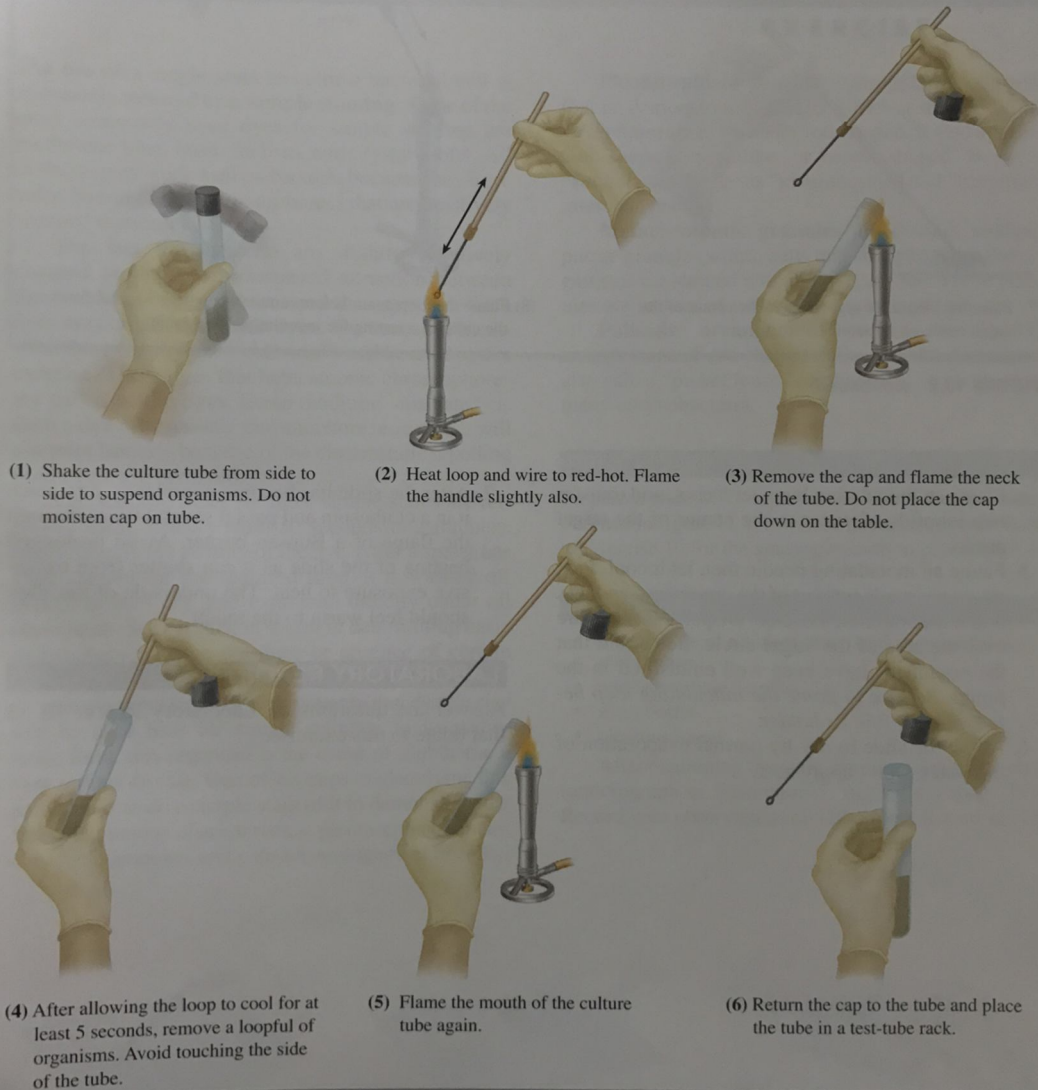
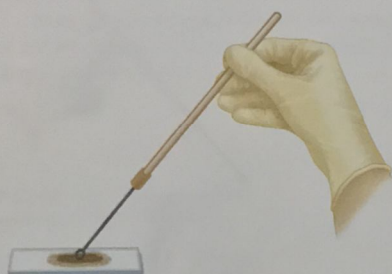


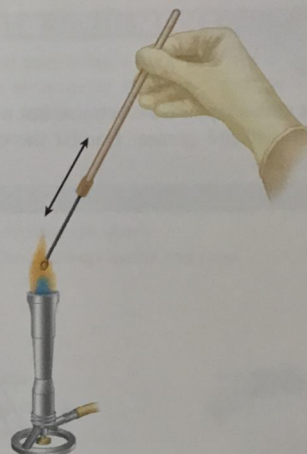
FIGURE 10.2 Aseptic procedure for organism removal

continued

EXERCISE 10 ■ Smear Preparation



(7) Place the loopful of organisms in the center of the target circle on the slide.



(8) Flame the loop again before removing another loopful from the culture or setting the inoculating loop aside.

FIGURE 10.2 (continued)

4. Flame an inoculating loop, let it cool, and transfer two loopfuls of water to the center of the target circle.
5. Flame an inoculating needle then let it cool. Pick up a *very small amount of the organisms*, and mix it into the water on the slide. Disperse the mixture over the area of the target circle. Be certain that the organisms have been well emulsified in the liquid. *Be sure to flame the inoculating loop before placing it in its holder.*
6. Allow the slide to dry by normal evaporation of the water. Don't apply heat.

7. After the slide has become completely dry, place it in a clothespin and pass it several times through the flame of a Bunsen burner. Avoid prolonged heating of the slide as it can shatter from excessive exposure to heat. The underside of the slide should feel warm to the touch.

LABORATORY REPORT

Answer the questions on Laboratory Report 10–13 that relate to this exercise.

Simple Staining

11

EXERCISE

The use of a single stain to color a bacterial cell is commonly referred to as **simple staining**. Some of the most commonly used dyes for simple staining are methylene blue, basic fuchsin, and crystal violet. All of these dyes work well on bacteria because they have color-bearing ions (*chromophores*) that are positively charged (cationic).

The fact that bacteria are slightly negatively charged produces a pronounced attraction between these cationic chromophores and the organism. Such dyes are classified as **basic dyes**. The basic dye methylene blue (methylene⁺ chloride⁻) will be used in this exercise. Those dyes that have anionic chromophores are called **acidic dyes**. Eosin (sodium⁺ eosinate⁻) is such a dye. The anionic chromophore, eosinate⁻, will not stain bacteria because of the electrostatic repelling forces that are involved.

The staining times for most simple stains are relatively short, usually from 30 seconds to 2 minutes, depending on the affinity of the dye. After a smear has been stained for the required time, it is washed off gently, blotted dry, and examined directly under oil immersion. Such a slide is useful in determining basic morphology and the presence or absence of certain kinds of granules.

An avirulent strain of *Corynebacterium diphtheriae* will be used here for simple staining. In its pathogenic form, this organism is the cause of diphtheria, a very serious disease. One of the steps in identifying this pathogen is to do a simple stain of it to demonstrate the following unique characteristics: pleomorphism, metachromatic granules, and palisade arrangement of cells.

Pleomorphism pertains to irregularity of form: that is, demonstrating several different shapes. While *C. diphtheriae* is basically rod-shaped, it also appears club-shaped, spermlike, or needle-shaped. *Bergey's Manual* uses the terms "pleomorphic" and "irregular" interchangeably.

Metachromatic granules are distinct reddish-purple granules within cells that show up when the organisms are stained with methylene blue. These granules are masses of *volutin*, a polymetaphosphate.

Palisade arrangement pertains to parallel arrangement of rod-shaped cells. This characteristic, also called "picket fence" arrangement, is common to many corynebacteria.

PROCEDURE

Prepare a slide of *C. diphtheriae*, using the procedure outlined in figure 11.1. It will be necessary to refer back to Exercise 10 for the smear preparation procedure.

MATERIALS

- slant culture of avirulent strain of *Corynebacterium diphtheriae*
- methylene blue (Loeffler's)
- wash bottle
- bibulous paper

After examining the slide, compare it with the photomicrograph in illustration 1, figure 14.4 (page 103). Record your observations on Laboratory Report 10–13.

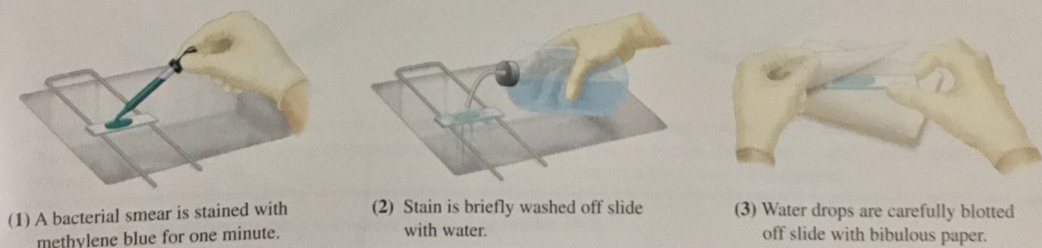


FIGURE 11.1 Procedure for simple staining

Negative Staining

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EXERCISE

Another stain that can be used to study the morphology of bacterial cells is the **negative stain**. Negative stains such as nigrosin and india ink do not penetrate the bacterial cell but rather cause the background area around a cell to be opaque or dark. Cells appear as transparent objects against the dark background. The negative stain reveals the shape of the cell and extracellular features such as capsules. The method consists of mixing the organism with a small amount of stain and spreading a very thin film over the surface of the slide. For the negative stain, cells are not heat fixed prior to the application of the negative stain.

The method can be useful for determining cell morphology and size of the cells. Because no heat fixation was performed, no shrinkage of the cells has occurred and size determinations are more accurate than those determined on fixed material. Avoiding heat fixation is also important if the capsule surrounding bacterial cells is to be observed because heating severely shrinks this structure. The negative stain is also useful for observing spirochaetes that do not stain readily with ordinary dyes.

THREE METHODS

Negative staining can be done by one of three different methods. Figure 12.1 illustrates the more commonly used method in which the organisms are mixed in a drop of nigrosin and spread over the slide with another slide. The goal is to produce a smear that is thick at one end and feather-thin at the other end. Somewhere between the too thick and too thin areas will be an ideal spot to study the organisms.

Figure 12.2 illustrates a second method, in which organisms are mixed in only a loopful of nigrosin instead of a full drop. In this method, the organisms are spread over a smaller area in the center of the slide with an inoculating needle. No spreader slide is used in this method.

The third procedure (Woeste-Demchick's method), which is not illustrated here, involves applying ink to a conventional smear with a black felt-tip marking pen. If this method is used, it should be done on a smear prepared in the manner described in exercise 13. Simply put, the technique involves applying a *single coat* of marking-pen ink over a smear.

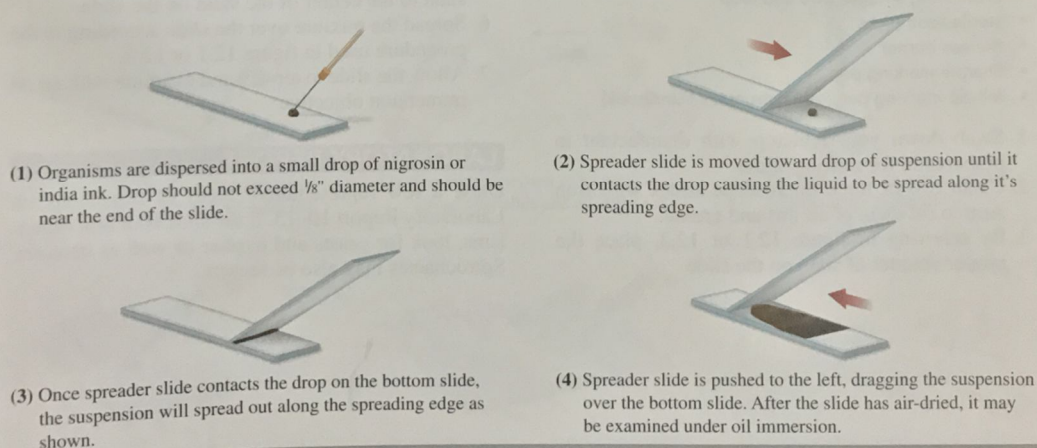


FIGURE 12.1 Negative staining technique, using a spreader slide

EXERCISE 12 ■ Negative Staining

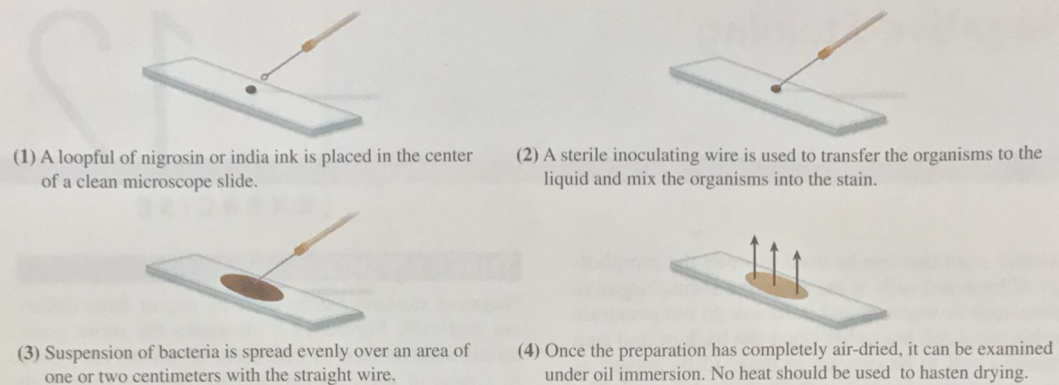


FIGURE 12.2 A second method for negative staining

Note in the procedure below that slides may be made from organisms between your teeth or from specific bacterial cultures. Your instructor will indicate which method or methods you should use and demonstrate some basic aseptic techniques. Various options are provided here to ensure success.

MATERIALS

- microscope slides (with polished edges)
 - nigrosin solution or india ink
 - slant cultures of *S. aureus* and *B. megaterium*
 - inoculating straight wire and loop
 - sterile toothpicks
 - Bunsen burner
 - Sharpie marking pen
 - felt-tip marking pen (see Instructor's Handbook)
1. Swab down your tabletop with disinfectant in preparation for making slides.
 2. Clean two or three microscope slides with Bon Ami to rid them of all dirt and grease.
 3. By referring to figure 12.1 or 12.2, place the proper amount of stain on the slide.

4. **Oral Organisms:** Remove a small amount of material from between your teeth with a sterile straight toothpick and mix it into the stain on the slide. Be sure to break up any clumps of organisms with the toothpick or a sterile inoculating loop. When using a loop, *be sure to flame it first to make it sterile.*

CAUTION If you use a toothpick, discard it into a beaker of disinfectant.

5. **From Cultures:** With a *sterile* straight wire, transfer a very small amount of bacteria from the slant to the center of the stain on the slide.
6. Spread the mixture over the slide according to the procedure used in figure 12.1 or 12.2.
7. Allow the slide to air-dry and examine with an oil immersion objective.

LABORATORY REPORT

Draw a few representative types of organisms on Laboratory Report 10–13. If the slide is of oral organisms, look for yeasts and hyphae as well as bacteria. Spirochaetes may also be present.

Capsular Staining

13

EXERCISE

Some bacterial cells are surrounded by an extracellular slime layer called a **capsule** or **glycocalyx**. This structure can play a protective role for certain pathogenic bacteria such as *Streptococcus pneumoniae*. The capsule prevents phagocytic white blood cells from engulfing and destroying this bacterial pathogen, enabling the organism to invade the lungs and cause pneumonia. The capsule is also a means for many bacteria to attach to solid surfaces in the environment. For example, *Streptococcus mutans* can attach to the surface of a tooth by its capsular material resulting in the formation of dental plaque, which contributes to the process of tooth decay in humans. Most capsules are usually composed of polysaccharides but in some cases a capsule can consist of polypeptides with unique amino acids. Evidence supports the view that probably all bacterial cells have some amount of slime layer, but in most cases the amount is not enough to be readily discernible.

Staining of the bacterial capsule cannot be accomplished by ordinary staining procedures. If

smears are heat fixed prior to staining, the capsule shrinks or is destroyed and cannot be seen in stains. If the cells are not heat fixed to the slide, they can wash off during washing procedures. However, the capsule can be demonstrated by combining the methods for the simple stain and the negative stain as shown in figure 13.1. You will use this method to stain the capsule of *Klebsiella pneumoniae*.

MATERIALS

- 36–48 hour milk culture of *Klebsiella pneumoniae*
- india ink
- crystal violet

Observation Examine the slide under oil immersion and compare your slide with illustration 2, figure 14.4 on page 103. Record your results on Laboratory Report 10–13.

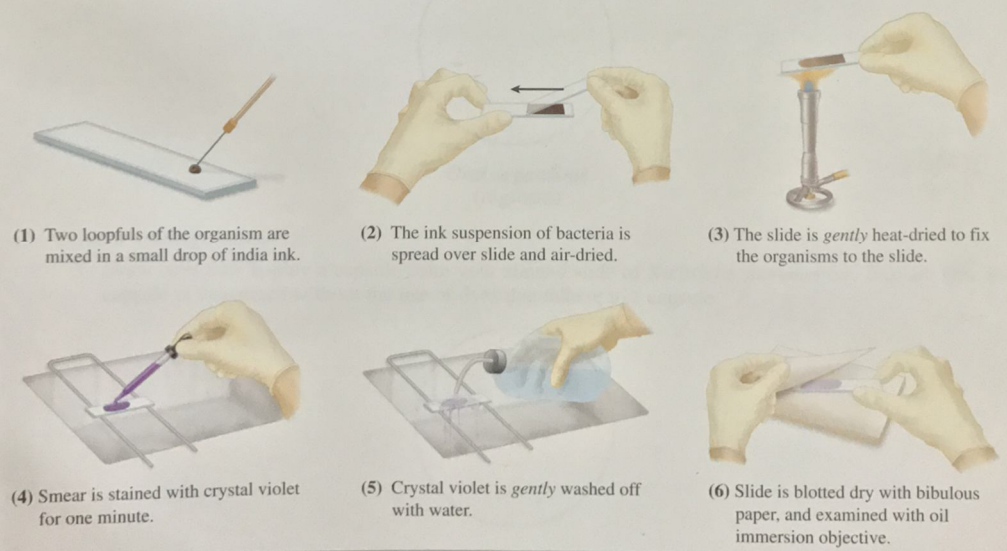


FIGURE 13.1 Procedure for demonstration of a capsule

Gram Staining

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EXERCISE

In 1884, the Danish physician Christian Gram was trying to develop a staining procedure that would differentiate bacterial cells from eukaryotic nuclei in stained tissue samples. Although Gram was not completely successful in developing a tissue stain, what resulted from his work is the most important stain in bacteriology, the Gram stain. This technique separates bacteria into two groups: gram-positive and gram-negative bacteria. The procedure is based on the fact that gram-positive bacteria retain a crystal violet-iodine complex through decolorization with alcohol or acetone. Gram-positive bacteria appear as purple when viewed by microscopy. In contrast, alcohol or acetone removes the crystal violet-iodine complex from gram-negative bacteria. These bacteria must, therefore, be counterstained with a red dye, safranin, after the decolorization step in order to be visualized by microscopy. Hence, gram-negative bacteria appear as red cells when viewed by microscopy.

Figure 14.1 illustrates the appearance of cells after each step in the Gram-stain procedure. Note that initially both gram-positive and gram-negative cells are stained by the **primary stain**, crystal violet. In the second step of the procedure, Gram's iodine is added to the smear. Iodine is a **mordant** that complexes with the crystal violet and forms an insoluble complex in gram-positive cells. At this point, both types of cells will still appear as purple. The dye-mordant complex is not removed from gram-positive bacteria but is leached from gram-negative cells by the alcohol or acetone in the **decolorization** step. After decoloriza-

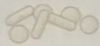
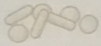
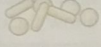
Reagent	Gram positive	Gram negative
None (Heat-fixed cells)		
Crystal Violet (20 seconds)		
Gram's Iodine (1 minute)		
Ethyl Alcohol (10–20 seconds)		
Safranin (1 minute)		

FIGURE 14.1 Color changes that occur at each step in the gram-staining process

tion, gram-positive cells are purple but gram-negative cells are colorless. In the final step, a **counterstain**, safranin, is applied, which stains the colorless gram-negative cells. The appearance of the gram-positive cells is unchanged because the crystal violet is a much more intense stain than safranin.

The mechanism for how the Gram stain works is related to chemical differences in the cell walls of gram-positive and gram-negative bacteria (figure 14.2). When viewed by electron microscopy, gram-positive cells have a thick layer of **peptidoglycan** that comprises the cell wall of these organisms. In contrast, the cell wall in gram-negative cells consists

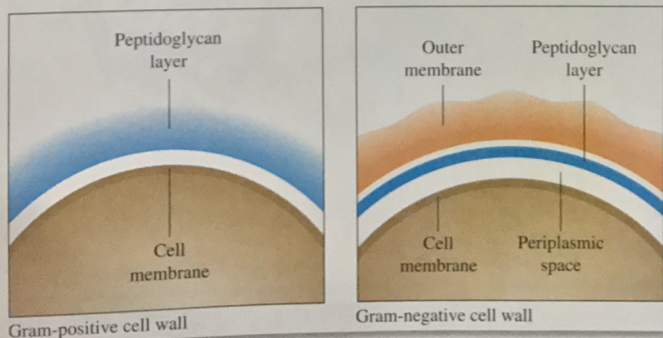


FIGURE 14.2 Comparison of gram-positive and gram-negative cell walls

EXERCISE 14 ■ Gram Staining

of an outer membrane that covers a much thinner layer of peptidoglycan. It is these significant differences in structure that probably determines whether the dye-mordant complex is removed from the gram-negative cell or remains associated with the gram-positive cell.

Of all the staining techniques you will use in microbiology, the Gram stain is one of the most important. It will be critical in identifying your unknown bacteria and you will use it routinely in many exercises in this manual. Although this technique seems quite simple, performing it with a high degree of reliability requires some practice and experience. Several factors can affect the outcome of the procedure:

1. It is important to use cultures that are 16–18 hours old. Gram-positive cultures older than this can convert to gram-variable or gram-negative and give erroneous results. (It is important to note that gram-negative bacteria never convert to gram-positive.)
2. It is critical to prepare thin smears. Thin smears allow the observation of individual cells and any arrangement in which the cells occur. Furthermore, the thickness of your smears can affect decolorization. Thick smears can entrap the primary

stain, which is not removed by alcohol or acetone. Cells that occur in the entrapped stain can appear gram-positive leading to erroneous results.

3. Decolorization is the most critical step in the Gram-stain procedure. If the destaining reagent is overapplied, the dye-mordant complex can eventually be removed from gram-positive cells, converting them to gram-negative cells.

During this laboratory period, you will be provided an opportunity to stain several different kinds of bacteria to see if you can achieve the degree of success that is required. Remember, if you don't master this technique now, you will have difficulty with your unknowns later.

STAINING PROCEDURE

MATERIALS

- slides with heat-fixed smears
- Gram-staining kit and wash bottle
- bibulous paper

1. Cover the smear with **crystal violet** and let stand for *20 seconds* (see figure 14.3).

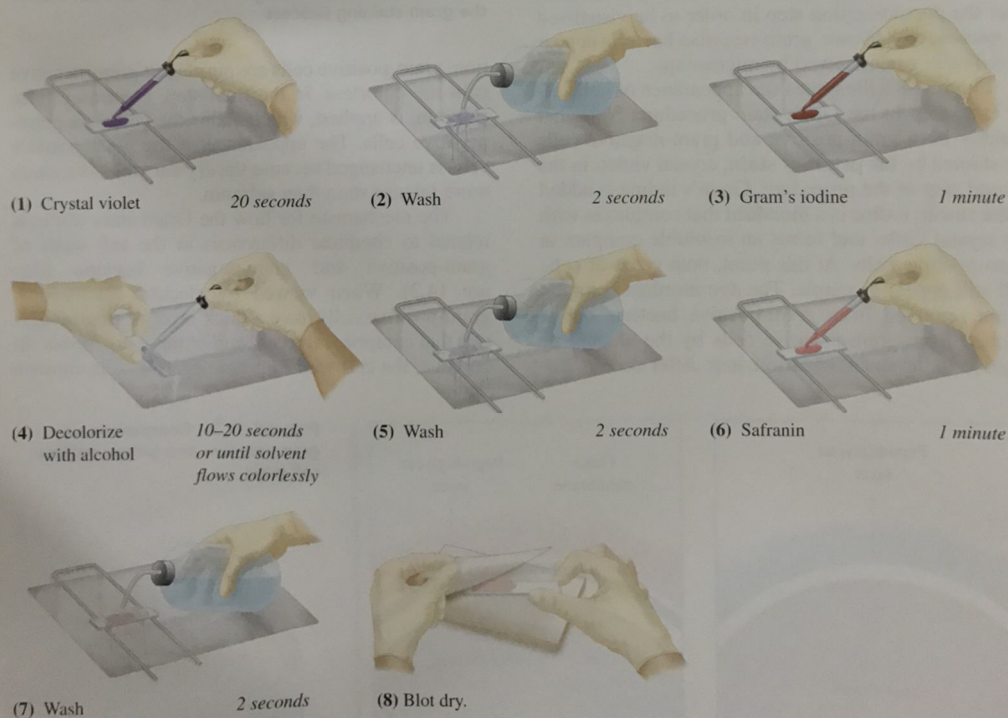


FIGURE 14.3 The Gram-staining procedure

- Briefly wash off the stain, using a wash bottle of distilled water. Drain off excess water.
- Cover the smear with **Gram's iodine** solution and let it stand for *one minute*. (Your instructor may prefer only 30 seconds for this step.)
- Wash off the Gram's iodine. Hold the slide at a 45-degree angle and allow the 95% alcohol to flow down the surface of the slide. Do this until the alcohol is colorless as it flows from the smear down the surface of the slide. *This should take no more than 20 seconds for properly prepared smears.* Note: thick smears can take longer than 20 seconds for decolorization.
- Stop decolorization by washing the slide with a gentle stream of water.
- Cover the smear with **safranin** for 1 minute.
- Wash gently for a few seconds, blot dry with bibulous paper, and air-dry.
- Examine the slide under oil immersion.

ASSIGNMENTS

The organisms that will be used here for Gram staining represent a diversity of form and staining characteristics. Some of the rods and cocci are gram-positive; others are gram-negative. One rod-shaped

organism is a spore-former and another is acid-fast. The challenge here is to make Gram-stained slides of various combinations that reveal their differences.

MATERIALS

- broth cultures of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Moraxella (Branhamella) catarrhalis*
- nutrient agar slant cultures of *Bacillus megaterium* and *Mycobacterium smegmatis*

Mixed Organisms I (Triple Smear Practice Slides) Prepare three slides with three smears on each slide. On the left portion of each slide make a smear of *Staphylococcus aureus*. On the right portion of each slide make a smear of *Pseudomonas aeruginosa*. In the middle of the slide make a smear that is a mixture of both organisms, using two loopfuls of each organism. **Be sure to flame the loop sufficiently to avoid contaminating cultures.**

Gram stain one slide first, saving the other two for later. Examine the center smear. If done properly, you should see purple cocci and pink rods as shown in illustration 3, figure 14.4.

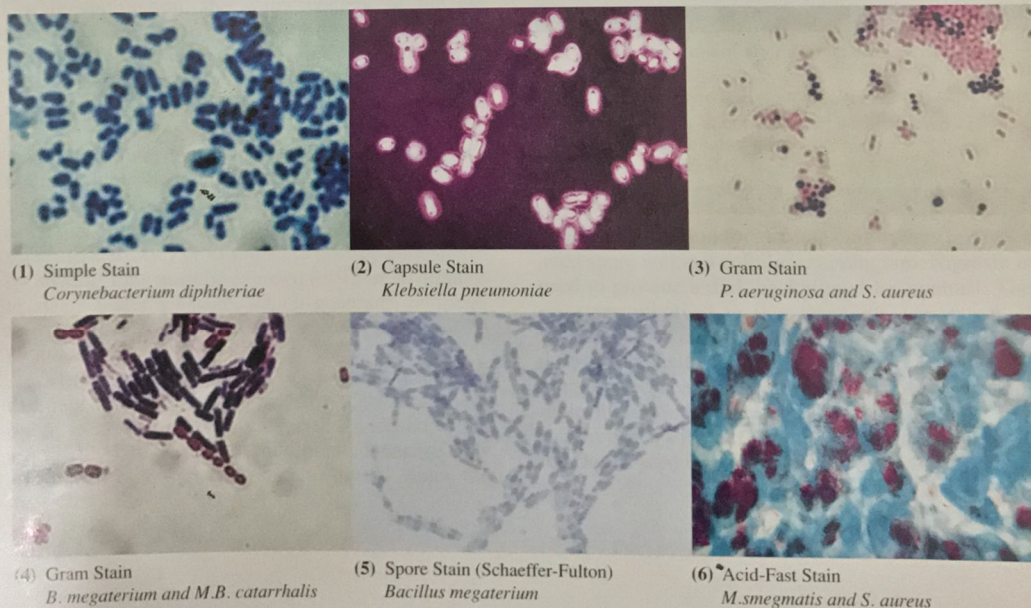


FIGURE 14.4 Photomicrographs of representative staining techniques (8000x)

(2) © Science VU/Visuals Unlimited (5) Courtesy of Lansing Prescott (6) © Science VU/Visuals Unlimited

Spore Staining: Two Methods

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EXERCISE

When species of bacteria belonging to the genera *Bacillus* and *Clostridia* exhaust essential nutrients, they undergo a complex developmental cycle that produces resting stages called **endospores**. Endospores allow these bacteria to survive environmental conditions that are not favorable for growth. If nutrients once again become available, the endospore can go through the process of germination to form a new vegetative cell and growth will resume. Endospores are very dehydrated structures that are not actively metabolizing. Furthermore, they are resistant to heat, radiation, acids, and many chemicals, such as disinfectants, that normally harm or kill vegetative cells. Their resistance is due in part to the fact that they have a thick protein coat, or **exosporium**, that forms a protective barrier around the spore. Heat resistance is associated with the water content of endospores. The higher the water content of an endospore, the less heat resistant the endospore will be. During sporulation, the water content of the endospore is reduced to 10–30% of the vegetative cell. This results because calcium ions complex with spore-specific proteins and a chemical, dipicolinic acid. The latter compound is not found in vegetative cells. This complex forms a gel that controls the amount of water that can enter the endospore, thus maintaining its dehydrated state.

Since endospores are not easily destroyed by heat or chemicals, they define the conditions necessary to establish sterility. For example, to destroy endospores by heating, they must be exposed for 15 to 20 minutes to steam under pressure, which generates temperatures of 121°C. Such conditions are produced in an **autoclave**.

The resistant properties of endospores also mean that they are not easily penetrated by stains. For example in exercise 14, you observed that endospores did not readily Gram stain. If endospore-containing cells are stained by basic stains such as crystal violet, the

spores appear as unstained areas in the vegetative cell. However, if heat is applied while staining with malachite green, the stain penetrates the endospore and becomes entrapped in the endospore. The malachite green is not removed by subsequent washing with decolorizing agents or water. In this instance, heat is acting as mordant to facilitate the uptake of the stain.

SCHAEFFER-FULTON METHOD

The Schaeffer-Fulton method, which is depicted in figure 15.1, utilizes malachite green to stain the endospore and safranin to stain the vegetative portion of the cell. Utilizing this technique, a properly stained spore-former will have a green endospore contained in a pink sporangium. Illustration 5, figure 14.4, on page 103 reveals what such a slide looks like under oil immersion.

After preparing a smear of *Bacillus megaterium*, follow the steps outlined in figure 15.1 to stain the spores.

MATERIALS

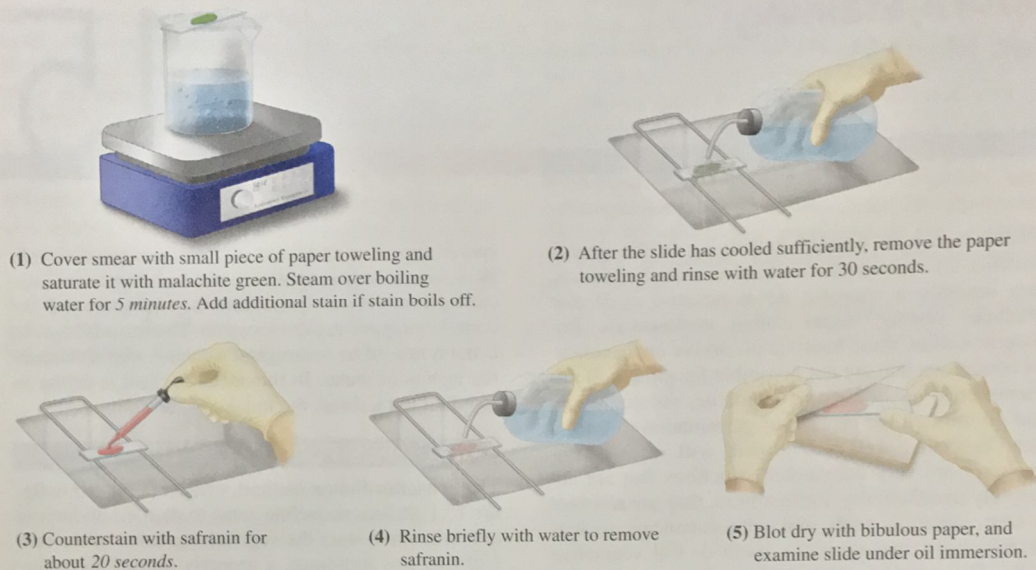
- 24–36 hour nutrient agar slant culture of *Bacillus megaterium*
- electric hot plate and small beaker (25 ml)
- spore-staining kit consisting of a bottle each of 5% malachite green and safranin

DORNER METHOD

The Dorner method for staining endospores produces a red spore within a colorless sporangium. Nigrosin is used to provide a dark background for contrast. The six steps involved in this technique are shown in figure 15.2. Although both the sporangium and endospore are stained during boiling in step 3, the sporangium is decolorized by the diffusion of safranin molecules into the nigrosine.

Prepare a slide of *Bacillus megaterium* that utilizes the Dorner method. Follow the steps in figure 15.2.

EXERCISE 15 ■ Spore Staining



(1) Cover smear with small piece of paper toweling and saturate it with malachite green. Steam over boiling water for 5 minutes. Add additional stain if stain boils off.

(2) After the slide has cooled sufficiently, remove the paper toweling and rinse with water for 30 seconds.

(3) Counterstain with safranin for about 20 seconds.

(4) Rinse briefly with water to remove safranin.

(5) Blot dry with bibulous paper, and examine slide under oil immersion.

FIGURE 15.1 The Schaeffer-Fulton spore stain method

MATERIALS

- nigrosin
- electric hot plate and small beaker (25 ml)
- small test tube (10 × 75 mm size)
- test-tube holder
- 24–36 hour nutrient agar slant culture of *Bacillus megaterium*

QUICK SPORE STAIN

A variation on the Schaeffer-Fulton method is a quick method that uses the same stains.

MATERIALS

- *Bacillus subtilis* slant cultures, older than 36 hours
- malachite green stain
- safranin stain
- staining racks
- clothespins

PROCEDURE

1. Prepare a smear of the organism and allow it to air-dry.
2. Grasp the slide with the air-dried smear with a clothespin and pass it through a Bunsen burner flame 10 times. Be careful not to overdo the heating as the slide can break.
3. Immediately flood the smear with malachite green and allow to stand for 5 minutes.
4. Wash the smear with a gentle stream of water.
5. Stain with safranin for 45 seconds. Spores will be green and the vegetative cell will be red.

LABORATORY REPORT

After examining the organisms under oil immersion, draw a few cells in the appropriate circles in Laboratory Report 14–16.

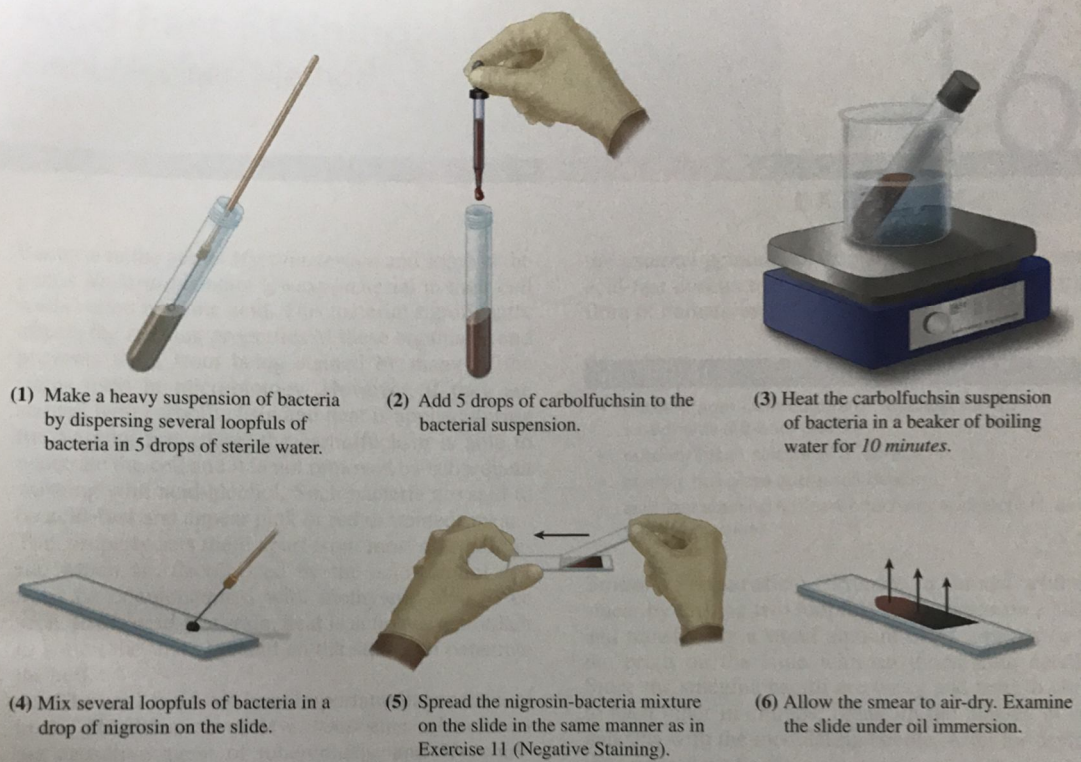


FIGURE 15.2 The Dorner spore stain method

Acid-Fast Staining: Ziehl-Neelsen Method

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EXERCISE

Bacteria in the genus *Mycobacterium* and some in the genus *Nocardia* contain a waxy material in their cell walls called **mycolic acid**. This material significantly affects the staining properties of these organisms and prevents them from being stained by many of the stains used in microbiology. However, if they are stained with carbolfuchsin and heat is applied during the staining procedure, the carbolfuchsin is able to penetrate the cell and it is not removed by subsequent washing with acid-alcohol. Such bacteria are said to be **acid-fast** and appear pink or red in stained smears. This property sets them apart from most other bacteria, which are decolorized by the acid-alcohol and must be counterstained with methylene blue to be seen. In the acid-fast stain, heat is acting as a mordant to soften the mycolic acid so the stain can penetrate the cell.

The acid-fast stain is an important diagnostic tool in the identification of *Mycobacterium tuberculosis* the causative agent of tuberculosis, and *Mycobacterium leprae*, the bacterium that causes leprosy in humans. When the stain is used in the diagnosis of these diseases, the mycobacteria appear as red rods whereas tissue cells and non-acid-fast bacteria are stained blue. An example of an acid-fast stain is seen in photo 6 in figure 14.4.

In the following exercise, you will prepare an acid-fast stain of a mixture of *Mycobacterium smegmatis* and *Staphylococcus aureus*. *M. smegmatis* is a nonpathogenic acid-fast rod that occurs in soil and on

the external genitalia of humans. *S. aureus* is a non-acid-fast coccus that can also be part of the normal flora of humans as well as a potential pathogen.

MATERIALS

- nutrient agar slant culture of *Mycobacterium smegmatis* (48-hour culture)
- nutrient broth culture of *S. aureus*
- electric hot plate and small beaker
- acid-fast staining kit (carbolfuchsin, acid-alcohol, and methylene blue)

Smear Preparation Prepare a mixed culture smear by placing two loopfuls of *S. aureus* on a slide and transferring a small amount of *M. smegmatis* to the broth on the slide with an inoculating needle. Since the smegma bacilli are waxy and tend to cling to each other in clumps, break up the masses of organisms with the inoculating needle. After air-drying the smear, heat-fix it.

Staining Follow the staining procedure outlined in figure 16.1.

Examination Examine under oil immersion and compare your slide with illustration 6, figure 14.4.

Laboratory Report Record your results in Laboratory Report 14–16.