Exercise 5

EXERCISE 5

Gram Staining

Objectives

After completing this exercise, you should be able to:

1. Explain the rationale and procedure for the Gram stain.
2. Perform and interpret Gram stains.

Background

The Gram stain is a useful stain for identifying and classifying bacteria. The **Gram stain** is a differential stain that allows you to classify bacteria as either gram-positive or gram-negative. The Gram-staining technique was discovered by Hans Christian Gram in 1884, when he attempted to stain cells and found that some lost their color when excess stain was washed off.

The staining technique consists of the following steps:

1. Apply **primary stain** (crystal violet). All bacteria are stained purple by this basic dye.
2. Apply **mordant** (Gram's iodine). The iodine combines with the crystal violet in the cell to form a crystal violet–iodine complex (CV–I).
3. Apply **decolorizing agent** (ethyl alcohol or ethyl alcohol–acetone). The primary stain is washed out (decolorized) of some bacteria, while others are unaffected.
4. Apply **secondary stain** or **counterstain** (safranin). This basic dye stains the decolorized bacteria red.

The most important determining factor in the procedure is that bacteria differ in their *rate* of decolorization. Those that decolorize easily are referred to as **gram-negative,** whereas those that decolorize slowly and retain the primary stain are called **gram-positive.**

Bacteria stain differently because of chemical and physical differences in their cell walls. Crystal violet is picked up by the cell. Iodine reacts with the dye in the cytoplasm to form a CV–I that is larger than the crystal violet that entered the cell. The CV–I cannot be washed out of gram-positive cells. In gram-negative cells, the decolorizing agent dissolves the outer lipopolysaccharide layer, and the CV–I washes out through the thin layer of peptidoglycan.

The Gram stain is most consistent when done on young cultures of bacteria (less than 24 hours old). When bacteria die, their cell walls degrade and may not retain the primary stain, giving inaccurate results. Because Gram staining is usually the first step in identifying bacteria, the procedure should be memorized.

Materials

Gram-staining reagents:

- Crystal violet
- Gram's iodine
- Ethyl alcohol
- Safranin

Wash bottle of distilled water

Slides (3)

Cultures

Staphylococcus epidermidis

Escherichia coli

Bacillus subtilis

Techniques Required

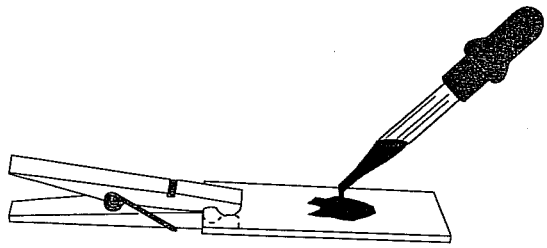
Compound light microscopy, Exercise 1

Smear preparation, Exercise 3

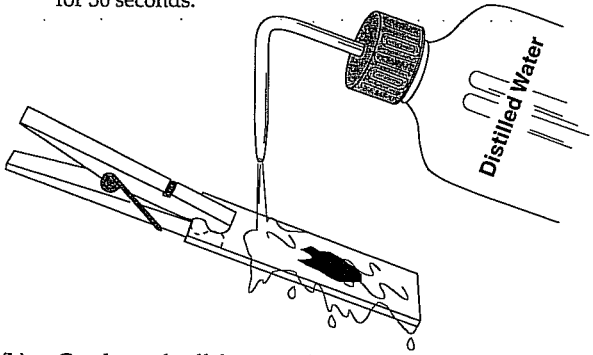
Simple staining, Exercise 3

Procedure (Figure 5.1)

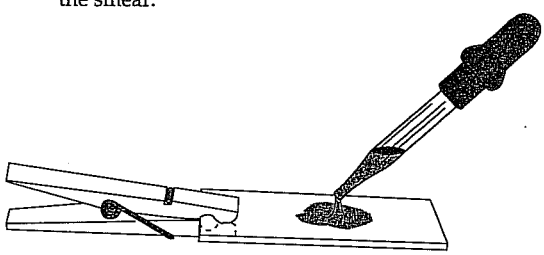
1. Prepare and fix smears (Figure 3.1). Clean the slides well, and make a circle on each slide with a marker. Label each slide for one of the cultures.
2. Prepare a Gram stain of one smear. Use a clothespin or slide rack to hold the slides.
   a. Cover the smear with crystal violet and leave it for 30 seconds (Figure 5.1a).
   b. Wash the slide carefully with distilled water from a wash bottle. Do not squirt water directly onto the smear (Figure 5.1b).
   c. Cover the smear with Gram's iodine for 10 seconds (Figure 5.1c).



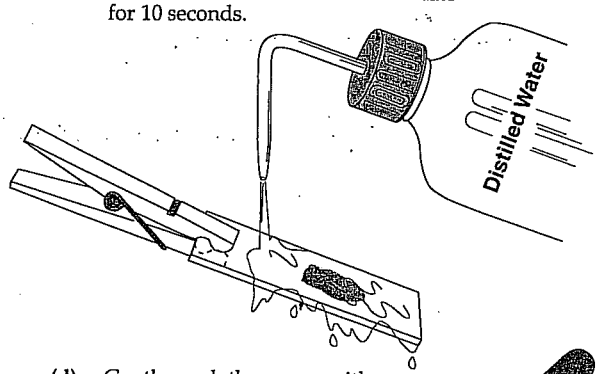
(a) Cover the smear with crystal violet for 30 seconds.



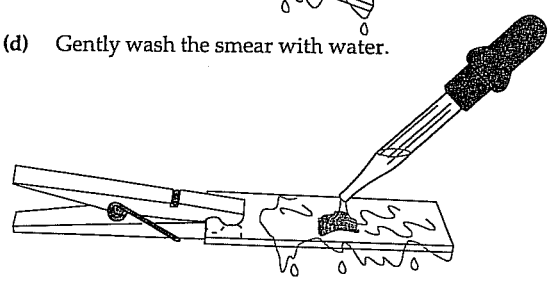
(b) Gently wash off the crystal violet with water by squirting the water so it runs through the smear.



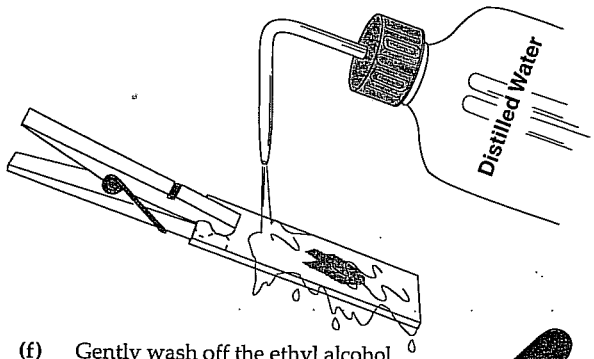
(c) Cover the smear with Gram's iodine for 10 seconds.



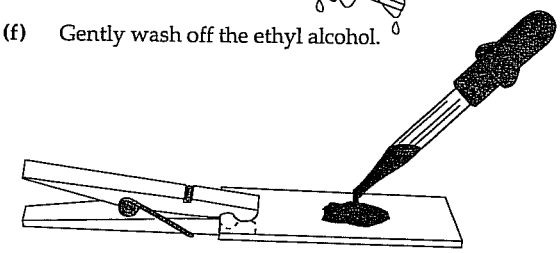
(d) Gently wash the smear with water.



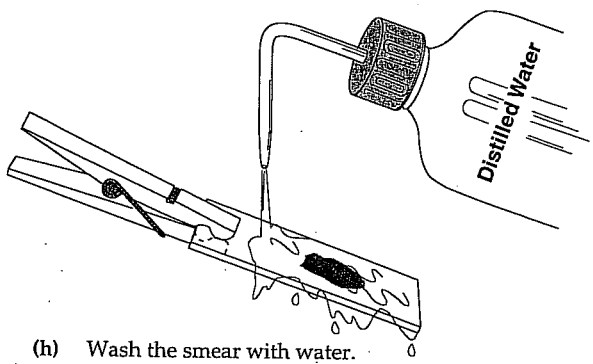
(e) Decolorize it with ethyl alcohol.



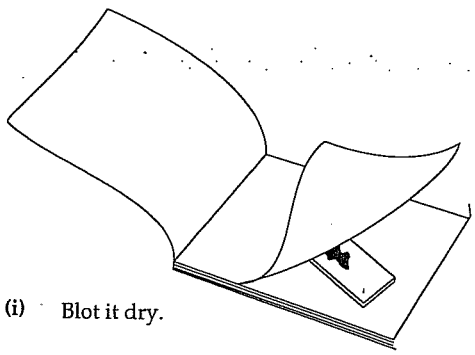
(f) Gently wash off the ethyl alcohol.



(g) Cover the smear with safranin for 30 seconds.



(h) Wash the smear with water.



(i) Blot it dry.

Figure 5.1
The Gram stain.

d. Wash off the iodine by tilting the slide and squirting water above the smear so that the water runs over the smear (Figure 5.1d).

e. Decolorize it with 95% ethyl alcohol (Figure 5.1e). Let the alcohol run through the smear until no large amounts of purple wash out (usually 10 to 20 seconds). The degree of decolorizing depends on the thickness of the smear. This is a critical step. *Do not overdecolorize*. However, experience is the only way you will be able to determine how long to decolorize. Very thick smears will give inaccurate results. Why? _____

_____

f. Immediately wash gently with distilled water (Figure 5.1f). Why? _____

g. Add safranin for 30 seconds (Figure 5.1g).

h. Wash the slide with distilled water and blot it dry with a paper towel or absorbent paper (Figure 5.1h and i).

3. Repeat step 2 to stain your remaining slides.

4. Examine the stained slides microscopically, using the low, high-dry, and oil immersion objectives. Put the oil directly on the smear. Record your observations. (See Color Plates I.2 and I.3.) Do they agree with those given in your textbook? _____ If not, try to determine why. Some common sources of Gram-staining errors are the following:
   a. The loop was too hot.
   b. Excessive heat was applied during heat fixing.
   c. The decolorizing agent (ethyl alcohol) was left on the smear too long.
   d. The culture was too old.
   e. The smear was too thick.

Exercise 5

Gram Staining

LABORATORY REPORT

NAME _____

DATE _____

LAB SECTION _____

Purpose

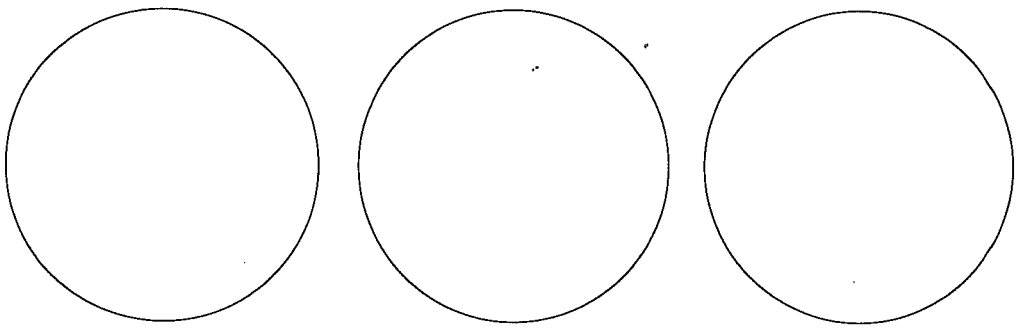
_____

_____

_____

Data

Sketch a few bacteria (oil immersion objective lens).



Bacteria:	*Staphylococcus epidermidis*	*Bacillus subtilis*	*Escherichia coli*
Total magnification:	___×	___×	___×
Morphology and arrangement:	_____	_____	_____
Color:	_____	_____	_____
Gram reaction:	_____	_____	_____

Which organism is the largest? _____ The smallest? _____

Questions

1. Did your results agree with the information in your textbook? _____ If not, why not?

   _____

   _____

2. Why will gram-positive cells more than 24 hours old stain gram-negative? _____

   _____

3. Can iodine be added before the primary stain in a Gram stain? _____

_____

_____

4. List the steps of the Gram-staining procedure in order (omit washings), and fill in the color of gram-positive cells and gram-negative cells after each step.

Step	Chemical	Appearance	
		Gram-Positive Cells	Gram-Negative Cells
1			
2			
3			
4			

5. Which step can be omitted without affecting determination of the Gram reaction? _____

Critical Thinking

1. Suppose you performed a Gram stain on a sample from a pure culture of bacteria and observed a field of red and purple cocci. Adjacent cells were not always the same color. What do you conclude?

2. Suppose you are viewing a Gram-stained field of red rods and purple cocci through the microscope. What do you conclude?

3. Considering you can't identify bacteria from a Gram stain, why might a physician perform a Gram stain on a sample before prescribing an antibiotic?

4. If you performed a Gram stain on human cells, what would happen?