

# LAB 2: Staining and Streaking

Protocols for Simple stain, Gram Stain, Streak Plate Technique and Culture Maintenance

## Lab 2a: INTRODUCTION To Staining

Live specimens are difficult to see with the bright field microscope. The contrast between a cell, which is *primarily* water, and the background, which *is* water, is poor. Staining is used to increase contrast and can be employed to provide information about the chemistry of a specimen.

Stains, or dyes, are salts in which one of the ions is colored. In a basic stain, the color is in the positively charged ion. In an acidic stain the color is in the negatively charged ion. Bacterial surfaces have a slight negative charge. Thus, there is an affinity between a positively charged color ion and the negatively charged bacterial cell. In the **Direct or Positive Staining Procedure** a cell takes up a positively charged dye and becomes stained. Methylene blue, crystal violet, and safranin, are all basic dyes. In the **Indirect or Negative Staining Procedure**, a cell is immersed in a negatively charged dye. As the cell will repel the dye, the cell appears clear in a background of color. Nigrosine is an example of an acidic stain.

Staining procedures that use *one* dye to increase contrast between specimen and background are **simple** staining procedures. **Complex** staining procedures employ a *series* of stains and chemical reagents to increase contrast and reveal information about the specimen. Any staining procedure that allows differentiation of one type of bacterium from another is a **differential** staining procedure.

For all staining procedures, it is first required that cells be **fixed** to the slide. In light microscopy fixing is completed through heating. In electron microscopy, fixation is accomplished using chemical fixatives. All fixatives kill cells. But their function in microscopy is to preserve bacterial structures while enhancing adherence of cells to the slide.

The **Gram stain**, the most widely used staining procedure in bacteriology, is a complex and differential staining procedure. Through a series of staining and decolorization steps, Bacteria are differentiated according to cell wall composition. **Gram-positive** Bacteria 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 stain is not used for Archeae or Eukaryotes as both lack peptidoglycan.

In the Gram stain, after heat fixing cells are stained with the first or **primary** stain, crystal violet. Both Gram-negative and Gram-positive cells bind this initial positive stain which forms large complexes inside the cell wall. After washing, a **mordant**, Gram's iodine, is added. Mordants increase the binding affinity of stains. Iodine binds with the crystal violet to form large CV-I<sub>2</sub> complexes within the cell wall. A **decolorizing agent**, alcohol, is added next. This is the crucial, differentiating step. The dehydrating effect of the alcohol causes the peptidoglycan-rich cell wall of the Gram

positive cells to shrink, holding the crystal violet-iodine complexes in the cells. The alcohol dissolves the lipid in the Gram-negative cell walls, leaving the walls porous. Crystal violet-iodine complexes leak out of the Gram-negative cells leaving them without color. At this point the Gram positive cells are purple and the Gram negative cells are clear. A **counterstain**, safranin, is added to allow visualization of the Gram negative cells.

### **Objectives:**

- Learn theory of staining bacterial cells.
- Learn to prepare a bacterial sample for staining.
- Learn to recognize bacterial shapes and arrangements.
- Learn to complete the Simple Stain Protocol effectively.
- Learn to complete the Gram Stain Protocol effectively.
- Learn to recognize Gram Positive from Gram Negative bacteria.
- Understand the role of the Gram Stain as an important differential stain.
- Learn the usefulness of a simple stain vs a differential stain

### **MATERIALS:**

- Control Cultures:      *Staphylococcus aureus* - Gram positive cocci  
                                 *Escherichia coli* - Gram negative rod
- 6 slides/group  
6 cover slips  
wax pencils  
Positive stains: Crystal violet, Safranin  
Gram stain reagents: Crystal violet, Gram's iodine, 95% alcohol, Safranin  
Demonstration slides: *Escherichia.coli* stained with simple and gram stain  
                                 *Neisseria subflava* stained with simple and gram stain  
                                 *Staphylococcus aureus* stained with simple and gram stain  
                                 *Bacillus subtilis* stained with simple and gram stain

### **Protocol: Preparation of sample for staining: heat fixing.**

1. Label slide: use permanent marker to write name of sample on one end of the slide.
2. a. For cultures from a plate or a slant:  
*TA's will demonstrate how to aseptically open and close the plate or slant culture tube.*
  - a. Add one drop of water to the slide.
  - b. Using a sterile loop , transfer a small amount of bacterial culture to the water and mix. Flame the loop and replace in receptacle.
  - c. Take care to transfer a small amount of culture! Too much culture will interfere with staining procedures. Be sure that the culture is well mixed in the water - there should be no large particles in the preparation.

*(Protocol: Preparation of Sample for staining, continued)*

- b. For broth cultures:  
*TA's will demonstrate how to aseptically open and close the broth culture tube.*
  - a. Use a wax pencil to mark a circle on the slide
  - b. Using a sterile loop remove one loopful of culture from the tube. Transfer culture to the center of the circled area. Flame loop and replace in receptacle.
  - c. Broth cultures may be dilute. The wax pencil circle will help you find your sample on the slide when the staining procedure is complete.
3. Allow the sample to AIR dry completely.
4. Hold slide carefully. Pass the slide through the upper most portion of a Bunsen burner flame. Do this three times to heat fix.

**Protocol: Simple Stain** In a straining tray, flood slide with a positive stain such as crystal violet. Allow stain to remain on culture for one minute.

1. Gently rinse slide with water, drain.
1. Place slide into the interior of a blotting paper tablet. Close tablet. Apply pressure to blot. Do not rub. The slide should be completely dry before microscopic examination.
1. Use Brightfield microscope to examine cell smear. Begin with low magnification.
1. Record observations of cells as they appear at 1000X magnification. Observe an area of the slide where individual cells are seen clearly. If no such field can be found on your slide, there are too many cells in your sample. Repeat the sample preparation with a smaller sample size.

**/ Assignment 1 Check Point :**

Practice the Simple Stain technique with one of the control cultures. Compare your work to your lab partners. Controls are used to demonstrate positive and negative results for a test.

The Simple Stain will allow you to see size, shape and arrangement of bacterial cells.

Can you determine that you have three cell types in your environmental isolate by using the simple stain procedure?

What are the general shapes of bacterial cells?

What are the names for bacterial arrangements?

What are the shapes of the cells in your mixed culture?

### **Protocol: Gram Stain**

Prepare slide as described above. *But for this stain you will add **two** samples to the slide.* Place the samples in distinct locations on the slide. The samples should not mix or overlap. The first sample will be a Gram stain control culture and the second sample will be your test sample. After air drying, heat fix the slide.

6. Place slide in staining tray, flood with crystal violet. Stain for 30 seconds
7. Gently rinse with water and drain.
8. Flood the slide with Gram's iodine. Let stand for 10 seconds to one minute.
9. Rinse with water and drain.
10. Flood slide with 95% alcohol for 30 seconds or until stain no longer washes from the slide when held at an angle.
11. Quickly rinse slide with water.
12. Flood slide with safranin. Allow stain to remain for 30 seconds.
13. Rinse with water and drain.
14. Blot slide within a pad of blotting paper. Do not rub. The slide should be completely dry before microscopic examination.
15. Observe bacterial cells at 1000x magnification.
16. Check the Gram Stain control. Focus on an area of the slide where individual cells can be seen clearly. Gram positive cells will stain purple, Gram negative cells will stain pink. If you have achieved the expected result with the Gram stain control, observe your test culture. If the Gram stain control does not stain as expected, repeat the procedure on a freshly prepared slide.

### **/ Assignment 1 Check Point :**

What additional information does the use of this stain give you with respect to your environmental isolate?

The Gram stain is a pivotal stain in microbiology.

This stain should be repeated when pure cultures are isolated.

## Lab 2b: Introduction to Streaking and Culture Maintenance

The immediate objective of the **streak plate** is to produce well separated colonies of bacteria from a concentrated suspension of cells. To achieve this result the bacterial stock is streaked with an inoculating loop onto solid media in a series of steps. Each streaking step is followed by a flaming step. As bacteria are streaked from one area of the plate to the next, a dilution occurs. At the last streak, the number of cells on the loop should be small. As these small number of cells are spread across the remaining area of the plate, the cells are physically separated. Each isolated cell will divide. As the dividing cells accumulate in the local region of the original isolated cell, a distinct mound of cells becomes visible. This mound of cells is a **colony**. All cells in a colony are descendants of one cell from the original sample.

Isolated colonies of different species will demonstrate distinct morphologies. The purity of a culture can be determined by observation of the morphology of isolated colonies. A streak plate of a mixed culture will show colony types of each culture. To isolate pure cultures of each culture found in a mixed culture, one distinct colony may be picked, restreaked on a fresh culture plate and incubated. Observation of identical colonies on this second plate will indicate a pure culture. Most microbiologists repeat streaking from an isolated colony two to three times before they guarantee culture purity. (~ Figures 4.21, 4.51, 4.54, 4.55)

The use of the streak plate technique requires knowledge of rules of **aseptic technique**. Whenever working with a culture, the microbiologist must proceed with care to ensure that the culture is not contaminated during the manipulations, and with caution that no culture escapes into the environment. In addition it is important that all culture vessels and laboratory media are free from bacteria - sterile. Sterilization procedures include treatment with high temperature and irradiation. Some culture media are filtered to remove all bacteria. Inoculating loops are exposed to a flame for sterilization.

When working with a mixed culture, the streak plate is used to isolated colonies of each individual culture in the mix. Once isolated, each colony can be re-streaked to prepare pure cultures. To maintain a pure culture, there are a number of options. Many cultures will survive in the refrigerator for short periods of time. The time will vary from weeks to months ( some will last years). Refrigeration is thus used for short term storage of cultures. Long term storage employs the use of -70°C freezers and lyophilization (see your text book). For cultures frequently used in the lab, one may maintain the culture safely by a combination of refrigeration and sub culture. For sub- culture, on a regular basis, refrigerated cultures are streaked on fresh media, grown and then replaced in the refrigerator.

### / *Assignment 1 Check Point :*

At this point you are working with a mixed culture containing three organisms. For the next labs it will be useful to *isolate* the three distinct bacterial types. To do this, a physical isolation procedure - **the streak plate technique** - is suggested.

**Objective:**

- Learn to complete the Streak Plate method effectively.
- Learn to recognize a bacterial colony.
- Learn to recognize a pure culture.
- Learn methods for culture maintenance: subculture.
- Learn the role of aseptic technique in bacterial culture.

**MATERIALS:**

	<b>Per group</b>	<b>Per section</b>
Day 1	3 Nutrient Agar plates	18 NA plates
Day 2	3 skim milk plates	18 Skim milk plates
Day 3	3 Nutrient Agar	18 NA plates
		1 plate each control culture

**Protocol: Subculture - transferring a culture**

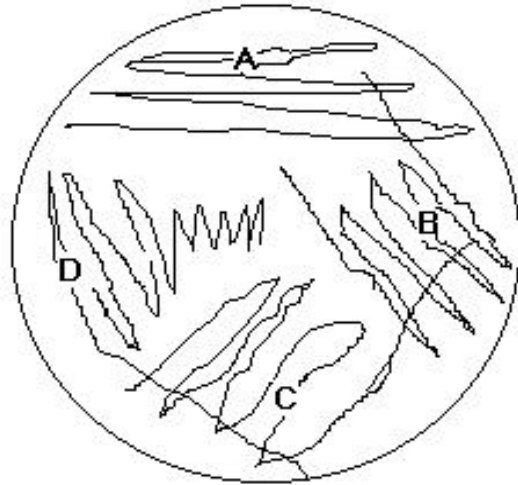
1. Light the Bunsen burner and do all work near the flame. Tie up loose hair and sleeves.
2. The inoculating loop should be heated to bright red, holding the loop straight down into the flame so the loop and lower part of the handle is heated. This will destroy all living organisms on the loop. After flaming, hold loop briefly in the air to allow cooling.
3. Hold culture tube in the left hand (or right hand, if left-handed) at an angle and remove the cap with the fourth and fifth fingers of the right hand that is holding the loop. Never lay a cap down. Keep the tube at a near horizontal angle to prevent dust, etc. from falling into the mouth of the tube.
4. Flame the mouth of the tube. This will kill organisms on the lip of the tube and create outward convection currents that will decrease the chance of contamination.
5. Insert the loop into the tube and remove a drop of broth.
6. Flame the mouth of the tube again and replace the cap.
7. After transferring the loopful of inoculum to another tube or to a plate, flame the loop. Care must be taken not to create a culture aerosol during the flaming. First bring the loop into the cool portion of the flame to heat the wet culture. Now move the loop to the hottest inner cone of the flame and heat the loop till red hot. After loop is heated, hold briefly in the air to allow cooling. Replace the loop in receptacle.

When transferring cultures from plate to plate, use a similar method of aseptic technique as demonstrated by your TA. Work quickly to discourage contamination of plates with airborne microbes. ~ Figure 4.54.

**Protocol: Streak Plate**

Day 1

1. Label bottom of plate with your name, date, and name of culture.
2. To streak a plate use the following procedure:



- ~ Streak the initial inoculum over an area corresponding to A.
  - ~ Flame the loop; cool it on an un-streaked area of the agar.
  - ~ Streak area B starting on a streak of A.
  - ~ Again flame the loop; cool it on the agar.
  - ~ Streak area C, going once through a streak of B.
  - ~ Flame the loop; cool it on agar.
  - ~ Starting from a streak of C, make a fourth series of streaks (D), ending in the middle of the plate.
3. Invert plates and incubate. (Most cultures that we will use in BSCI 223 should be incubated at 37<sup>0</sup>C for 48 hours).

Refrigerate original broth culture.

Day 2

4. Observe streak plates. Record observations. Are isolated colonies visible? Observe colony morphology. Is this a mixed culture or a pure culture?

## / *Assignment 1 Check Point*

At this point you have a broth that contains a mixed culture.  
Use the streak plate to isolate pure cultures.

Day 1: Each person can streak one plate from the group's mixed culture. All environmental isolates should be incubated at 37°C for 48 hours.

Day 2: After observation of plates use the group's three plates to isolate colonies and re-streak. Each person should streak one plate. The goal will be to isolate three cultures, eventually one group member will *work* with one culture and everyone will observe results of *all* the group work.

One Day 2 use Skim milk plates for the streak plate. Bacteria that form protein capsules will use the protein in the milk to grow and form capsules for the Capsule Stain used in Lab 3.

Be sure to label the plates appropriately (eg. Unknown 1, Unknown 2, and Unknown 3 along with your name and the date).

Once you have a pure culture, repeat your Gram stain until you are confident of the result for each organism. Gram stain results should also help confirm the purity of your cultures.

Day 3: ( On this day you will also Complete Lab 3)

Look at your plates. On each plate, are all colony types the same?

IF so, you have a pure culture. Choose one well isolated colony from each plate. Restreak each to a fresh Nutrient agar plate. Incubate at 37°C until the next lab period. This will serve as a culture maintenance measure.

IF you find that you do not have three pure cultures. Use cultures provided by your TA. Gram stain these cultures and proceed with these as your isolates.

Proceed with lab 3.

Can you begin to recognize characteristics of your three organisms? (Gram reaction? Shape? Colony morphology?) Are they beginning to be identifiable to you? Record your data carefully.

~ Chapters 2, 3, and 4



**Exercise 2 \_\_\_\_\_ Laboratory Report Questions**

**Staining and Streaking**

Name \_\_\_\_\_

Results:

Draw observations:

Simple Stain of mixed culture - Environmental Isolate:

Gram Stain of mixed culture - Environmental Isolate:

Gram stain of Individual colonies of environmental isolate:

Questions:

1. While performing a streak plate, you forget to flame the loop between streaks. Draw the predicted result.
2. While performing a Gram stain, you forget to decolorize. If the organism was a Gram negative cell, what result do you predict? Why?
3. Draw the Gram negative cell wall. Label all of the structures. Show how the mordant acts to increase the binding of crystal violet in the Gram stain procedure.

Define:

counterstain

differential stain