

Background

In microbiology, it is very important to be able to identify bacteria and its characteristics. One of the major characteristics of bacteria is its morphology. In order to view morphology of a particular bacterium to determine what it is, we would need a prepared specimen and a microscope. If a bright-field microscope is used, the specimen would need to be stained to make it visible.

Staining of a bacterial film called a **smear** will allow us to view the bacterium's shape, size and arrangement. There are two types of stains: **simple** and **differential**. Simple stain will reveal the morphology and external features of bacterium. The simple stain uses one reagent. Differential stains reveal morphology as well as the chemical composition of the bacterium (ex. Cell wall). Differential stains involve treating the smear with a series of reagents.

All stains used to stain bacterium are made from dyes. These dyes may contain a chemical grouping called the **chromophore**, which exhibits its color. Chromophores can have a positive or negative charge. If the chromophore is positively charged, the stain is called a **basic** or **direct** stain. If the chromophore is negatively charged, the stain is called an **acidic** or **negative** stain. To understand this better, we would need to remember that a bacterial cell surface is negatively charged. So, opposite attract! The basic stain would stain the cell wall and the acidic stain would be repelled resulting in the staining of the background.

Protocol

First Lab Period – Smear Preparation

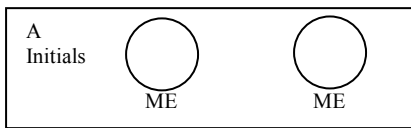
During this lab session, we will be preparing the smears to later on be stained. To prepare a smear, we will need to spread the bacterial cells onto a slide and allow it to **air-dry**. Air-drying is important to prevent lysis from boiling. Once the slide is dry, we will **heat fix** it to adhere the cells to the slide and to kill the bacterial cells. If we do not heat fix, the smear will wash away during the staining procedure.

1. Break up into groups of two to three students. You will be **SHARING** cultures with the person next to you. There are only enough for 1 culture per every two people.
2. Each group will retrieve the following:
 - 12 slides (18 slides for groups of three)
 - Bottled water
 - MS: *Mycobacterium smegmatis*
 - SA: *Staphylococcus aureus*

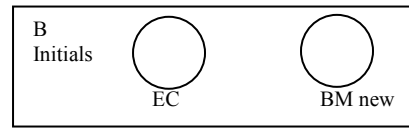
BIOL 2250L – Microbiology for the Health Sciences
Lab 3 and 4 - Smear Preparation and Staining

- EC: *Escherichia coli*
- PA: *Pseudomonas aeruginosa*
- BS old: Old Culture of *Bacillus subtilis*
- BM new: New Culture of *Bacillus megaterium*
- MX: mixed culture
- ME: Any culture that you saved previously and want to stain and look at under the scope (From Microbes in Environment)

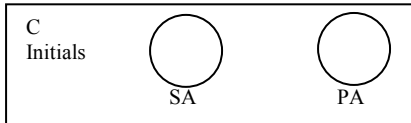
3. Each group member will have 6 total slides with 2 smears on each side of the slide (**except for D which will have only 1 smear**). Label with your initials, letter corresponding to slide below, culture description under each smear (see below).



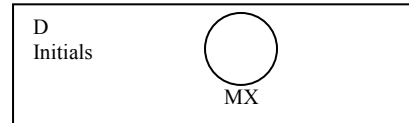
Slide A will be **simple stained**.



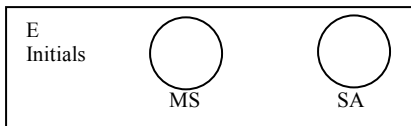
Slide B will be **Gram stained**.



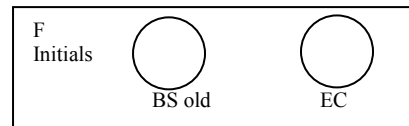
Slide C will be **Gram stained**.



Slide D will be **Gram stained**.



Slide E will be **acid fast stained**.



Slide F will be **spore stained**.

4. As your instructor demonstrated, you need to aseptically transfer a loopful of bacteria to the slide. Be sure to spread in a circular motion within the circle to get a thin smear. Do all six slides before you begin to heat fix.
5. Be sure to obtain an **A+ smear**. An A+ smear would be a thin whitish film and not thick and turbid when air dried.
6. Heat fix all the slides by passing through the flame two or three times.
7. Place the slides into the slide box in your lab drawer. Be sure to keep your slides together and not spread out since you will be sharing the slide box.

Second Lab Period - Staining

Simple Stain

The simple stain is used to provide contrast to observe the external features such as morphology using the bright-field microscope.

1. Obtain some gloves, clothes pin, paper towel and bibulous paper.
2. Retrieve slide A from slide box. As demonstrated, clamp your clothes pin near your initials on the slide. Place the slide on the paper towel in front of you.
3. Cover the smears with methylene blue for 1 minute.
4. Wash the methylene blue off the smear gently with water in the sink on your bench.
5. Blot dry with the bibulous paper. Be very gentle so you do not break the slide.
6. Place slide back into slide box.

Gram Stain

The Gram stain is a differential stain used for diagnostic purposes to determine what type of cell wall the bacterium has. The Gram stain divides bacteria into two groups: **Gram-positive** or **Gram-negative**. Gram-positive bacteria have thick peptidoglycan layers that retain the **primary stain** called **crystal violet**. So, they appear purple under the microscope. Gram-negative bacteria have thin peptidoglycan layers which does not retain the primary stain. So they appear pink due to **safranin** which is a **counterstain**.

1. Retrieve slide B - D from slide box. Be sure to carry out all four steps for each slide individually. Clamp your clothes pin near your initials on the slide. Place the slide on the paper towel in front of you.
2. Cover the smears with crystal violet for 30 seconds.
3. Wash the crystal violet off the smear gently with water.
4. Cover the smears with Gram's Iodine (**mordant**) for 1 minute.
5. Rinse with water gently.
6. Cover the smears with alcohol (**decolorizer**) for 30 seconds.
7. Rinse with water gently.
8. Cover the smears with safranin for 30 seconds.
9. Rinse with water gently.
10. Blot dry with the bibulous paper.
11. Place slides back into slide box.

Acid-Fast Stain

The Acid-Fast stain is a differential stain used on certain species of bacteria such as *Mycobacterium*, that cannot be stained with basic stains such as the Gram stain. *Mycobacterium* have waxy cell walls that contain mycolic acids. These cell walls are hydrophobic so they repel water-based stains. The acid-fast stain involves the use of a reagent called **carbol fuchsin** (red dye) which is lipid soluble and has the ability to penetrate the cell wall.

The Ziehl-Neelsen Method

1. Retrieve slide E from slide box. Clamp your clothes pin near your initials on the slide. Place the slide on the paper towel in front of you.
2. Cover the smears with a strip of blotting paper. Be sure the paper does not extend beyond the edges of the slide.
3. Saturate the paper with carbol fuchsin.
4. Place the slide onto the beaker on the hot plate to be heated for 5 minutes. Steam will be heating the slide. **Do not allow the slide to dry.** Add more carbol fuchsin if necessary.
5. Remove the blotting paper and wash the slide with water.
6. Cover the smears with **acid alcohol** for 30 seconds.
7. Rinse with water gently.
8. Cover the smears with methylene blue for 30 seconds.
9. Rinse with water gently.
10. Blot dry with the bibulous paper.
11. Place slide back into slide box.

The Kinyoun's Cold Procedure Method

1. Retrieve slide E from slide box. Clamp your clothes pin near your initials on the slide. Place the slide on the paper towel in front of you.
2. Cover the smears with carbol fuchsin for 5 minutes.
3. Rinse with water gently.
4. Cover the smears with **acid alcohol** for 1 minute.
5. Rinse with water gently.
6. Cover the smears with methylene blue for 1 minute seconds.
7. Rinse with water gently.
8. Blot dry with the bibulous paper.
9. Place slide back into slide box.

The Spore Stain

Certain genera such as *Bacillus* and *Clostridium* carry out a process called sporulation in response to unfavorable environmental conditions. This is a survival mechanism which forms endospores which are resistant to any hazardous conditions (ex. High temperature, chemicals, etc). Since endospores are quite resistant, a special staining procedure must be used.

12. Retrieve slide F from slide box. Clamp your clothes pin near your initials on the slide. Place the slide on the paper towel in front of you.
13. Cover the smears with a strip of paper towel. Be sure the paper does not extend beyond the edges of the slide.
14. Saturate the paper with malachite green.
15. Place the slide onto the beaker on the hot plate to be heated for 5 minutes. Steam will be heating the slide. **Do not allow the slide to dry.** Add more malachite green if necessary.
16. Remove the paper towel and wash the slide with water.
17. Cover the smears with safranin for 30 seconds.
18. Rinse with water gently.
19. Blot dry with the bibulous paper.
20. Place slide back into slide box.

Third Lab Period – View Smears

1. Remove your slides from the slide box.
2. View all of your slides under oil immersion.
3. Sketch pictures of each slide in your lab notebook.
4. Place slides back into slide box.