

BACTERIAL SMEARS AND SIMPLE STAINING

Principle and Purpose

The visualization of bacteria under the bright-field microscope usually requires that contrast be provided since prokaryotes are often colorless. There are different means of providing such contrast. The simplest is staining cells with a single type of dye. Not only can one more readily observe bacterial cells, but the dye helps distinguish among different cell types (Fig. 1).

Prior to staining the cells, however, a thin lawn of bacteria must be fixed in place on a support, such as a glass slide. The lawn of cells is termed a 'bacterial smear'. The creation of a smear is an essential skill necessary to obtain a proper staining result. If the lawn is too thick, the cells are crowded and may not stain well or can be visually resolved well. Too thin a smear and searching for cells under the microscope becomes a frustrating task.

In the procedure below, students will have the opportunity to master the important techniques of making and staining bacterial smears. This skill set is important for later experiments involving differential staining as well as for microbial identifications.

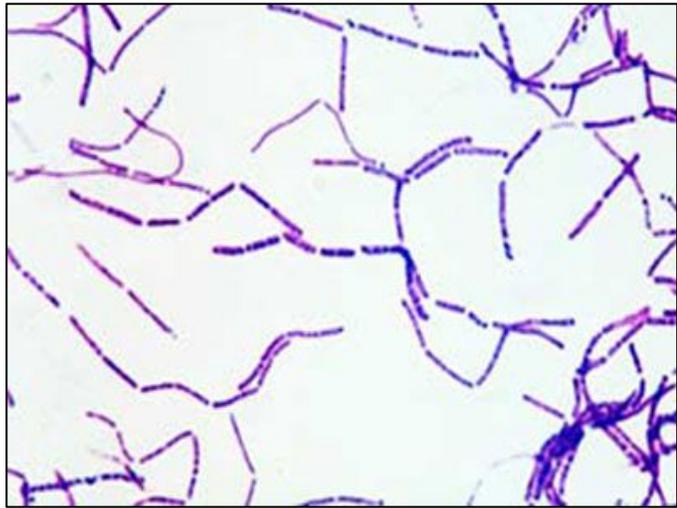


Figure 1. Simple stain of *Bacillus cereus*, a bacterial rod. (<https://notasprensa.info/8135862-bacillus-subtilis-simple-stain.html>)

Note: Students are strongly encouraged to view two potentially helpful videos depicting the making of a bacterial smear (<https://youtu.be/on5-5oQNNqo>) and the process of simple staining (<https://youtu.be/n5fXlpJUgD4>).

Learning Objectives

Upon completion of this exercise, a student should be able to:

- Prepare a proper bacterial smear from both a solid and broth culture;
- Properly stain and observe microbes using a single, simple dye; and
- Differentiate between different cell shapes.

Materials Required

The following materials are necessary to successfully conduct this exercise:

Organisms

- The following organisms should be provided as 24-48 hour TSA slant cultures:
 - *Staphylococcus aureus* (ATCC 25923) [abbreviated as *S. aureus*]
 - *Escherichia coli* (ATCC 25922) [abbreviated as *E. coli*]

- Mixed TSB culture of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922)

Reagent

- 1% (w/v) Crystal Violet

Procedures

Students shall review and use the BIOL 3702L Standard Practices regarding the labeling, incubation, and disposal of materials.

Part A: Making a Bacterial Smear from a Culture Grown on Solid Medium

This procedure uses TSA agar slant cultures of *S. aureus* and *E. coli* to produce two slides of heat-fixed, bacterial smears – one for each species. These smears will be subjected to simple staining in Part C.

The steps described below produce a fixed smear for one of the above cultures. The goal of this portion of the exercise is to generate a fixed smear for each bacterium. Hence, this procedure can be repeated to produce the second smear or both cultures can be processed simultaneously.

- 1) Obtain a glass slide and label it appropriately at one end. (If the end of the slide is frosted, then mark the slide in this area.) Using a bacteriological loop or a disposable plastic bulb pipette to obtain water from the labeled water bottle on the bench top, place a *tiny* drop of water (about a quarter or less the size of a finger nail) in the center of the slide.
- 2) Sterilize the bacteriological loop and, using aseptic technique, obtain a very small mass of cells on the loop from the surface growth on the TSA agar slant. The cellular mass obtained should be barely visible on your loop.
- 3) Transfer the cells to the water on the glass slide and make a bacterial smear by spreading the water suspension of cells using the loop such that the liquid is about the size of a quarter.

Note: The cell mass should not produce a suspension the consistency of paste nor should the cells be clumped. If this occurs, repeat the procedure being sure to discard the “bad” smear in the sharps container.

- 4) Sterilize the bacteriological loop and place it aside.
- 5) The wet smear must now be dried. The slide may be allowed to air dry or set the slide on a hot plate operating on the lowest setting to facilitate drying.

Note: If a hot plate is used, do not allow the smear to remain on it longer than necessary.

- 6) The dry slide now needs to be heat fixed. There are two methods to do so. One uses the flame of a gas burner (6a) and the other uses a hot plate (6b). Each is described below.
 - a) [PREFERRED METHOD] Using slide holders, tongs, etc., pass the slide containing the bacterial smear through the flame of a gas burner three times. Each pass should be of moderate speed. The slide should not linger in the flame, nor should it pass through so quickly that it is not warmed. Once heated, allow the slide to cool on the bench top.

Note: Exercise caution when using this method due to the extreme heat, the possibility of burns, and the presence of an open flame.

- b) [ALTERNATIVE METHOD] Set the slide containing the bacterial smear on a hot plate operating on the highest (hottest) setting. Use slide holders, tongs, etc., to place the

slide on the hot plate surface for 3 seconds. Remove the slide immediately and allow it to cool on the bench top.

Note: Exercise caution when using this method due to the extreme heat produced by the hot plate and the possibility of burns.

At this point, if the above procedure was performed properly, the slide is heat fixed and the bacterium is no longer viable. The slide can be placed in a slide container for storage/transport and stained at a later time.

Part B: Making a Bacterial Smear from a Culture Grown in Broth Medium

This procedure uses a mixed TSB culture of *Staphylococcus aureus* and *Escherichia coli* to produce a heat-fixed, bacterial smear. This smear will be subjected to simple staining in Part C. This same procedure would be used to make fixed smears from broth cultures of individual bacterial species.

- 1) Obtain a glass slide and label it appropriately at one end. (If the end of the slide is frosted, then mark the slide in this area.)
- 2) Ensure that the bacterial cells in the mixed broth culture are fully mixed by rolling the tube between both palms ten times or more to suspend any sediment of cells that may have formed. Roll the tube quickly, but not so harshly that the broth splashes onto the tube cap or such that it rolls out of the hands causing leakage or breakage.
- 3) Using a sterilized microbiological loop, transfer loopfuls of broth from the bacterial culture to be stained to the glass slide. Be sure to sterilize the loop between each transfer (the glass slide is not sterile!).

Note: The volume of broth to be transferred is somewhat dependent upon the turbidity (i.e., cloudiness) of the culture. A slightly cloudy broth will require more broth transferred to the slide than a very cloudy broth. Typically, 5-6 loopfuls of a slightly turbid culture should be placed on the slide, whereas 2-3 loopfuls are usually sufficient from a cloudy broth culture.

- 4) Using the microbiological loop, make a bacterial smear by spreading the broth such that the liquid is about the size of a quarter.
- 5) Sterilize the bacteriological loop and place it aside.
- 6) The wet smear must now be dried. The slide may be allowed to air dry or set the slide on a hot plate operating on the lowest setting to facilitate drying.

Note: If a hot plate is used, do not allow the smear to remain on it longer than necessary.

- 7) The dry slide now needs to be heat fixed. There are two methods to do so. One uses the flame of a gas burner (7a) and the other uses a hot plate (7b). Each is described below.
 - a) [PREFERRED METHOD] Using slide holders, tongs, etc., pass the slide containing the bacterial smear through the flame of a gas burner three times. Each pass should be of moderate speed. The slide should not linger in the flame, nor should it pass through so quickly that it is not warmed. Once heated, allow the slide to cool on the bench top.

Note: Exercise caution when using this method due to the extreme heat, the possibility of burns, and the presence of an open flame.

- b) [ALTERNATIVE METHOD] Set the slide containing the bacterial smear on a hot plate operating on the highest (hottest) setting. Use slide holders, tongs, etc., to place the slide on the hot plate surface for 3 seconds. Remove the slide immediately and allow it to cool on the bench top.

Note: Exercise caution when using this method due to the extreme heat produced by the hot plate and the possibility of burns.

Note: At this point, if the above procedure was performed properly, the slide is heat fixed and the bacterium is no longer viable. The slide can be placed in a slide container for storage/transport and stained at a later time.

Part C: Simple Staining of a Bacterial Smear

Perform the following staining procedure using the heat-fixed, bacterial smears generated in Parts A and B above. The staining of the slides can be conducted individually or all at once.

- 1) Place a heat-fixed bacterial smear on the slide support rails sitting across the sink.
- 2) Obtain the vial of crystal violet and flood the area of the smear. Allow the stain to remain for one minute (the exact time is not critical for this procedure).
- 3) Lifting the slide at an angle, use the water bottle on the bench to rinse both sides of the slide until the dye has been removed.
- 4) Gently blot (do not rub or scrape) the slide dry with bibulous paper or paper towel.

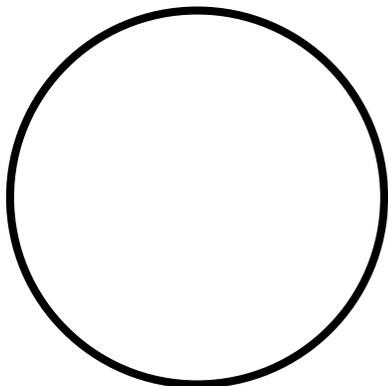
Note: The bibulous paper/paper towel can be discarded in the regular trash receptacle.

- 5) Examine the slide under the microscope using both the high dry and immersion oil objective lenses.

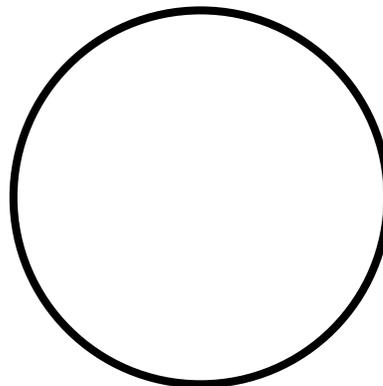
Record any observations on the data report sheet attached to this document.

Student Name: _____

DRAWINGS OF SIMPLE STAIN OF *STAPHYLOCOCCUS AUREUS*

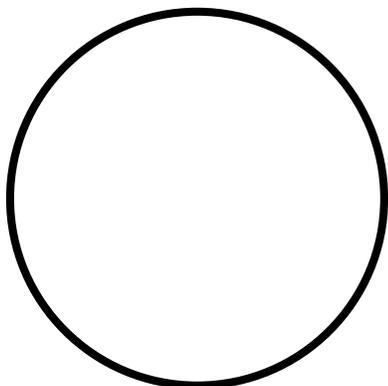


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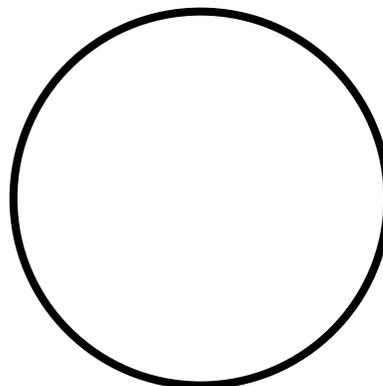


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DRAWINGS OF SIMPLE STAIN OF *ESCHERICHIA COLI*

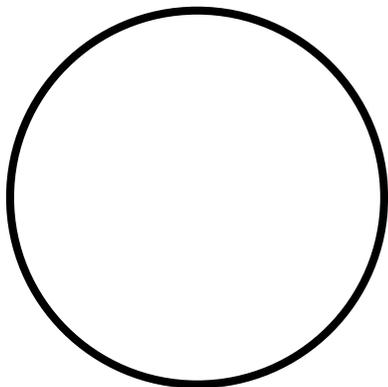


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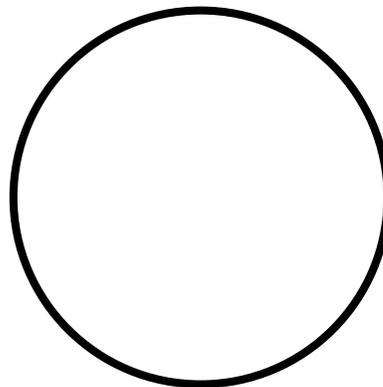


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DRAWINGS OF SIMPLE STAIN OF *E. COLI* AND *S. AUREUS* MIXTURE



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