

Exercise 6-C

STAINING OF MICROORGANISMS

ACID-FAST STAIN

Introduction

The acid-fast stain is a **differential stain** that separates bacteria on the basis of the lipid content of their cell walls. Bacteria categorized as acid-fast (members of the genus *Mycobacterium* and some other Actinobacteria) have Gram-positive walls with thick peptidoglycan containing large quantities of a wax-like lipid called **mycolic acid**. These cells are resistant to staining with normal procedures, and require severe treatment, e.g., heat and potent dyes, in order to be stained. Once stained, their walls do not readily decolorize, and so can be differentiated from non-acid-fast cells. The acid-fast stain is used primarily in the diagnosis and study of diseases caused by acid-fast bacteria (Tuberculosis and Leprosy), however it can also be used on a variety of non-pathogenic organisms. The basic steps in this technique are as follows:

1. Bacteria cells are heat-fixed and stained with a potent primary staining reagent called carbol fuchsin while being subjected to steam heat. An alternative procedure uses a different formulation of carbol fuchsin (“Kinyoun’s Acid-Fast Reagent”) and does not require the application of steam heat.
2. The stained cells are subjected to decolorizing with an acid-alcohol solution; 95% ethyl alcohol containing 2.5% concentrated nitric or hydrochloric acid (HNO₃ or HCl). This step will remove the primary stain from cells that are not acid-fast, rendering them colorless.
3. Non-acid-fast cells are counterstained with a basic dye of a contrasting color (e.g., methylene blue).

When a mixed culture containing both acid-fast and non-acid-fast cells is subjected to this procedure, the acid-fast cells will be stained red and the non-acid-fast cells will be stained blue. The presence of purple-colored cells usually indicates the procedure has been improperly completed; however, some cells are only partially acid-fast and others may contain acid-fast structures. The endospores formed by bacteria in the genus *Bacillus* will often stain red when subjected to the acid-fast stain.

Standard Procedure Using Steam Heat

1. Place about 200 mL of water in a 400 mL beaker on a wire gauze platform over a Bunsen burner and heat the water to generate steam.
2. At one end of a clean glass slide, prepare a small, **mixed smear** containing a *Mycobacterium* species **and** *Staphylococcus aureus*. For best results, thoroughly mix a small quantity of the *Mycobacterium* with a loopful of water and break up the cell clusters before adding the *Staphylococcus*. At the other end of the slide, prepare a small smear of your morphological unknown.
3. Allow the smears to air-dry and then heat-fix them. It is important to insure that the cells used are dead, but do not apply excess heat.
4. Place a wire mesh platform over the beaker, place the slide on the platform smear side up, and cover each smear with a **SMALL** section of paper towel (just cover the smear). Make certain the paper does not extend over the edge of the slide in any direction, as this will allow most of the stain reagent to run off, potentially staining the bench top.

5. Apply several drops of carbol fuchsin to the smear (thoroughly wetting the paper towel) and allow it to act over steam-heat for 10-15 minutes (apply more reagent as needed). This will color all cells in the smears red. **Caution** - Do not allow the paper towel to dry out during the staining procedure, and avoid excess flooding.
6. Remove the slide from the stain rack, hold it in the sink and rinse the smears thoroughly with tap water. The paper towel sections covering the smears will be rinsed off during this process, and can be removed from the sink when staining is completed.
7. Decolorize each smear with acid-alcohol (95% ethyl alcohol containing 2.5% concentrated hydrochloric acid) by allowing the reagent to flow across the smear. Watch the color run off, and when it stops moving, rinse immediately with water. This decolorizing step will remove the red color from non-acid-fast cells leaving them colorless. **Caution – do not over decolorize!**
8. Rinse the smears thoroughly with water to be certain the action of the decolorizing reagent has been stopped.
9. Counterstain the smears by placing the slide on a stain rack over the sink, applying Loeffler's methylene blue and allowing it to act for 60 seconds without heat. This counterstain will color the non-acid-fast cells blue.
10. Rinse the slide thoroughly with tap water, remove excess water from the slide bottom and allow the smears to air dry (place near the base of a lit Bunsen burner).
11. Examine both smears under oil immersion (focus with your 10X objective first) and compare the data and results. In a properly prepared acid-fast stain, the rod-shaped *Mycobacterium* cells will appear red and the non-acid-fast *Staphylococcus* cells will appear blue. Make sketches of the cells present and record their characteristics. Cells from an unknown culture can be expected to appear either red (acid-fast) or blue (non-acid-fast); however, *Bacillus* cells containing endospores will often appear blue with red-colored endospores.

Alternative Procedure Using Kinyoun's Acid Fast Reagent

1. At one end of a clean glass slide, prepare a small, **mixed smear** containing a *Mycobacterium* species **and** *Staphylococcus aureus*. For best results, thoroughly mix a small quantity of the *Mycobacterium* with a loopful of water and break up the cell clusters before adding the *Staphylococcus*. At the other end, prepare a small smear of your morphological unknown.
2. Allow the smears to air-dry and then heat-fix them. It is important to insure that the cells used are dead, but do not apply excess heat.
3. Place the slide on a stain rack and stain each smear by covering it with Kinyoun's Acid-Fast Reagent. Allow the stain to act for **10 minutes**.
4. While holding the slide in the sink, rinse the smears thoroughly with tap water.
5. Decolorize each smear with acid-alcohol (95% ethyl alcohol containing 2.5% concentrated hydrochloric acid) by allowing the reagent to flow across the smear. Watch the color run off, and when it stops moving, rinse immediately with water.

6. The decolorizing step (above) will remove the red color from non-acid-fast cells leaving them colorless; however, if the decolorizing process is not stopped, it will also remove the red color from the acid-fast cells too. Rinse the smears thoroughly with water to be certain the action of the decolorizing reagent has been stopped.
7. Counterstain the smears by applying Loeffler's methylene blue and allowing it to act for 60 seconds. This counterstain will color the non-acid-fast cells blue.
8. Rinse the slide thoroughly with tap water, remove excess water from the slide bottom and allow the smears to air dry.
9. Examine both smears under oil immersion (focus with your 10X objective first) and compare the data and results. In a properly prepared acid-fast stain, the rod-shaped *Mycobacterium* cells will appear red and the non-acid-fast *Staphylococcus* cells will appear blue. Make sketches of the cells present and record their characteristics. Cells from an unknown culture can be expected to appear either red (acid-fast) or blue (non-acid-fast); however, *Bacillus* cells containing endospores will often appear blue with red-colored endospores.

Questions:

1. In what ways is the acid-fast stain similar to the Gram stain?
2. What is mycolic acid and how does it influence staining?
3. Are Gram-negative bacteria likely to be acid-fast?
4. What would you expect to observe if you decolorized an acid-fast stain preparation with acetone-alcohol instead of acid-alcohol?
5. What color do acid-fast cells appear when stained with the acid-fast stain preparation?

NOTES, OBSERVATIONS & ADDITIONAL INFORMATION

Name _____

Lab Section _____

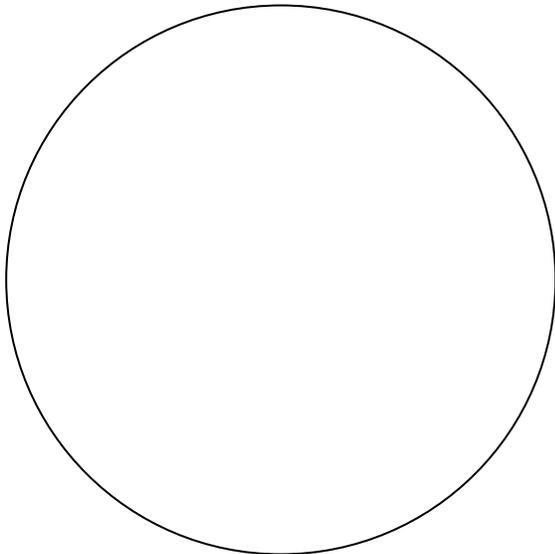
WORKSHEET
Exercise 6C
Staining of Microorganisms: Acid-Fast Stain

Goals: _____

Materials & Methods:

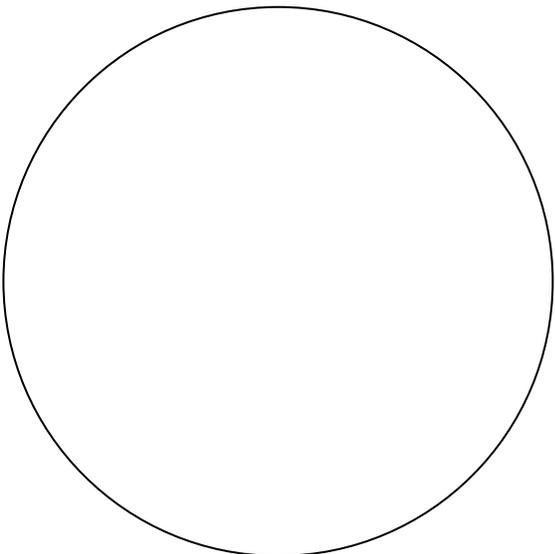
Age of Unknown: _____ Reagents used: _____

Data & Results:



A) Acid-Fast Positive & Negative Controls

Specimen:			
Total Magnification:			
Length:	units x	$\mu\text{m}/\text{unit} =$	μm
Width:	units x	$\mu\text{m}/\text{unit} =$	μm
Notes:			



B) Morphological unknown

Specimen:			
Total Magnification:			
Length:	units x	$\mu\text{m}/\text{unit} =$	μm
Width:	units x	$\mu\text{m}/\text{unit} =$	μm
Notes:			

Acid-Fast Results Summary:

Specimen	Data	Result

NOTE: If your controls do not appear as expected, **SOMETHING IS WRONG**. Consult your instructor.

Conclusions:

Based on your results, what can you conclude about the cell walls of your Morph. Unknown? _____

Does the cell morphology of your Morphological Unknown match what you saw for Exercise 6A (indirect stain) and 6B (Gram stain)? _____

Additional Comments: _____

