

Lab Exercise #3a**Identification of Unknown Bacteria:**

- Streak Plating
- Preparing Bacterial Smears for Differential Staining

Unknown Number _____

I. OBJECTIVES:

- ✓ Provide student with opportunity to perform bacterial smear, with controls and unknown.
- ✓ Practice performing isolation streak plates and aseptic technique.
- ✓ Use terminology correctly.

II. TERMINOLOGY:

Acid-Fast	counterstain	<i>Mycobacterium</i>
vegetative cell	differential stain	negative control
Acid-Fast Stain	Gram negative	Nonacid- fast
bacilli	Gram positive	positive control
cocci	Gram stain	primary stain
Endospore stain		isolation streak plate
<i>Escherichia coli</i> (<i>E. coli</i>)	<i>Staphylococcus epidermidis</i> (<i>S. epi</i>)	
<i>Mycobacterium smegmatis</i> (<i>M. smeg</i>)	<i>Bacillus subtilis</i> (<i>B. sub</i>)	

III. INTRODUCTION:

In this lab, you will be given a numbered bacterial culture, the identity of which you don't know. The 'unknown' is only unknown to you; your instructor knows the identity and it is your job to match the identity correctly based on the stains and other tests you will perform. It is imperative that you record your "**unknown number**". Your instructor will check the identity of the unknown for accuracy. Over the next two weeks, your job will be to narrow down the possible identity of this unknown bacteria using specialized media and differential stains (Gram, Acid fast and Endospore stains).

Isolation Streak Plates

Today will learn to use aseptic (sterile) technique to prepare streak plates of your unknown bacterium. Streak plating allows you to spread out a specific sample of bacteria on a medium, so that the bacteria are sparse enough to grow into individually distinct colonies. It is important that you do not contaminate this sample while you are transferring it from the unknown source plate to the specialized media plates. Your instructor will show you how to implement this technique. You will interpret these plates next week, after they have had a chance to incubate and grow.

Gram Stain

In the late 1800's, Danish bacteriologist Christian Gram developed a method for staining bacterial cells that seemed to separate the cells into two groups. These groups, known as Gram negative and Gram positive, were separated on the basis of the color of the bacteria after a series of stains were applied to them. The Gram positive cells stain purple (the color of the primary, first dye used in the staining procedure), and the Gram-negative cells stain pink (the color of the counterstain, second dye used in the staining procedure).

Since two different color dyes are used, the Gram stain is considered a **differential stain**, (i.e. it illustrates differences between bacterial cells). The identification of an unknown organism typically begins with a Gram stain. Gram stains quickly tell not only if a bacterium is Gram-positive or Gram-negative, but also staining the cells reveals the shape of the bacterium (its cell morphology). Clinically, Gram stain results allow for rapid intervention with appropriate antibiotics.

In today's lab exercise, you will be preparing a bacterial smear for Gram staining. The smear will include your unknown and both a (+) and (-) control.

Acid Fast Stain

You will then prepare a smear for an **Acid-fast stain**. This stain is used in the identification of bacillus-shaped bacteria of the genera *Mycobacterium* & *Nocardia*. *Mycobacterium tuberculosis* is the causative agent of tuberculosis and *Mycobacterium leprae* causes leprosy. The acid-fast stain is most commonly used on the **clinical sample** sputum when tuberculosis is suspected. In lab we will use *Mycobacterium smegmatis* (*M. smeg*), which is a non-pathogenic acid-fast bacterium.

Mycobacteria have a cell wall that is different from both Gram-negative and Gram-positive bacteria. *Mycobacteria* can stain either Gram-positive or Gram-negative depending on the age of the culture. They are said to be "**Gram variable**." The cell wall of *Mycobacteria* contains a waxy substance called **mycolic acid**. The presence of this waxy material means that it is difficult to get stains into and out of *Mycobacteria*. Acid-fast staining involves using heat to drive the hot-pink, carbol fuchsin primary stain into the waxy cell wall of acid-fast bacteria. Bacteria that do not have a waxy cell wall are considered nonacid-fast, and retain the counterstain, which is purple (crystal violet).

In today's lab exercise, you will be preparing a bacterial smear for Acid-fast staining. The smear will include your unknown and both a (+) and (-) control.

Endospore Stain

Clostridium and *Bacillus* are two genera of bacteria that produce endospores. Endospores are resistant structures made through a process known as sporulation by members of these genera in response to extreme environmental conditions. Extreme environments include high temperatures, lack of food, harsh chemicals. The endospores are inert structures meaning they do not metabolize and do not reproduce. They simply exist, much like plant seeds, until placed in appropriate environmental conditions. When environmental conditions are favorable, the endospore germinates. Upon germination, the cell returns to its normal metabolic state, capable of reproduction.

Normal staining techniques will not stain the resistant endospores. In the endospore stain, the dye malachite green is forced into the spore with heat much like the carbol fuchsin was forced through the waxy mycolic acid layer of *Mycobacterium*. Once the endospore is stained, the counter stain, Safranin, provides color for the vegetative (i.e. metabolically active) cells. At the end of this differential staining process the vegetative cells are pink and the endospores, if present, are green.

In today's lab exercise, you will be preparing a bacterial smear for Endospore staining. The smear will include your unknown and both a (+) and (-) control.

Bacterial Controls

For most of the experiments that you perform in this course, you will include controls. **Controls** are samples whose identity you already know that are subjected to the same procedures and motions to which to submit your unknown sample. For example, when you perform a Gram stain, you will always include samples of *Staphylococcus epidermidis* (*S. epi*), which is known to be Gram positive, and *Escherichia coli*, which is known to be Gram-negative. If the Gram stain procedure works as it should, *S. epi* will be purple and *E. coli* will be pink. For stains and other procedures, controls also provide you with a basis of comparison and tell you if you performed the stain correctly. For example, most of you have not observed cocci and/or bacillus-shaped bacteria before. *S. epi* is a coccus and *E. coli* is a bacillus, so you can look at your unknowns and compare them to your controls to help you decide the shape and Gram-stain result of the unknowns. Your instructor will explain which controls you will use for your Acid fast and Endospore stains.

IV. MATERIALS (In addition to supplies found in your supply drawer):

Cultures of *Mycobacterium smegmatis*, *E. coli* and *Staphylococcus epidermidis* and *Bacillus subtilis*
 Numbered “unkown” bacterial cultures
 Microincinerator
 Sterile Mannitol Salt plate & MacConkey’s plate

V. PROCEDURE:**A. Streak Plating onto Specialized Media**

Obtain an unknown bacterial culture from your instructor. Record the unknown number. The bacteria are growing in thin film on the agar’s surface. Obtain the sample gently and do not gouge the media or get agar in your sample. Perform an isolation streak of your unknown on MacConkey’s and Mannitol Salt. Store the streak plates in the ‘save’ bin until next week.

B. Gram Stain Bacterial Smear

1. Draw three circles on the microscope slide with a wax pencil. Write letter G in upper right corner of slide. Turn the slide over and transfer 1 drop of water to each of the three circles.
3. Aseptically transfer *Staphylococcus epidermidis* (*S. epi*) to the circle on the left.
2. Aseptically transfer a sample of your unknown bacterial colony to the middle circle.
4. Aseptically transfer *Escherichia coli* (*E. coli*) to circle on right. Allow slide to heat fix.

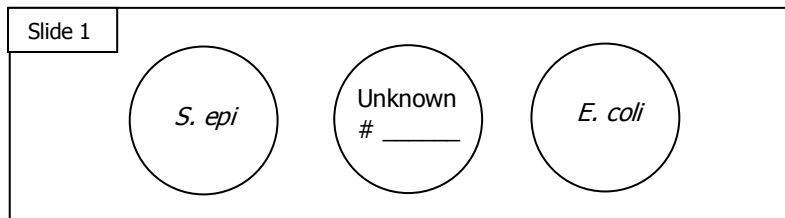


Figure 1: Gram Stain Slide Set-up

C. Acid-fast Stain Bacterial Smear

Prepare and heat fix a slide marked with three circles organized as follows:

1. Draw three circles on the microscope slide with a wax pencil. Write letter A in upper right corner of slide. Turn the slide over and transfer 1 drop of water to each of the three circles.
2. Aseptically transfer a sample of *M. smegmatis* (*M. smeg*) into the circle on the left.
3. Aseptically transfer a sample of your unknown to the center circle.
4. Aseptically transfer a sample of *S. epidermidis* (*S. epi*) to the circle on the right.

D. Endospore Stain Bacterial Smear

1. Prepare and heat fix a slide marked with 3 three circles using the same technique described in B & C. Write letter E in the upper right corner of slide, and use the following samples:
2. *Bacillus subtilis* in the circle on the left.
3. Your unknown in the center circle.
4. *E. coli* in the circle on the right.

E. Wrap your bacterial smears in a paper towel that you have labeled with your name, and place them in the metal “Save” tray.

**Always wash down your bench
with disinfectant at the end of the lab.**