Lab Exercise #2 - INSTRUCTIONS

Identification of Unknown Bacteria

Gram Stain & Isolation Streak Plate Technique

Unknown Number_____

I. OBJECTIVES:

- ✓ Provide student with opportunity to perform a Gram stain, with controls and an unknown
- ✓ Practice evaluating Gram stain results
- ✓ Perform an isolation streak plate technique
- ✓ Aseptically pour media plates
- ✓ Use terminology correctly

II. TERMINOLOGY:

Acid-Fast Stain	differential stain	negative control
Agar	*Escherichia coli (E. coli)*	positive control
Aseptic Technique	Gram negative	primary stain
Autoclave	Gram positive	*Staphylococcus epidermidis (S. epi)*
bacilli	Gram stain	vegetative cell
cocci	isolation streak plate	
counterstain	Media/Medium	

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.

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. You instructor will show you how to implement this technique. This culture of bacteria will be available next week, after the bacteria have had a chance to grow on the media.

Growth media (singular: **medium)** are used to cultivate bacteria. Media are mixtures of nutrients that the microbes need to live. They also provide the necessary moisture and pH to support microbial growth. Bacterial cultures are incubated at temperatures known to be best for their growth. The bacteria that live on your body surfaces are mesophiles – they grow at temperatures between 25 and 45 ^{o}C

The medium that we use most often is designated "TSY" (**T**ryptic **S**o**y** **A**gar) and it is a complex nutrient medium which supports the growth of a wide variety of microbes. When the lab personnel make a medium, they measure out a designated quantity of dry powdered medium, add a designated amount of water and check the pH. They dispense the medium into bottles, cap it and autoclave it. Autoclaving is a process similar to home canning techniques of food preservation. Once the medium is **autoclaved** (or pressure cooked) it is considered sterile. The autoclave exposes the medium to high temperature (121°C) and pressure (15 psi) for 20 minutes. This

exposure has been demonstrated to result in **sterilization** (Sterilization is the process of killing all life forms).

Many normal flora and clinically important microbes can be grown either in liquid medium (sometimes called **broth**), or on Petri dishes (also called Petri plates, or just "plates"). When grown on plates, **agar** is added to the liquid medium so that, when cool, the medium has the consistency of very stiff Jello®. Agar is an inert seaweed extract that solidifies at room temperature.

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 Gram positive (GP) and Gram negative (GN) 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 future differential staining techniques.

The controls are in test tubes containing media. When the racks of test tubes containing the media are removed from the hot autoclave, they are set on their side. The media 'comes to temperature,' meaning it cools down to room temperature and solidifies with the expanded surface area of the slant.

IV. MATERIALS (In addition to supplies found in your supply drawer)**:**
Slant Cultures of *E. coli* and *Staphylococcus epidermidis*
Numbered "unkown" bacterial cultures
Microincinerator
Sterile TSY plates

V. PROCEDURE:

A. Aseptically Pour Media

Aseptically pour 1 TSY plate using the following technique:

1. Obtain a bottle of TSY media from the warming oven. Careful, it is *warm/hot. You may need an insulated glove to protect your hand.*
2. Get the Bunsen burner out of your supply drawer, connect it to the gas supply, then arrange the six Petri dishes, and the one arm plate, so that they are near the Bunsen burner.
3. Carefully light the Bunsen burner on your table. (Care should be taken that there are no papers in the area and all long hair and loose clothing has been secured prior to ignition.)
4. Swirl the bottle of media to re-suspend the mixture.
5. Open the cap as demonstrated, flame the top of the bottle.
6. Open the Petri dish as demonstrated, pour about a ¼ inch of media into the plate.
7. Flame the bottle and return the cap.
8. Return the bottle to the warming oven. If the bottle is empty set it in the "discard" bin on the side bench in the lab.
9. Do not disturb the Petri dish until it has come to room temperature.

B. Streak Plating onto TSY Media

Obtain an unknown bacterial culture from your instructor. Record the unknown number on your lab card. The bacteria are growing in thin film on the agar's surface. Obtain the sample gently, as demonstrated:

1. Sterilize the inoculation loop in the bactoincinerator.
2. Remove the cap from the slant tube and flame the top of the tube by passing the top of the test tube through the flame.
3. Insert your inoculating loop into the test tube without touching the sides of the tube and gently drag the loop along the slant for about ¼ of an inch. Careful not to gouge the media or get agar in your sample.
4. Remove the inoculating loop from the tube again using care not to touch ANYTHING. This loop contains live bacterial cells and is VERY HAZARDOUS.
5. Pick up the media side of the TSY plate you poured that is now at room temperature. Inoculate quadrant #1 as indicated in Figure 2:

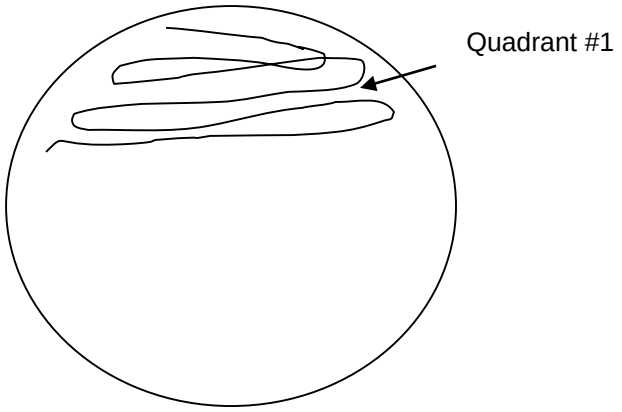
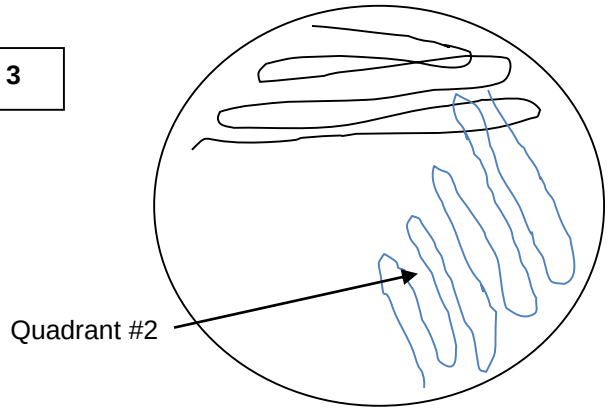


FIGURE 2

6. Sterilize your inoculating loop.

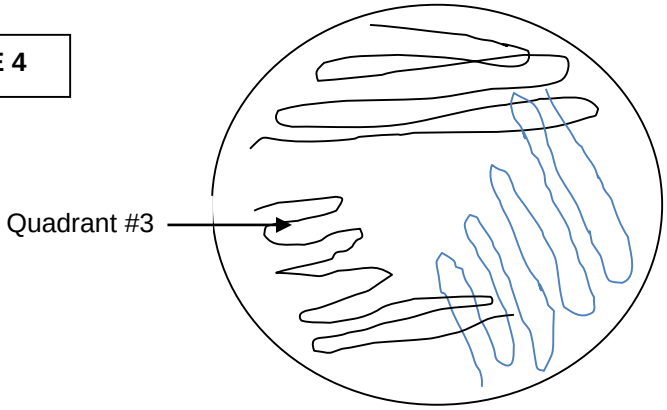
7. Allow the loop to cool and then tease cells out of quadrant #1 into quadrant #2 by beginning the streak in quadrant #1, and then moving into quadrant #2 as demonstrated and indicated in Figure #3:

FIGURE 3



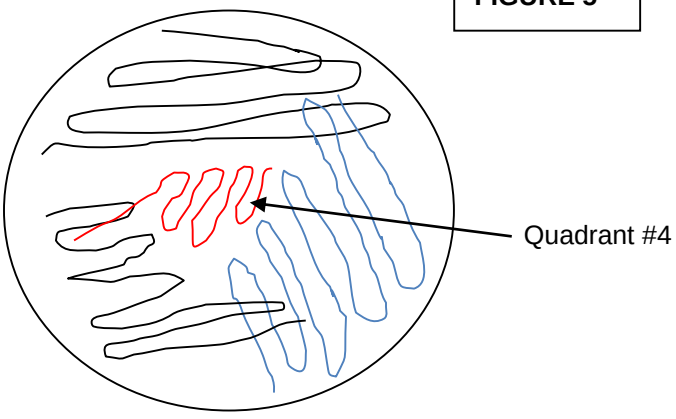
8. Sterilize your loop and streak quadrant #3 in the same manner as quadrant #2 (as demonstrated and as indicated in Figure 4). Notice the loop did not touch quadrant #1.

FIGURE 4



9. Sterilize your loop and use the same technique to inoculate Quadrant #4 as indicated in Figure 5 (note the loop only contacts quadrant #3 one time and does not come in contact with any other quadrant).

FIGURE 5



10. Label your plates. (Identifying specimens in the clinical setting is very important. Observe that when writing on media plates, specimen collection cups or culturettes, you are directed to write on the portion of the plate that cannot be displaced. This same technique is used throughout the clinical setting. Writing on a lid that can be exchanged for a lid from a different culture plate would lead to error.) See Figure 1.

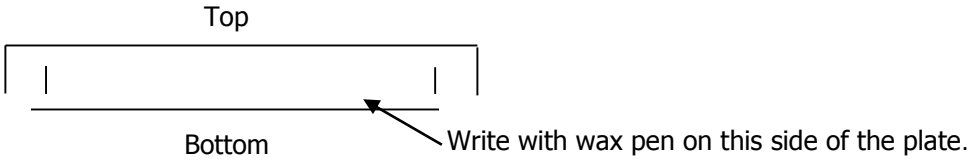


Figure 1: Plate Labeling

11. Place the plate upside down (media side up) in the green bin in the back of the lab. It will be incubated at 37° C for 24 hours and stored until you lab next week.

C. Prepare the 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.

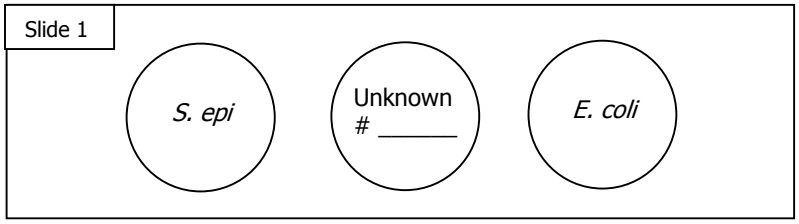


Figure 6: Gram Stain Slide Set-up

D. Perform the Gram Stain

1. Take the heat fixed Gram slide to one of the staining racks located at the benches with sinks. Flood each slide with **crystal violet** and allow to stand for **one minute**. Rinse the slide with water.
2. Cover the smears with **Gram's iodine** solution and let stand for **one minute**. Rinse.
3. Flood the smear with **95 % acetone alcohol** for **15-20 seconds**; rinse. This is a critical step. Decolorization occurs when the alcohol flows colorless from the slide. Thick slide will require more time (closer to 20 seconds) than thin ones (10-15 seconds). Over decolorization may result and then all the bacteria will appear Gram negative.
4. Flood the slides with **safranin** for **one minute**. Rinse and blot dry.
5. Examine slide under oil immersion. See "Viewing Bacteria Under Oil Immersion" below, or the PPT slide of Instructions for Viewing Bacteria Under Oil Immersion.

E. Observe the Bacteria Under Oil Immersion

You will be viewing your differential stain slides using the microscope. You will need to view each differential stain (Gram, Acid-fast and Endospore) using the oil immersion objective of the compound light microscope. When looking at your differential stain slides, always start by viewing

the controls, since you know what these "knowns" should look like. Look at the unknown after examining the controls. Below is a review of how to view objects under oil immersion.

1. Place the specimen on the slide and secure the slide to the microscope using the stage clip. Check that the slide is held by the stage clip by moving it with the mechanical stage control.
2. Click the 10X objective lens (yellow band, total magnification 100XTM) in place.
3. Use the mechanical stage control to position the specimen over the light source.
4. Use the coarse adjustment to raise the stage to its highest position. Looking through the microscope ocular, turn the coarse adjustment slowly away from you, lowering the stage until your specimen comes into focus. Remember to adjust the iris diaphragm for the best image.
5. "Fine tune" your image with the fine adjustment knob.
6. Make sure the 40X objective (blue band, total magnification 400XTM) is covered with a finger cot to protect it from oil.
7. (**Note**: When focusing on a bacterial sample, always start at low power (100xTM) and work your way up. This not only helps find and focus on specimens quickly but also alleviates the potential of ramming a long, oil immersion objective through a slide when trying to focus with the course adjustment knob under high power. Pay attention to the **working distance** of the lens. This is the distance between the lens and the slide when the specimen is seen in sharp focus. The higher the magnification, the smaller the working distance. To avoid ramming a long objective into a slide, observe the **Working Distance Rule**: Use the coarse adjustment knob on low power only.)
8. You will use the oil immersion objective (100X) **after** you have focused your scope on your specimen using the 10X objective lens. If you have any difficulty finding a specimen at 100X, **always** go back to low power (10x) and begin the focusing process again.

    a. Move the nosepiece so that you are in between the high power and oil-immersion objective.

    b. Before moving the oil immersion objective into position, place a small drop of immersion oil on the slide over the area where the light source illuminates the specimen.

    c. Click the oil immersion objective into place. When the objective is moved into position it should be in contact with the immersion oil. Check that this is so. Using **fine focus only**, sharpen the image. Other helpful hints:

    - do not use immersion oil that is cloudy
    - keep your eyepiece and objectives clean; use only lens paper when cleaning
    - do not tilt your scope

    You should be very cautious when using oil immersion objectives and immersion oil. First, be sure you are using immersion oil, and not another kind of oil. Only the oil objective should come in contact with the oil. The other non-oil objectives on your microscope will not work properly if they get oil in them, which will happen if they are dragged through oil on a slide. Be sure you do not move from an oil immersion lens to a 40X objective, because the 40X objective will drag through the oil, and be ruined by the oil. The oil needs to be cleaned from the oil-immersion objective after use, and this is done using a special paper (lens paper) that will not scratch the lens.

9. Take micrographs of specimens viewed at 1000xTM where instructed to in your Lab Report.

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