

SOP-103

---

# Aseptic Technique

---

Procedures for working safely with biologics.

---

Agar plates with required antibiotics

## Bacterial Stocks

Glycerol stocks stored at -80 C

Lyophilized stocks that have to be re-suspended in media

Liquid cultures

## Gram Stain

crystal violet

safranin

## **Common Laboratory Techniques**

During the course of these laboratory sessions, you will be expected to become proficient in the performance of the following laboratory techniques:

- Isolation of pure bacterial colonies
- Gram stain.

Development of competency in these techniques requires that you also know how to:

- Flame a loop
- Streak a plate for isolated colonies
- Use a light microscope.

Each person is responsible for his microscope and is expected to clean the oil immersion lens at the end of each lab in which it is used.

The methods on the following pages will be a useful reference for each laboratory session.

## **Aseptic Technique**

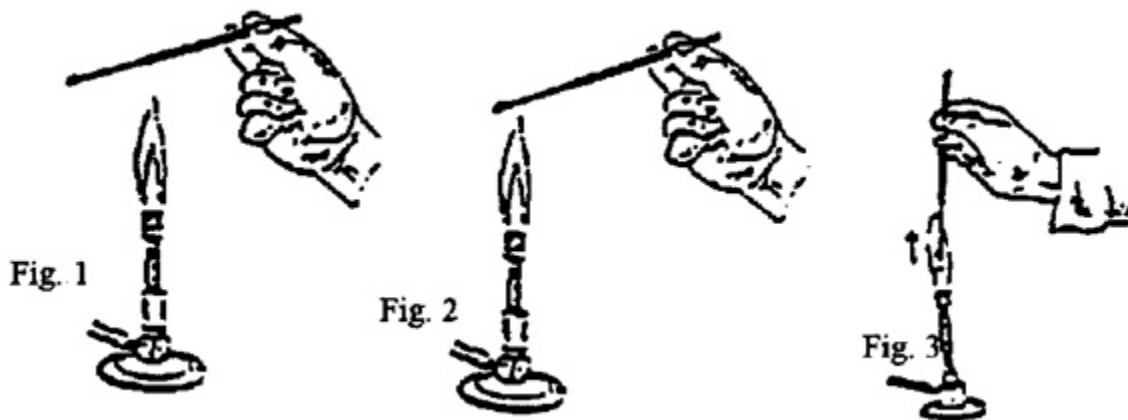
Aseptic techniques will be used in these laboratories. In the preparation of a microscope slide for staining and in plating a specimen for isolated colonies, it is necessary to observe the rules of aseptic technique to assure that contaminants are not introduced into a specimen. On a more personal note, adherence to aseptic technique assures that infectious agents are not spread to you, fellow students, or the laboratory surfaces.

The following general rules should be adhered to when working in the microbiology laboratory.

1. The inoculating loop is usually used for making transfers of bacterial cultures. Instructions for the proper technique used to flame a loop with a Bunsen burner are provided on the following page. Allow the loop to cool sufficiently so that any organisms to be tested will not be killed by the hot wire, but do not allow the loop to contact anything during the cooling period or contamination will result.
2. Learn to remove and replace the closures (usually caps) of the test tubes with the same hand that holds the loop. The caps must be held during the entire procedure and never placed on the desktop or contamination will result.
3. After the transfer is completed the loop must be sterilized again. Follow the procedure outlined on the following page in Figs. 1-3 to prevent splattering of infectious materials.
4. Always work sitting down.
5. Attention to details and practice will allow you to work both rapidly and accurately.

## FLAMING A LOOP

Heat from the **base** of the wire first (Fig. 1) and slowly move towards the loop (tip) (Fig. 2). Heat the wire until it is red-hot (Fig. 3).



Remove caps from liquid specimens and replace the caps of the test tubes with the same hand that holds the loop. The caps must be held during the entire procedure, as shown below in Figures 4-6, and not placed on the desktop or contamination may result.

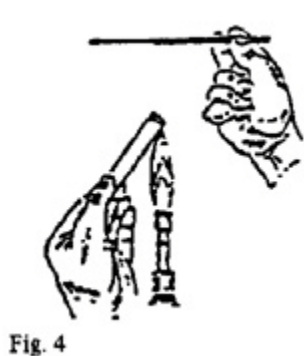


Fig. 4



Fig. 5

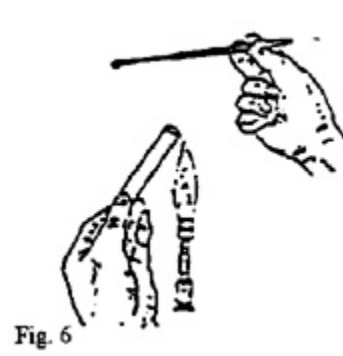


Fig. 6

Flame the neck of the tube (as in Fig. 4). If using a plastic tube, flame briefly to avoid melting the opening of the tube. Remove a loop full of the culture with the cooled loop (Fig. 5) and briefly flame the opening of the tube again (Fig. 6).



Fig. 7

Return the cap to the specimen tube (as in Fig. 7) and transfer the inoculum from the loop to the surface of an agar petri dish or microscope slide. Then flame the loop as in Figures 1-3 above before placing on a counter or in a rack.

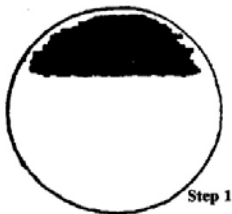
## LABORATORY CULTIVATION AND ISOLATION OF BACTERIA

Since accurate studies of the biochemical and the antigenic properties of a bacterial species are possible only through the use of pure cultures, it is necessary to have a reliable and rapid method that will permit the isolation of organisms. An inoculum from the specimen is streaked on solid agar in a manner, which physically separates most of the bacterial types, permitting them to form discrete colonies. This procedure is facilitated whenever possible by the use of either a selective medium that inhibits the growth of species not sought or by the use of a differential medium, which imparts a recognizable appearance to the colonies of the type sought. It is possible that colonies of

the bacterial type selected for by the selective medium will be contaminated with bacteria of a different type that are inhibited from growing but not killed by the selective medium. Upon transfer of this mixed colony to a medium without the inhibitors, both types of bacteria may grow, and a pure culture will not be obtained. Consequently, it is often necessary to streak a second plate of the same selective medium with a colony from the first selective plate in order to obtain a pure culture of the bacterial species that you are attempting to isolate.

The method used most often for colony isolation from a specimen or mixed culture utilizes the four-phase streaking pattern described in the following page.

## COLONY ISOLATION



**Step 1.** Using a sterile loop, streak cultures (liquid broth or isolated colonies picked from plates) over one-fourth of the surface of an agar plate. Then flame the loop as described on the preceding page.



Swabs containing an inoculum should be rolled over a small area of the agar surface to deposit the inoculum and then distributed over one-fourth of the surface with a sterile loop. The swab can then be used to prepare a stain or discarded.

**Step 2.** Air cool a flamed loop or cool it by touching an unstreaked area of agar on the same plate.



**Step 3.** Pass the cooled loop three or four times over the initial streaked portion of the plate. Streak it, without overlap, to the next quadrant.

**Step 4.** Flame the loop and allow it to cool as described above in Step 2.



**Step 5.** Pass the loop over the streaked portion of the second quadrant two or three times and then streak the material without overlapping over the third quadrant of the plate.

**Step 6.** Repeat Step 5 to streak the last quadrant.

Most bacteria do not move appreciably from the sites of inoculation but give rise there to clones of bacteria called colonies. Isolated colonies should arise in the third and fourth quadrants depending on the concentration of bacteria in the initial inoculum.

## **THE INITIAL EVALUATION OF BACTERIAL GROWTH ON AGAR SURFACES**

Some media support the growth of selective organisms (e.g. only gram-negative or only gram-positive). Growth on certain media gives preliminary information about specific biochemical characteristics (e.g. ferments or does not ferment a particular sugar) that lead to subsequent steps to take in the definitive identification of the organism.

Early preliminary information regarding a culture results can often be provided after observing the growth and colony morphology on a specific agar media.

Microbiologists routinely make note of the colony characteristics of bacterial growth on agar plates. The success or failure of bacterial identification procedures often depends on the accuracy of the initial observation of colony characteristics. Some of the criteria frequently used to characterize bacterial growth on agar media include:

- Colony size (using relative terms such as pinpoint, small, medium, large)
- Colony color
- Colony shape (describing the form, elevation, and edges of a colony)
- Colony surface appearance (e.g. dull, opaque, moist/glistening)
- Changes in agar media resulting from growth (e.g. hemolytic pattern on blood agar, changes in media or colony color due to a change in pH indicators, pitting of agar surface)
- Odor (certain bacteria produce distinct odors)

## **GRAM STAIN**

### **Importance of a Gram Stain**

A Gram stain of a direct smear is a rapid, relatively inexpensive and productive way to initially screen specimens. Gram stains also play a key role in the characterization of organisms that have been cultivated in the laboratory.

## Gram Stain Principle

The Gram stain divides bacteria into two classes, gram-positive and gram-negative. The thick peptidoglycan layer of Gram-positive organisms allows these organisms to retain the crystal violet-iodine complex and stain purple. Gram-negative bacterial cell walls consist of a thinner layer of peptidoglycan and lose the crystal violet-iodine complex during the alcohol rinse. Gram-negative bacteria stain with safranin and appear red.

Most clinically important bacteria can be detected using the Gram stain. Exceptions are organisms that exist almost exclusively within host cells (e.g. chlamydia), those that lack a cell wall (e.g. mycoplasma and ureaplasma), and those too small to be resolved by light microscopy (e.g. spirochetes). Factors that may alter the true Gram reactivity of a bacteria include loss of cell wall integrity because of antibiotic treatment, age of the bacteria, action of autolytic enzymes, and as mentioned in Step 3 of Gram Stain Procedure, overheating the slide during heat fixation.

## Gram Stain Procedure

1. Use a sterile slide or pass one side of a clean glass slide through a flame several times. Allow the slide to cool before smearing with a specimen.
2. For bacterial suspensions in broth: Apply 1 loop of broth onto the cleaned slide. Dilute heavy suspensions by applying less than one loop to a drop of sterile water or saline on the slide. Spread the fluid to form a film about one centimeter in diameter. Excessive spreading may result in disruption of cellular arrangement. A satisfactory smear will allow examination of the typical cellular arrangement and isolated cells.

For bacterial colonies: Use water or saline to emulsify a colony or portion of colony on the previously flamed side of the slide. Only *a very small* amount of material from an isolated colony is needed for a gram stain (a loopful of a colony is excessive).

For inoculum on swabs: *Roll* the swab over the cleaned surface of a glass slide. To avoid contamination of culture media, discard the swab used to make the slide and use a second swab containing the inoculum to inoculate media.

The most common flaw in smear preparation is application of too much material on the slide. Broth cultures with visible growth contain at least  $10^6$  bacteria/ml. Similarly, depending on size, one bacterial colony can be equivalent to  $10^5$  bacteria. Excessive material interferes with the passage of light through the specimen, prevents adequate decolorization, and interferes with the ability to view single cells and to determine the cellular arrangement present in the specimen or culture.

3. Air dry and heat fix the slide by passing it through a flame two or three times.



DO NOT OVERHEAT the slide as protein in the specimen can coagulate and cellular morphology may appear distorted from excess heat.

4. Using the slide holder provided (it looks like a big clothespin), clamp the slide and suspend it, with specimen side up, over the sink. Flood the slide with crystal violet and allow it to remain for 1 minute. Rinse the slide gently with cold tap water.
5. Apply Gram's iodine and allow it to remain for 1 minute.
6. While holding the slide at a slight angle, allow drops of alcohol to run over the smear (3-4 seconds). Quickly rinse well with water.
7. Apply safranin and allow it to counterstain for 30 seconds. Rinse with water until all free stain is removed. **Blot** (DO NOT WIPE) the slide dry with bibulous paper.
8. Examine the smear under oil immersion (you must use oil). NEVER determine the morphology or staining reaction of bacteria with any objective other than oil immersion.

## Gram Stain Examination

Using the oil immersion lens, examine the smear for presence of bacterial cells. Note the Gram reaction (e.g. positive or negative), morphology (e.g. cocci or bacilli), and arrangements (e.g. single cells, pairs, clusters, or chains) of the cells seen.

### Gram-positive organisms (stain purple)

#### 1. Gram-positive cocci

- a. Staphylococci -spheres occurring in irregular clusters, singly, in pairs, or in short chains.
- b. Streptococci – round or oval-shaped usually occurring in pairs and short, or long chains. These cocci are generally slightly elongated.
- c. Pneumococci –resemble streptococci. Distal ends of paired organisms may be lancet shaped. In direct smears and sometimes from a culture, a halo (capsule) may be observed.
- d. Yeast- large ovoid to spherical forms often occurring in clusters. Budding forms may be observed.

#### 1. Gram- positive rods

- a. Corynebacterium- occur as straight or slightly curved rods. They vary greatly in dimensions and can occur in parallel, or "V", "L", and "Y" arrangements. Often referred to as diphtheroids.
- b. Bacillus spp. (aerobic spore-formers)- large, fat bacilli occurring singly, in pairs or chains. They frequently stain gram-variable (both pink and purple). Spores generally do not stain but appear clear and can give the bacilli a "moth-eaten" appearance.

### 3. Gram-negative organisms (stain red)

#### a. Gram-negative bacilli

Examples: Enterobacteriaceae, *Pseudomonas* spp. and *Haemophilus* spp.

The Gram-negative bacilli often have similar characteristics in the Gram stain; therefore, it is impossible to even presumptively identify them strictly on the basis of stained appearance. Generally, the *Haemophilus* can be coccoid or bacillary (sometimes short round-ended rods referred to as a coccobacillus) and the Enterobacteriaceae and *Pseudomonas* spp. are larger and more elongated in shape.

#### b. Gram-negative cocci

Spheres occurring singly and in pairs. *Neisseria* and *Moraxella* spp. often have a "coffee bean" shape where the adjacent surfaces of the pair are flattened.

## USE OF THE MICROSCOPE

### Focusing Procedure

1. Place slide on stage.
2. Rotate the nosepiece to the 10X objective (low power lens).
3. Using the coarse adjustment, lower the nosepiece to its lowest limit.
4. Using the fine adjustment control knob, bring the image of the slide into focus.
5. Swing the low power lens out of position; place a drop of immersion oil on the slide; swing the oil immersion lens (100X objective) into position.
6. Focus with the fine adjustment knob.
7. Raise the condenser as high as it can go to improve the image contrast.

### Adjust the diaphragm

8. Pull out the eyepiece.
9. Look down through the barrel; close the condenser diaphragm until its edge can just be seen. The aperture of the condenser and objective are then approximately equal. This position gives the optimum optical image. Replace the eyepiece.

### Cleaning the microscope

When finished, clean the oil immersion lens with lens paper. Never touch the lens with anything else. The lens paper can be moistened with lens cleaner if necessary.

Section

3

## References

---

### References