The graphic consists of a vertical rectangle on the left with the word "Appendix" written vertically in white on a dark teal background. To its right is a larger teal square containing a white letter "A".

# General Microscopy and Aseptic Techniques

## **INTRODUCTION**

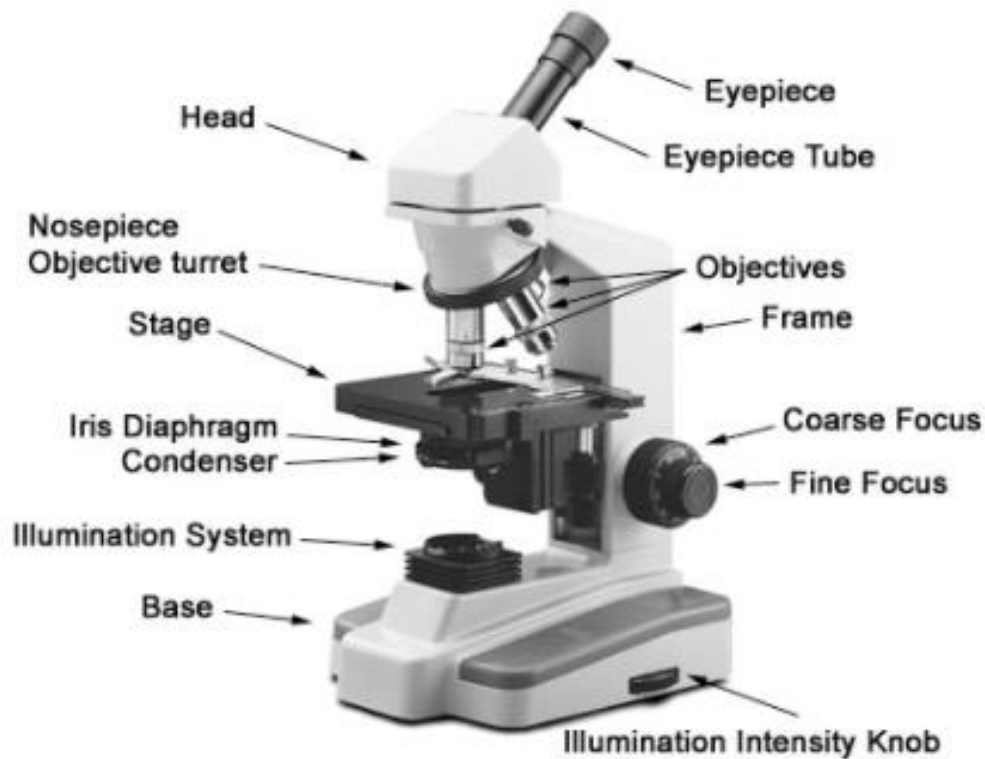
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One of the most important aspects of the microbiology lab is safety, for yourself and your lab partners. Besides the hazards of chemicals and fire, you must also be aware of the potential hazards of microbes if and when they are mishandled.

Throughout the course, you must assume that **all** microbes are potentially hazardous. Microbes enter the body primarily by entering the mouth. It is therefore imperative to avoid drinking and eating in the lab. Microbes may also enter the body by way of the bloodstream. As a result, wearing protective clothing and proper protective equipment, such as gloves, may help protect you against any contamination risks. It is also paramount to sanitize/disinfect your work area **before** and **after** each laboratory sessions in order to prevent the spread of contamination.

### **A.1 MICROSCOPY OVERVIEW**

The microscope is a major component of this course where we are required to observe organisms that are significantly smaller than human cells. To view small structures, we use a bright field, binocular, compound microscope. These microscopes use a series of lenses to achieve the proper magnifications of up to 1000 times to better observe shapes, sizes, and structures. It is therefore essential that this instrument is used and cleaned properly. Figure 1 shows a detailed image of a compound microscope; make sure to familiarize yourself with every component.



**Figure 1.** The compound light microscope

The compound light microscope we use in the lab consists of four **objective lenses**. The objective lens can be swiveled into place and allow an observer to see objects magnified by the magnification indicated. The microscopes used in this lab have four objective lenses, 4X, 10X, 40X, and 100X. The 4X and 10X objectives are considered the *low-power* lens, which are used as primary lens to initially focus objects on the slide. The *high-dry* 40X lens is used to select a field of interest before increasing to the 100X lens. Finally, the 100X objective oil immersion lens allows you to see 1000 times magnification and is the only lens requiring oil to properly see.

The **ocular lens**, or **eyepiece**, typically has a magnification of 10X that will magnify objects 10 times its size. In order to calculate the exact magnification of an object being observed, you must multiply the constant ocular lens magnification of 10X by the magnification of the objective lens.

**Calculating total magnification:**

Objective lens magnification X Ocular lens magnification

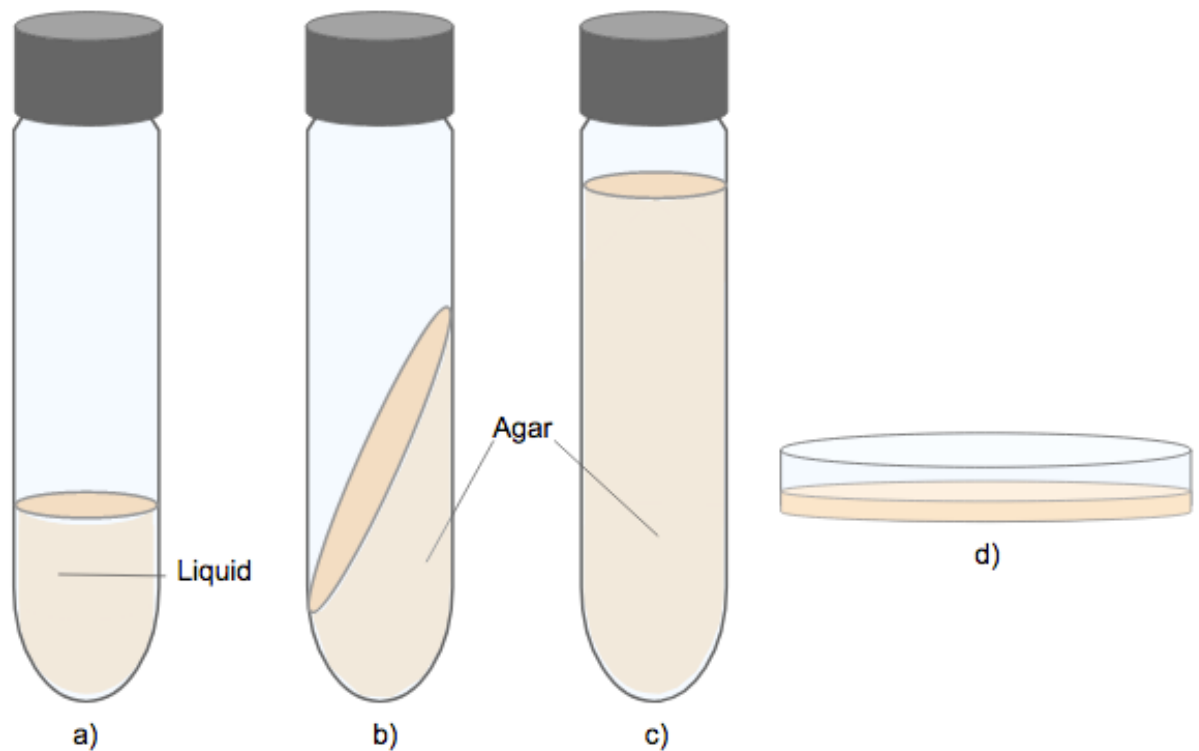
The **coarse** and **fine adjustments** are knobs located below the microscope stage. The coarse knob is the large of the two and is **only to be used when the lower power lens are in place**. If the coarse knob is used with higher power lens damage to the lens and the slide will occur.

The fine adjustment knob is the smaller knob located on top of the coarse knob and used only when using high powered lens such as the 40X and 100X objective lens.

## A.2 CULTURAL CHARACTERISTICS OF BACTERIA

Bacteria in the lab are often grown in either **broth** (liquid) culture or on solid agar (Figure 2). Depending on the goal of the experiment, specific growth media is used. Broths are used to grow large numbers of bacteria while **agar slants** are used for long term storage. **Agar deep** tubes are used to grow organisms that require low oxygen, and finally **agar plates** are used when a large surface area is needed in order to streak and separate bacteria.

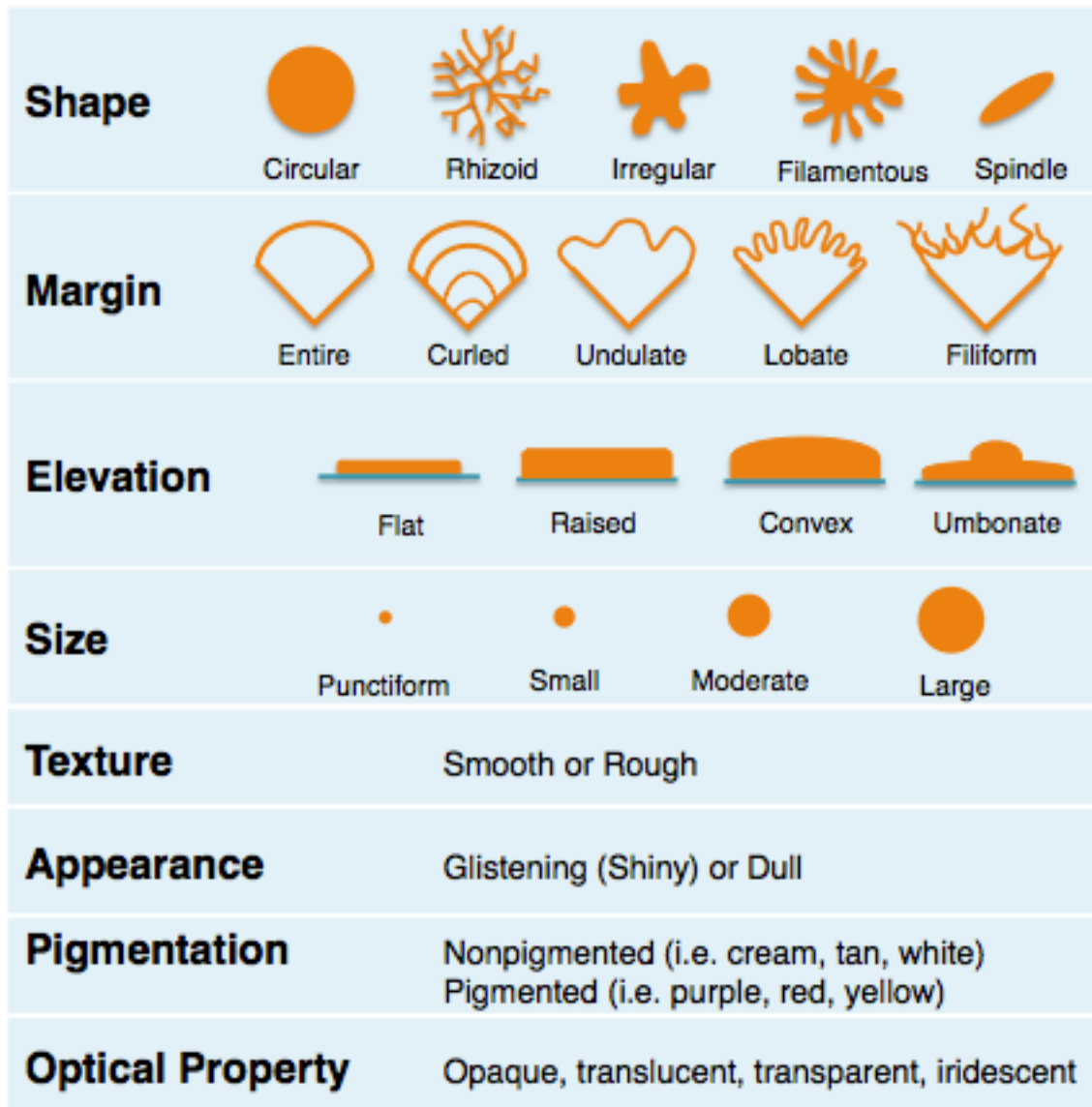
Bacteria have various morphologies, or shapes, both microscopically and macroscopically on various types of media. In other words, depending on the media type (Figure 2) bacteria will display different types of growth patterns especially on plates (Figure 3) and in broth (Figure 4).



**Figure 2.** Diagram of culturing media in different forms including a) liquid broth, b) agar slant, c) agar deep, and d) agar plate.

On agar plates bacteria can take on various interesting shapes, margins, elevations, sizes, textures, colors, and even appearances (shiny or

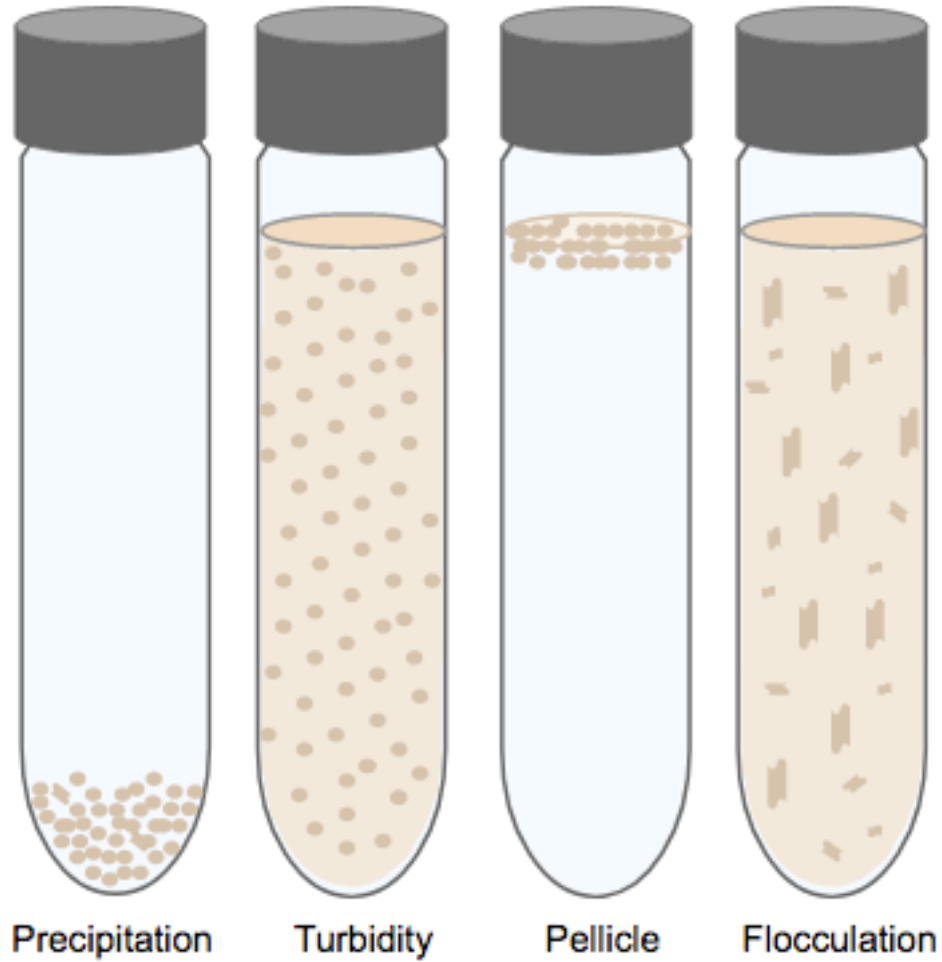
reflective). *Figure 3* depicts a few characteristics that will be important in describing bacterial colonies.



**Figure 3.** Bacterial colony morphology on solid agar plate.

In liquid broth, bacteria can take on various growth patterns as well. Depending on the nature of the broth, temperature incubated, and relative

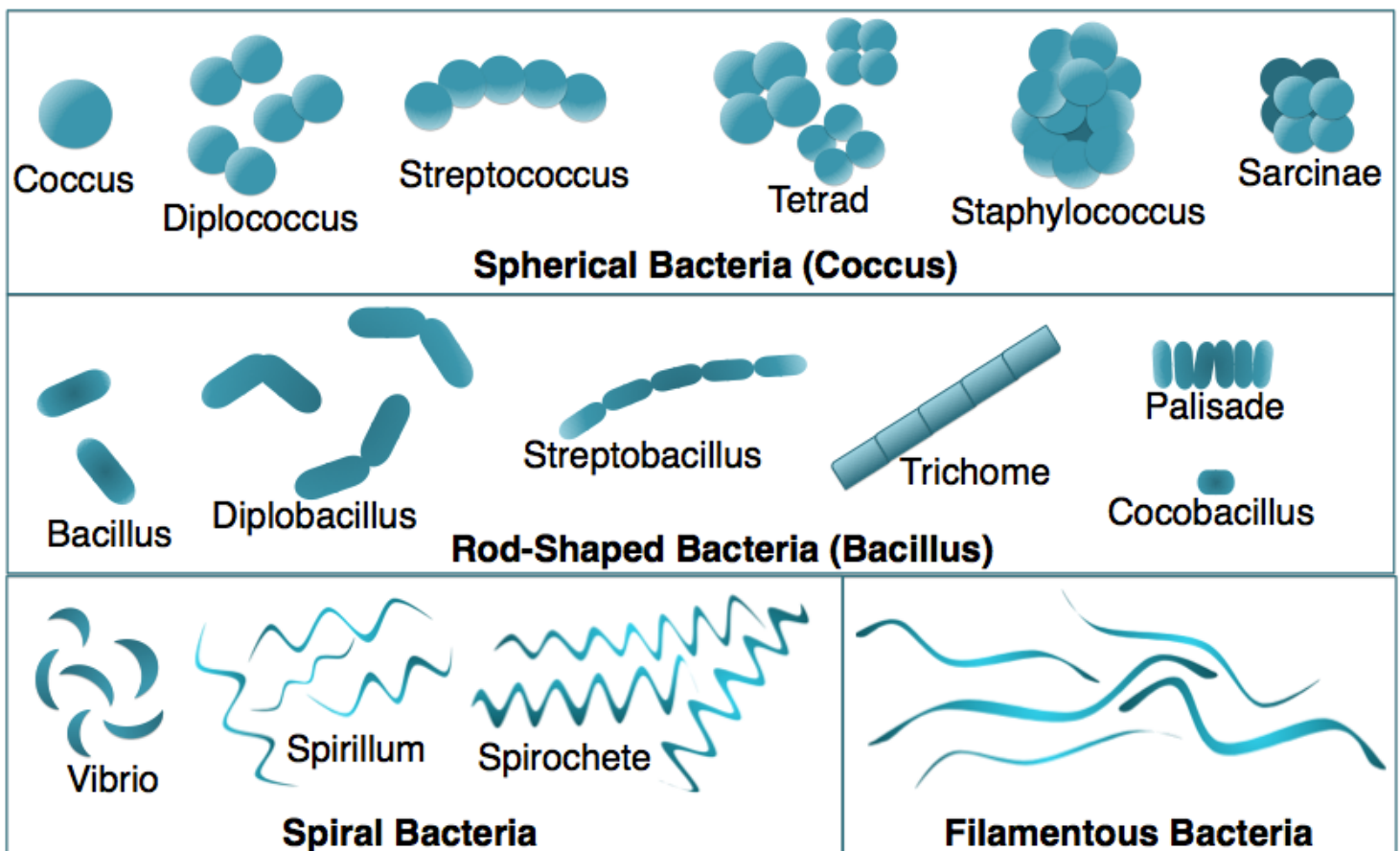
growth of the bacteria different patterns emerge including precipitation, turbidity, flocculation, and pellicle or ring formation. Figure 4 depicts the different growth pattern you may encounter.



**Figure 4.** Bacterial growth characteristics in broth.

### A.3 MICROBIAL CELLULAR MORPHOLOGY

A good method of distinguishing between different groups of bacteria is to use cellular morphology and arrangement. Bacteria can be separated into three groups depending on cellular morphology, or basic shape of a single cell, including coccus, bacillus, and spirillum. These general groupings can be further distinguished using their cellular arrangements, or pattern formed by a collection of cells. Figure 5 depicts the varying cellular morphologies and arrangements among common bacterial groups.



**Figure 5.** Cellular morphology and arrangement of various bacterial groups.

## A.4 ASEPTIC TECHNIQUES

In the lab, you must work in a very clean manner in order to keep bacteria, fungi, algae, viruses, and other microbes under control. **Aseptic** (sterile) techniques involve the use of different careful procedures designed to prevent microbial contamination. There are different methods of rendering your work area safe and to minimize the risk of any microbial threats including sterilizing your loop, properly heat fixing a slide, properly transferring microbes, and flaming your tubes. The following sections discuss each technique in detail. Aseptic techniques include: 1) Loop Sterilization, 2) Heat Fixation, 3) Culture Transfers, 4) Plate Streaking, and 5) Plate Spreading 6) Plate Counting.

### I. Loop Sterilization

A **loop**, or **inoculator**, is a thin, twisted metal wire that is attached to a handle used to transfer the inoculum into various growth media. The loop must be flamed before and after all procedures. Proper loop sterilization destroys the all microbes present on the loop. Figure 6 demonstrates the proper way to sterilize your loops. During sterilization, the loop is heated until it turns red hot to ensure complete elimination of microbes. Make sure to never set the hot loop down or it will burn through the material and become contaminated as well.

**Figure 6.** Loop sterilization



### II. Heat Fixation

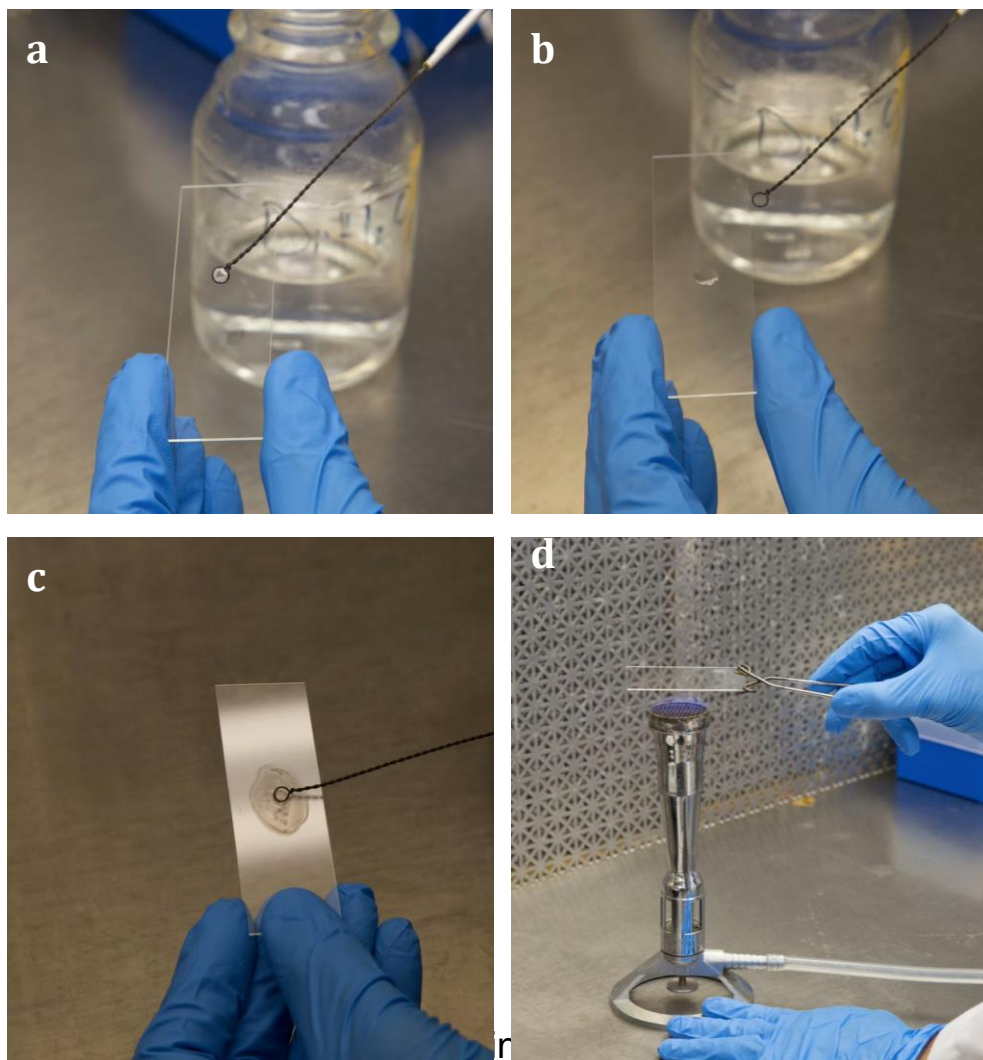


In order to work effectively and safely with microbes, the bacteria must first be secured onto the slide using the **heat fixed smear** protocol (Figure 7). A heat fix smear involves smearing the bacteria colony onto a slide and allowing to first air dry. This drying step is vital in that if a wet bacterial mount is heated, the cells will boil away and destroy the cell walls. Once dry, the slide is then heated through a flame so that the bacteria are killed and secured on the slide. This fixation process renders potentially pathogenic bacteria on a slide safe for staining procedures.

When preparing a slide with bacterial cultures, you will encounter bacteria growing in different types of medium including culture slants, plates, and broth (Figure 2). Each type of medium requires a different method of preparation onto a slide.

***Procedure for a Heat Fixed Smear:***

1. Obtain a clean dry slide. For bacteria cultures growing on slants and plates, place a drop of water onto the slide using a loop (Figure 7a, b).
2. Use a sterile loop to then pick up the bacterial growth and smear it onto the drop of water to properly dissolve, then let air dry (Figure 7c).
3. For liquid broth medium, use a sterile loop to pick up a loopful of culture broth and transfer directly onto a dry slide, and then air dry completely.
4. Quickly pass the slide through the flame several times (Figure 7d).

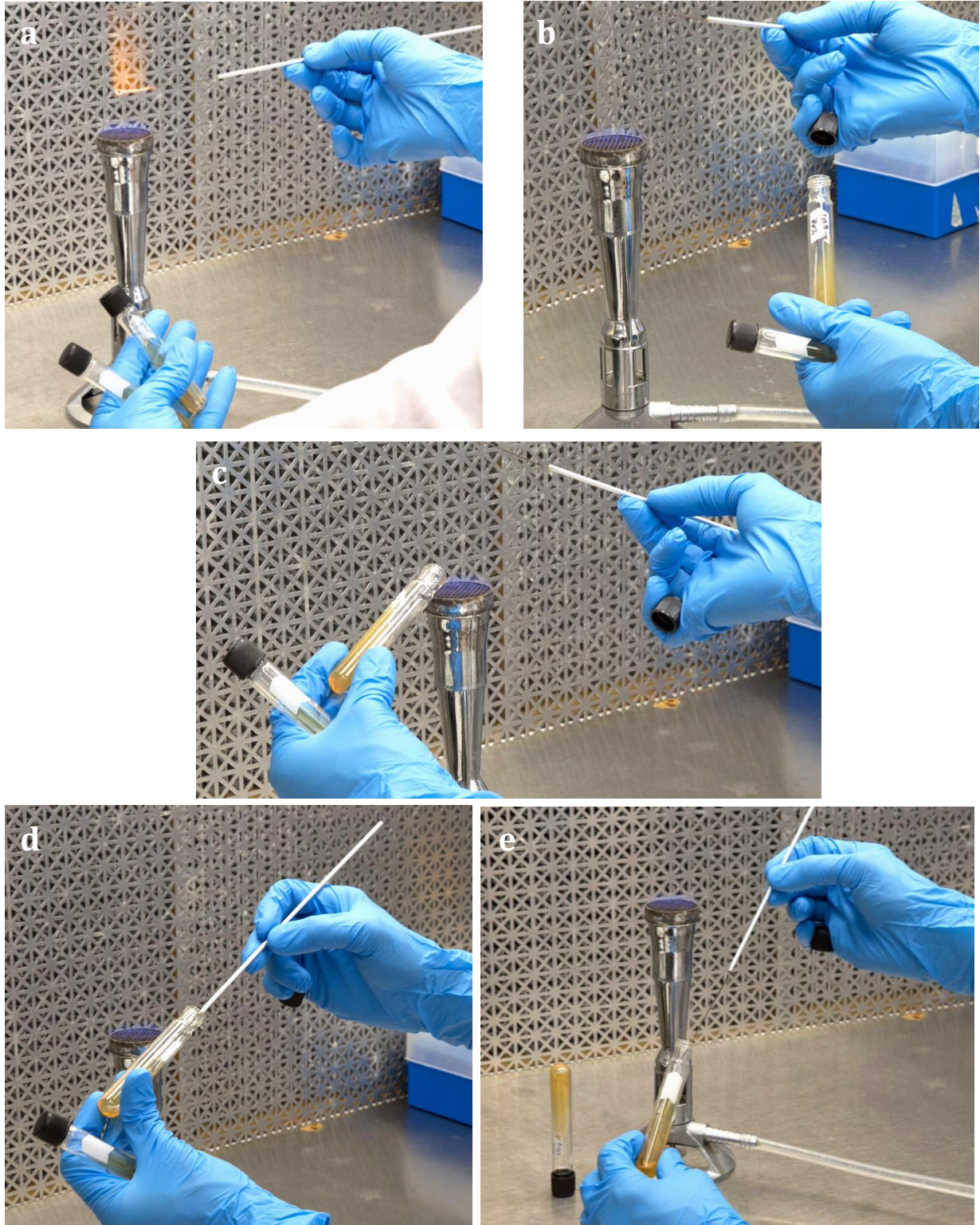


### III. Culture Transfer

In this lab, when transferring microbes such as bacteria or fungi from growth medium, the media utilized will often vary, and the technique used to transfer the microbe must be correct. Based on the form of culture adopted (Figure 2) there can be tube-to-tube, broth-to-broth, broth-to-slant, and slant-to-broth transfers. For the most part, the techniques are very similar where you must hold caps with your pinky finger, transfer, and flame the tubes in each case.

#### ***Culture Transfer Procedures:***

1. Hold both tubes in your left (non-dominant hand). Make sure the tube caps are loosened for easy removal.
2. With dominant hand, grab inoculator loop and flame until red (Figure 8a).
3. Open the tubes, and hold the tube caps with your pinky finger of your dominant hands (Figure 8b).
4. Briefly flame the lips of the tubes, and carefully place cooled loop into tube. DO NOT touch the lips of the tubes (Figure 8c).
5. Carefully place the loop with the inoculum into the sterile tube (Figure 8d). May also transfer aseptically from a cultured agar plate.
6. If the tube contains broth, gently stir the broth with the loop containing the microbe. If the tube is a slant, gently scribble back and forth onto the agar surface with the loop containing the inoculum (Figure 8d).
7. Flame the mouth of the test tubes again and replace the proper caps onto each tube.
8. Flame your loop until red-hot again.



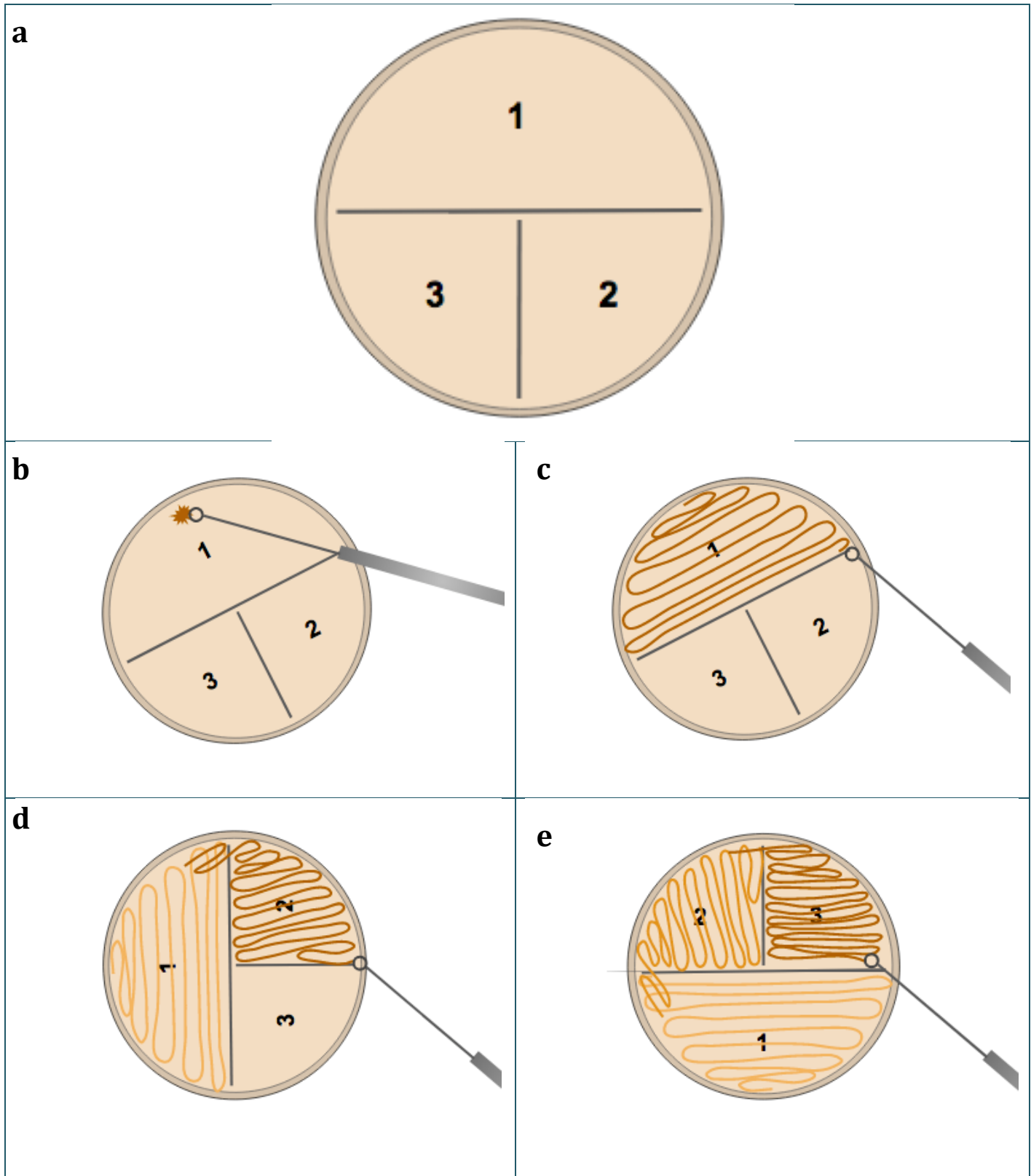
**Figure 8.** Procedure for transferring cultures

## IV. Plate Streaking

When working with microbes, microbiologists must be certain that the test organism is always be free of contamination. Therefore, we must continually make sure that our cultures are pure cultures. **Pure cultures** are those derived from one microbe type where all the microbes are the exact same in the medium. In order to make sure our cultures are pure, in this lab, we use the streaking methods to separate individual cells from a large sample of microbes. The purpose of this technique is to get isolated colonies onto the agar surface.

### ***Streaking for Isolation Procedure:***

1. Place the agar plate upside down. With a marker, carefully label your plate into three quadrants as indicated in (Figure 9a).
2. Place a loopful of bacterial *broth* onto the first quadrant and allow to evaporate completely. Or if using a *slant* or *colony*, grab a loopful of bacteria and place on top of quadrant one (Figure 9b).
3. With the plate placed onto your lab table, use a sterilized loop to gently draw the loop across the surface of the agar in a zig-zag pattern. Make sure to avoid crossing the previous streak.
4. Continue to zig-zag until you reach the end of the first quadrant (Figure 9c).
5. Flame your loop and cool.
6. Draw the loop across the first quadrant once or twice, and continue to streak the second quadrant until you reach the end (Figure 9d).
7. Flame your loop and cool.
8. Draw your loop across the second quadrant once and continue to streak the third quadrant (Figure 9e). Flame your loop.



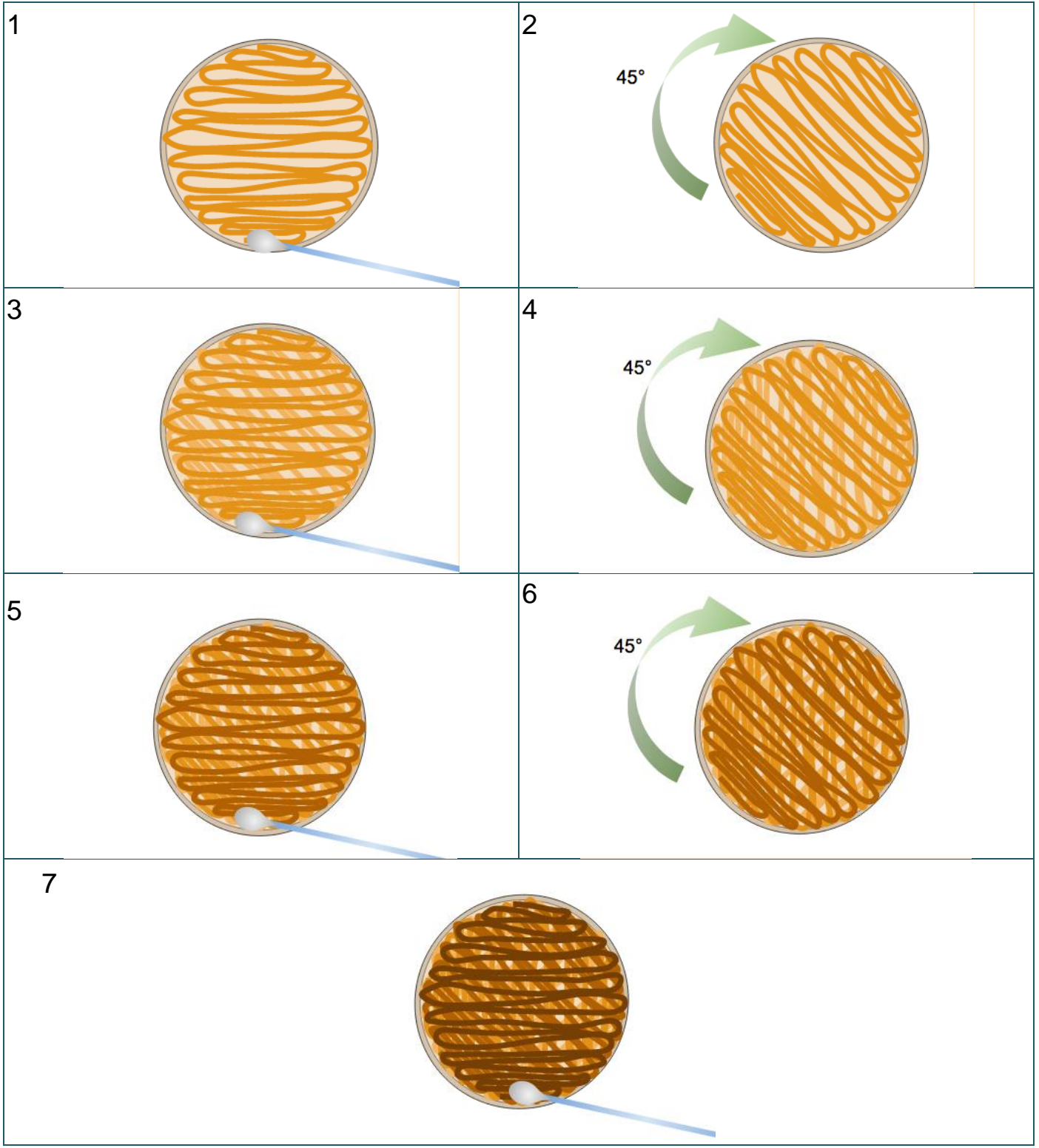
**Figure 9.** Procedure for Plate Streaking for Isolation

## V. Plate Spreading

At times, instead of using a streaking procedure, where the bacterium are isolated and separated on the agar surface, a plate spreading procedure is required. Plate spreading involves forming a single layer of bacteria inoculum over an agar surface in order to achieve a uniform “lawn” of growth. Spreading bacteria this way is useful for various tests including antibiotic susceptibility tests and others. Spreading a plate can be done using a cotton swab (Figure 10) soaked with the bacterial in broth, or alternatively using a plate spreader.

### *Procedure for Plate Spreading:*

1. Obtain a sterile cotton swab and dip into the broth medium containing bacteria.
2. “Color in” the agar surface using the cotton swab, making sure to cover the entire surface in a zig-zag motion.
3. Turn the plate 45° degrees right or left.
4. Swab the entire surface of the agar plate in a zig-zag motion, making sure to cover every inch. Turn the plate 45° degrees in the same previous direction. Repeat this procedure two more times until the entire plate surface has been equally swabbed. Refer to Figure 10. You will swab the plate the total of **FOUR** times, as depicted in the figure.



**Figure 10.** Procedure for plate spreading



## VI. Plate Counting; Enumeration of Titer

A common procedure in microbiology is to measure the total **viable titer** in a sample. The viable titer is the count of living cells in a given sample. The procedure involves making a dilution or serially diluting a sample and directly inoculating each dilution onto a plate and spread evenly using a spreader. A **dilution** is when a sample of interest is thinned out by adding another solvent. The dilution is calculated by dividing the volume of a sample being diluted into the diluent by the total volume amount of the sample added and the diluent.

$$\text{Dilution} = \text{Sample} / (\text{Sample} + \text{Diluent})$$

For example if you take 1 ml of a sample and add it to a 9 ml diluent you will have a 1:10 dilution or  $10^{-1}$

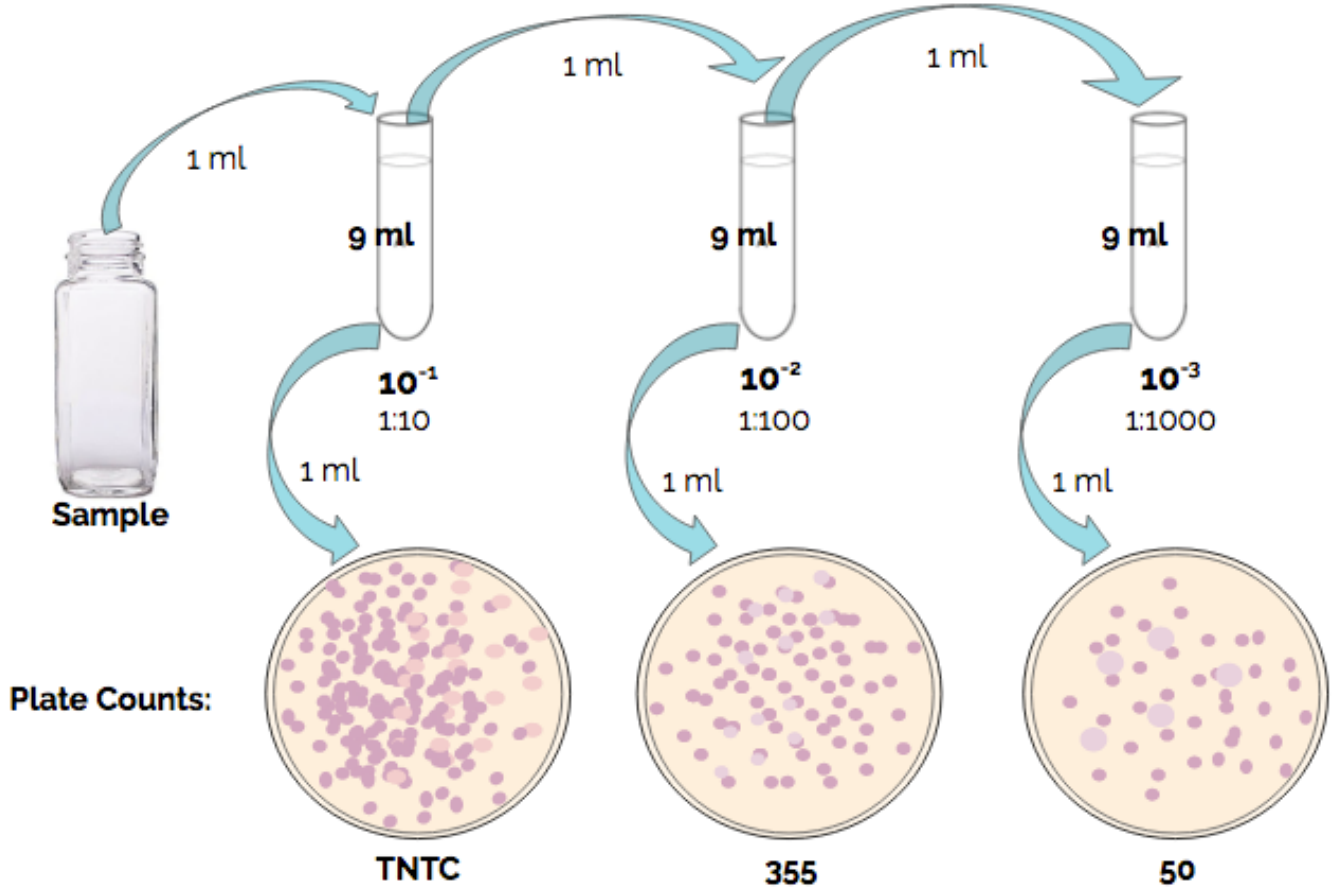
The Dilution Factor will be 10 divided by 1; which equals **10**

The Viable Titer is determined by counting the number of colonies on a plate, or colony-forming units (CFUs) per volume plated and multiply by the **dilution factor**. The dilution factor is the reciprocal or inverse of the **Dilution**.

$$\text{Viable Titer} = (\text{CFU} / \text{volume plated}) \times \text{Dilution Factor}$$

In order to reduce the concentration of the cells in a sample and facilitate counting the colonies, a stepwise dilution process called **serial dilution** is used. After proper incubation of the agar plates they are used to enumerate the total number of clearly separated colonies of bacteria. The viable titer is only properly determined if the inoculated plates are countable. A **countable plate** is one that comes from a dilution that yields at least 20 and no more than 300 colonies. Overcrowding results when some bacteria grow very fast and may take over the other slow growers, and results in incorrect viable titer. Figure 11 indicates how a serial dilution is calculated and performed. From Figure 11, the best countable plate that falls between the allowable CFUs (20-300) is the third dilution plate with 50 CFUs. To calculate this viable titer, one would divide the CFUs from the countable plate, 50, by the amount plated, 1ml which yields  $5.0 \times 10^1$  CFU/ml. The countable

plate came from a dilution of  $10^{-3}$ , therefore the dilution factor, or reciprocal, is  $10^3$ . And thus, the Viable titer of Figure 11 is  $5.0 \times 10^1 \times 10^3$ ; which yields  $5.0 \times 10^4$  CFU/ml.



**Figure 11.** Diagram of a serial dilution. (The volume of sample diluted and plated varies)