PURE CULTURE TECHNIQUES

I. OBJECTIVES

- To demonstrate good aseptic technique in culture transfer or inoculation and in handling sterile materials.
- To demonstrate skill in isolation of organisms from a mixed culture using selective and differential media.
- To isolate microorganisms from a wide variety of sources and describe their colonial morphology.

II. INTRODUCTION

Most environments carry a mixed microbial population. To fully appreciate the contribution of each group of organisms to the ecology of the mass, one must first dissect this mixed culture to obtain single colonies. The single colony is transferred (picked) to a fresh medium to obtain a larger, homogeneous culture that may be studied and characterized by a variety of techniques. One such technique is called aseptic technique. Microbiologists and health workers use this technique to prevent contamination of cultures from outside sources and to prevent the introduction of potential disease agents into the human body (infection can occur through contamination of your hands and clothing with material from your bacterial cultures).

Aseptic Techniques

Aseptic techniques (also called sterile techniques) are defined as the processes required for transferring a culture from one vessel to another without introducing any additional organisms to the culture or contaminating the environment with the culture. The following conditions must exist for aseptic technique to be successful:

1. The work area must be wiped with an antiseptic to reduce the number of potential contaminants.
2. The transfer instruments must be sterile.
3. The work must be accomplished quickly and efficiently to minimize the time of exposure during which contamination of the culture or laboratory worker can occur.

Developing a thorough understanding and knowledge of aseptic techniques and culture transfer procedures is a prerequisite to working with microbiological cultures. You will save yourself a lot of time and energy and avoid erroneous results if a few simple and common sense rules are observed when working with cultures.

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- Always sterilize your inoculating loop by flaming before using it to enter any culture material.

- Always flame the lip of the culture tube before inserting your sterile loop into the culture. This destroys any contaminating cells that may have been inadvertently deposited near the lip of the tube during previous transfers or by other means.

- Keep all culture materials covered with their respective caps and lids when not making transfers. Do not lay tube caps or petri dish lids on the tabletop, thereby exposing cultures to possible contamination. When transferring colonies from petri plates, use the lid as a shield by slightly raising it enough so that your loop can be inserted but the agar surface is still protected from contaminants falling upon it.

- Do not allow tube closures and petri dish lids to touch anything except their respective culture containers. This will prevent contamination of closures and therefore of cultures.

Bacteria are found in all parts of the laboratory environment--on the workbench, in the air, on your hands, etc. The precise methods for handling sterile materials, for taking samples, for making cultures, and for disposing of contaminated materials after use are all designed to prevent the spread of bacteria from one area to another. Close attention to details in the written procedure and in your instructor's demonstrations will prevent contamination and infection and will be of practical value in the future.

General, Selective and Differential Media

In addition to using sterile technique, you will have the opportunity to utilize differential and selective media to assist you in the pursuit of the pure culture. A selective medium "selects" for the growth of specific microbes (while inhibiting the growth of others) by virtue of some distinguishing nutritional or environmental factors (e.g., ability to utilize lactose as the sole carbon source, survival at a low or high pH, presence of selective inhibitors such as bile, crystal violet, antibiotics). A differential medium enlists a particular bacterial property to allow visual differentiation of one organism from another (e.g., ability to ferment a particular carbohydrate like lactose alters a pH indicator and a lactose-fermenter colony has a distinctive color compared to lactose non-fermenters which are not colored). Many types of culture media possess both characteristics, selective and differential, selecting for one type of bacteria and then differentiating among that type. There are hundreds of selective and differential media, but a few of the more common ones as well as those used for general purposes are described below:

- Nutrient Agar (**NA**), Supplemental Nutrient Agar (**SNA**), Luria or Luria-Bertani agar (**L** or **LB**), Trypticase Soy Agar (**TSA**): These media are general cultivation and maintenance media used for many environmental organisms that do not have special growth requirements, such as *E. coli, Staphylococcus* sp. and *Bacillus* sp.

- Brain Heart Infusion (**BHI**): This is used to cultivate and maintain the more fastidious bacteria such as *Neisseria* sp. and *Streptococcus* sp. However, it can also be used as a general cultivation and maintenance medium for the species which are not

fastidious. Fastidious organisms are those which have specific nutritional requirements for growth.

- Sheep Blood Agar (**SBA**): This is a medium with TSA, SNA or BHI as a base and 5% sheep blood. Blood is incorporated into the medium to provide growth factors required by fastidious pathogens. Bacteria may also be characterized by their ability to cause hemolysis of the blood cells. Alpha (α) hemolytic bacteria produce a zone of partial clearing (greening) around single isolated colonies; beta (β) hemolytic bacteria produce a complete zone of clearing surrounding isolated colonies and gamma (γ) hemolytic bacteria produce no hemolysis around colonies.

- Columbia Nalidixic Acid Agar (**CNA**): This is sheep blood agar with nalidixic acid. Nalidixic acid inhibits the growth of Gram negative cocci and rods and most Gram positive rods.

- MacConkey Agar (**MAC**): This is used to isolate *Enterobacteriaceae* and other related enteric Gram negative rods. Included bile salts and crystal violet inhibit growth of Gram positive bacteria, Gram negative cocci and fastidious Gram negative rods. Lactose is the sole carbohydrate source. Lactose-fermenters produce colonies in varying shades of red due to the conversion of neutral red indicator dye (red below pH 6.8) from the products of mixed acids. Bacteria which do not ferment lactose appear colorless or transparent.

- Mannitol Salt Agar (**MSA**): This medium contains 7.5% NaCl, inhibitory to the growth of most bacteria other than the staphylococci. It also contains mannitol as the carbohydrate source and a pH indicator, phenol red, for detecting acid produced by mannitol-fermenting staphylococci. Mannitol-fermenting bacteria produce a yellow zone surrounding their growth while other staphylococci do not produce a color change.

Ubiquity of Microorganisms

Although bacteria are ubiquitous in nature, beginning microbiology students are unaware of the extent of their presence. This lab will introduce you to a variety of different types of bacteria, the variation in types and numbers found in different habitats, and the physical conditions necessary for growth. You will take samples from a variety of sources--your own body, the laboratory environment, the outside, natural environment--and inoculate each sample to a solidified agar culture medium containing nutrients (sugars, amino acids, vitamins, and minerals) necessary for bacterial growth. These cultures will then be incubated at two different temperatures. Within one or two days each bacterium will reproduce by many cell divisions resulting in a single visible colony that contains millions of cells. Each bacterial species has its own characteristic type of colonial growth, which will vary from that of other bacteria in size, shape, color, and consistency.

Noting the Observable Characteristics of Bacteria

Here are the more common culture characteristics of bacteria on agar plates:

1. Odor Production: Some bacteria produce a unique *aroma* (gas) as a result of their metabolic processes. An astute microbiologist will always make note of the specific aroma of the organism under study. Examples of aroma are ammonia (NH_3), rotten egg (H_2S), alcoholic (-OH), fecal, etc.

2. Pigment Production: Pigments produced by bacteria are either *cell bound* (limited to cells and the colony), *extracellular* (pigment is released into the medium) or *both*. The color of the pigment should be noted, as this characteristic could be very helpful in the identification of the organism.

3. Colonial Morphology: This category includes the colony density, texture, elevation, cohesiveness, shape and margin.

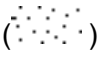
Note: Although we do not require you to learn these terms by heart, it is a good idea to get familiar with them.

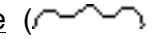
a. Colony density: Hold the plate in front of a light source to determine if the colonies are clear, opaque (no light passes through the colony) or translucent.

b. Colony texture: This is best observed when direct light is reflected off the colonies. The texture is usually either smooth (even surface) or rough (irregular, nonsmooth surface).

c. Colony elevation: Colony elevation can be observed under direct light. It can be either flat (), raised ( ), convex ( ), pulvinate (), or umbonate ().

d. Colony cohesiveness: This can be grouped into stringy, creamy, mucoid and dry. The dry colonies can be lifted off the plate entirely and they are hard to suspend in liquid.

e. Colony shape: If viewed from above, the colony shape could be categorized as punctiform (), which are very small pointed shapes, filamentous (), spindle-shaped (), irregular (), circular (), or rhizoid ().

f. Colony margin: Colony edge or margins could likewise be described as entire (), undulate (), filamentous (), curled (), lobate () or erose ( ).

4. Colony size: The relative diameter (in mm) of average individual colonies growing on a certain medium can be a useful characteristic, especially when one is comparing different species.

By observing the total number and the number of different types of colonies, the different growth responses, different incubation temperatures, and results of culturing the same source from different individuals, you will find answers to the following questions:

- In what type of environments are bacteria found?
- Which environment has many bacteria and which has few?
- How many different species of bacteria, as revealed by different colony types, are found in any given environment?
- How do different areas of the human body vary in type and number of the bacteria present? Are the areas the same or are they different in different individuals?
- How does a single environmental factor, temperature, affect the number and type of colonies found in the culture?

III. LABORATORY SUPPLIES

Mixed culture in broth tubes	1/table
MAC plate	1/tableside
BHI plates (First Lab)	4/tableside
BHI plates (Second Lab)	3/tableside
CNA plate	1/tableside
SBA plates	2/table
Sterile water Eppendorfs	2/table
Lettuce sample	1/lab
Urine sample	1/lab
Soil sample	1/lab
Swabs, sterile	2/table + 4/lab

IV. PROCEDURE (The students at each tableside will work together as a group).

First Session

Streaking For Isolation by the Quadrant Method

1. Obtain one MacConkey agar (MAC), two Brain Heart Infusion (BHI) and one Columbia Nalidixic Acid Agar (CNA) plates. Turn these culture media dishes bottom side up and label the perimeter of the dishes with your initials, date, section number and table number, temperature of incubation, type of medium and specimen.

2. Draw two perpendicular lines with a marker on bottom of the plate to divide the circle into 4 quadrants.

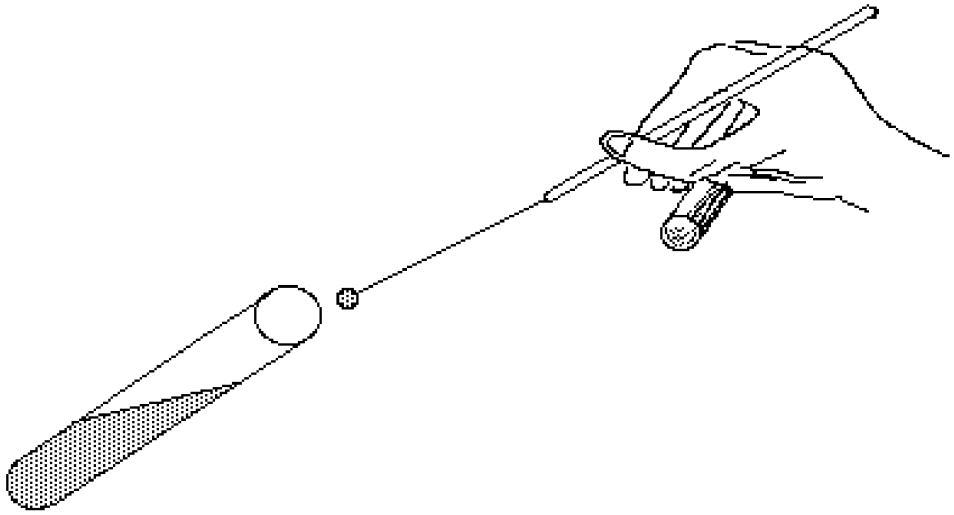
   Note: After you become proficient in streaking, you could visualize each petri dish divided into quarters instead of actually drawing lines.

3. Holding an inoculating loop between your thumb and index finger, insert the wire portion into the Bunsen burner flame, heating the entire length of the wire until it

is red and glowing. Allow the wire to cool before doing the next step. Do not wave the loop in the air.

Note: The wires of your loops are made of special alloy that makes them heat fast and cool fast. Still, the loop takes about a minute to get down to room temperature after being in the flame. If your loop is not sufficiently cooled down, it may kill the organisms that it comes in contact with and you may observe no growth on your plates.

4. Using your free hand, pick up the tube containing the mixed culture and gently shake it to disperse the culture. Remove the tube cap or plug with free fingers of the hand holding the sterile inoculating loop and carefully flame the lip of the tube in the Bunsen burner flame.



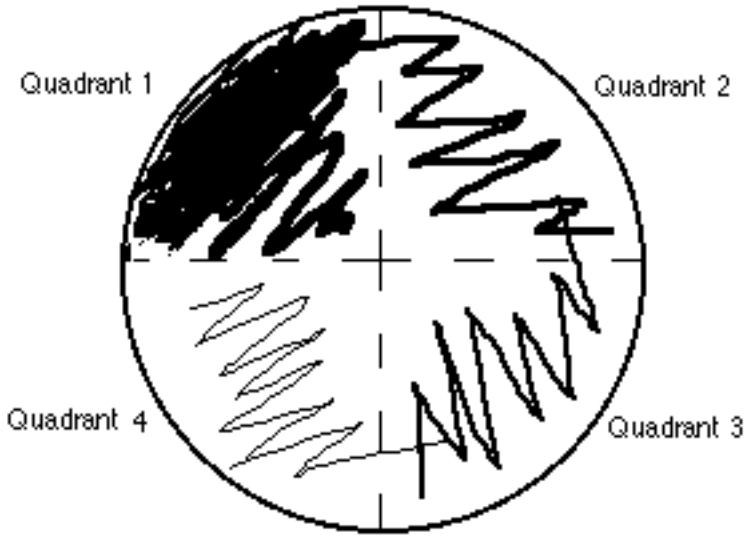
5. Tilt the tube to bring the broth culture within 1 inch from the lip of the tube. Insert the sterile loop and remove a small amount of growth; a loopful is usually sufficient. Try not to touch the sides of the tube with the loop.

6. Flame the tube lip again, carefully replace the tube cap or plug, and return the culture tube to the test tube rack.

7. Expose the agar surface of each plate for inoculation by raising the lid at an angle over the agar, thus keeping the plate surface protected from aerial contamination.

8. Apply the mixed culture on the loop onto the first quadrant by sweeping the area of this quadrant. Spread the specimen out well.

9. Flame the loop and allow it to cool. You may cool the loop in an uninoculated area of the medium. DO NOT wave it in the air to cool!



10. Now streak the inoculum from quadrant 1 into quadrant 2. Use smooth, non-overlapping strokes. Utilize the entire quadrant 2 as shown in the figure below. Flame the loop when done.

11. Let the loop cool. Now streak the inoculum from quadrant 2 into quadrant 3 by smooth, non-overlooking strokes again.

12. Flame your loop one more time and let it cool. Now bring some inoculum from quadrant 3 into quadrant 4 in the same manner as for other previous quadrants.

    Note: In this procedure, the number of times you enter back into the preceding quadrant depends on how heavy the initial inoculum is. If the initial inoculum comes from a plate, slant or a heavy broth culture, enter the preceding quadrant only once (as shown in the above figure). However, if the inoculum is obtained from food material, very light broths or any other source where you expect to have few bacteria, you may need to bring the inoculum from the previous quadrant to a new quadrant a few times.

13. Flame your loop and cool.

14. Invert the plates and incubate the MAC and one BHI plate at 37°C and CNA and the other BHI plate at 30°C. The reason the plate is inverted is the fact that the air space between the dish lid and the agar surface is saturated with moisture; during incubation the moisture condenses on the upper lid as droplets. As these droplets collect into a large drop, the water drips onto the agar surface causing the spread and mixing of colonies. Inversion of the plate eliminates this problem.

    Note: Plates are always incubated inverted, even (especially) in the refrigerator.

Human and environmental swabs

1. Each tableside will be assigned one environmental and one human swab. The list of specimens, appropriate media to be used and the incubation temperatures are given in the table that follows.

2. After you have received your assignment, take two BHI or two blood plates (SBA) and label properly.

3. Aseptically remove the swab from its container. Unless the source itself is moist, wet the swab as follows. Take a tube of sterile water in the left hand, open the lid, carefully insert the swab into the water and press against the wall of the tube to squeeze out excess water. The swab should be damp and not soggy.

		Medium to be used	
Table	Specimen	BHI	SBA
1	Throat	-	30°C, 37°C
	Coin	20°C, 30°C	-
2	Nose	-	30°C, 37°C
	Soil	20°C, 30°C	-
3	Skin	-	30°C, 37°C
	Tap water	20°C, 30°C	-
4	Urine	-	30°C, 37°C
	Lettuce	20°C, 30°C	-

4. Inoculate the swab according to the specific instructions given below for each source and streak quadrant 1 of plates:

   - Throat specimen: Have the subject open his/her mouth wide, lower the tongue and gently touch far back in the tonsil area on both sides with a dry sterile swab. Inoculate 2 blood plates.

   - Specimens from dry surfaces: Rub the moistened swab over about one square inch of surface. For skin, try various areas such as forearm, armpit, foot, etc. Inoculate the 2 appropriate plates.

   - Nose specimen: Touch the back of the interior nostril area. Inoculate 2 blood plates.

- Soil specimen: Pick up a sample of earth with a moistened swab, put in a test tube with about 1 ml of sterile water and agitate to break up soil particles and completely suspend the soil. Let the larger particles settle down. Use a fresh, dry swab to take a sample of the supernatant fluid above the settled-out soil particles. Squeeze swab inside the tube and inoculate 2 BHI plates.

- Urine specimen: Moisten a dry swab with sample, squeeze out extra liquid and inoculate 2 blood plates.

5. Finish streaking other quadrants with a loop as instructed before. Label and invert all plates and place in incubation racks. The instructor will place racks in the incubators.

Second Session

Streaking for isolation

1. When your plates from the mixed culture have been sufficiently incubated, look for growth and the number of different colony types. Always make descriptions about colonial morphology from well-isolated single colonies. These colonies usually appear in quadrants 3 and 4. Colonies in quadrants 1 and 2 overlap (confluent) and may look different because of competitive inhibition phenomena.

2. Inoculate a BHI plate with a representative colony of each different organism and incubate at 30°C for 48 hours.

Human and environmental swabs

1. Examine the plates you prepared in the previous session and record your observations on the Report Sheets provided. Count the number of colonies in the last quadrant of the plate where the colonies are isolated rather than showing confluent growth. This will be a very rough estimate of the number of organisms from various sources.

2. Look for different colony types, sizes and color. REMEMBER that on the agar surface where the number of bacteria is too high, either the colonies will grow into each other (in such a confluent growth, it is not possible to see individual colonies) or the colonies will be very small, restricted in growth and atypical. Characteristics are always determined from the well-isolated individual colonies.

3. Note the differences in number and type of colonies at the two temperatures and record your observations.

4. Examine the cultures prepared by other students from different habitats and grown at different temperatures. Record approximate numbers of colonies in the 4th quadrant and note the colony characteristics of each colony type. Do the same with different habitats at the same temperature. Record all of your observations on the Report Sheet.

Third Session

1. Examine your BHI plates and confirm your original observations on colonial morphology.

Results of the Pure Culture Lab Exercise

NAME _____DATE _____ GROUP NAME _____

Mixed Culture: You have spread a culture containing several species of bacteria on different media. Write below what your observations are and make drawings.

Medium Used	Description	Drawing
1. MAC at 37°C		
2. CNA at 30°C		
3. BHI at 37°C		
4. BHI at 30°C		

Do you see great differences between the 2 BHI plates grown at different temperatures? Explain.

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Results of the Pure Culture Lab Exercise

NAME _____DATE _____ GROUP NAME _____

Pure culture: You have purified each species of the mixed culture onto a single medium (BHI). Write the detailed colony morphology of each of these species on this medium.

Organism	Description on BHI plate
Staphylococcus epidermidis	
Bacillus cereus	
Escherichia coli	

Results of the Pure Culture Lab Exercise

NAME _____DATE _____ GROUP NAME _____

PARTNER(S) _____

Human and Environmental Swabs: This part of the lab is done to observe the diversity of bacteria from different sources (specimen). Briefly describe your observations from different sources considering colony shape, morphology, color and number. Write the most obvious and clear characteristics. Compare both growth temperatures in the same box.

Specimen source	Description
Throat:	
Coin:	
Nose:	

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Specimen source	Description
Soil:	
Skin:	
Tap water:	
Urine:	
Lettuce:	

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