AN OVERVIEW OF SOME GENERAL LABORATORY TECHNIQUES CARRIED OUT BY MICROBIOLOGISTS

IDENTIFICATION
One goal of these procedures is to attach a name or identity to the microbe, using information gathered from inspection and investigation. Identification is accomplished through the use of keys, charts, and computer programs that analyze the data and arrive at a final conclusion.

INOCULATION
The sample is placed into a container of medium that will support its growth. The medium may be solid or liquid, and held in tubes, plates, flasks, and even eggs. The delivery tool is usually a loop, needle, swab, or syringe.

INFORMATION GATHERING
Additional tests for microbial function and characteristics may include inoculations into specialized media that determine biochemical traits, immunological testing, and genetic typing. Such tests will provide specific information unique to a certain microbe.

SPECIMEN COLLECTION
Microbiologists begin by sampling the object of their interest. It could be nearly anything or place on earth. Very common sources are body fluids, foods, water, soil, plants, and animals, but even places like icebergs, volcanoes, and rocks can be sampled.

INSPECTION
Cultures are observed for the macroscopic appearance of growth characteristics. Cultures are examined under the microscope for basic details such as cell type and shape. This may be enhanced through staining and use of special microscopes.

ISOLATION
Some inoculation techniques can separate microbes to create isolated colonies that each contain a single type of microbe. This is invaluable for identifying the exact species of microbes in the sample, and it paves the way for making pure cultures.
Certain *isolation* techniques are based on the concept that if an individual bacterial cell is separated from other cells and provided adequate space on a nutrient surface, it will grow into a discrete mound of cells called a **colony** that consists of just one species.
It generally requires the following materials:

1. a medium that has a relatively firm surface contained in a clear
2. flat covered plate called a Petri dish
3. inoculating tools
GOAL OF ISOLATION

THE GOAL - ISOLATED COLONIES TO START PURE CULTURES
a) A mixed culture of *Micrococcus luteus* and *Escherichia coli* can be readily differentiated by their colors.

b) This plate of *Serratia marcescens* was overexposed to room air and has developed a large white colony. Because this intruder is not desirable and not identified, the culture is now contaminated.

c) Tubes containing pure cultures of E. coli (white), M. luteus (yellow), and S. marcescens (red) made by subculturing isolated colonies.
A mixed culture is a container that holds two or more easily differentiated species of microorganisms, not unlike a garden plot containing both carrots and onions.
A pure culture is a container of medium that grows only a single known species or type of microorganism.

This type of culture is most frequently used for laboratory study, because it allows the precise examination and control of one microorganism by itself.
A contaminated culture has had contaminants (unwanted microbes of uncertain identity) introduced into it, like weeds into a garden.

Because contaminants have the potential for disrupting experiments and tests, special procedures have been developed to control them, as you will no doubt witness in your own laboratory.
Can an isolated colony be considered pure?

• This is generally assumed, however....
  • some colonies are very slow growers and may be too small to see.
  • some colonies may be growing under another colony
  • selective media may be preventing reproduction of some bacteria so they may be present but not visible
  • condensed water, capsules, slime, all represent areas where individual contaminant cells hide out.
Any special considerations?

• Different species of microbes represent challenges....
  • Encapsulated bacteria are sticky and don’t separate well.
  • Some species are motile and do not stay where you streak them spreading across the plate.
  • Fungal spores easily contaminate cultures within a plate.
  • Organisms can gain entrance to a Petri dish through water or the edges, or from the air currents while you are streaking.
**STREAK PLATE METHOD**

In the streak plate method, a small droplet of sample is spread with a tool called an inoculating loop over the surface of the medium according to a pattern that gradually thins out the sample and separates the cells spatially over several sections of the plate.

Because of its ease and effectiveness, the streak plate is the method of choice for most applications.
The streak plate method is the most common way to get pure cultures of bacteria. A device called an inoculating loop is sterilized and dipped into a culture of a microorganism or microorganisms and then is "streaked" in a pattern over a nutrient medium. The last cells that are rubbed off the loop onto the medium are far enough apart to allow isolation of separate colonies of the original culture.
Streak Plate Isolation Principle

- An original inoculum containing a mixture of bacteria is spread into 4 quadrants on solid media.

- The goal is to reduce the number of bacteria in each subsequent quadrant.

- Colonies are masses of offspring from an individual cell therefore streaking attempts to separate individual cells.

- Discrete colonies form as the individual cells are separated and then multiply to form isolated colonies in the later quadrants.
STREAK PLATE METHOD

Making a Streak Plate

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STEPS IN A STREAK PLATE
THIS ONE IS A FOUR-PART OR QUADRANT STREAK

Steps in a quadrant streak plate
resulting isolated colonies of bacteria
Streaking the Quadrants

Flame between each quadrant.

Quadrant 1

Quadrant 2

Quadrant 3

Quadrant 4
Quadrant 1- Streak with broad narrow strokes in the upper half of the first quarter of the plate.
Incinerate and cool the loop between the quadrants
Quadrant 2 — Rotate the plate, enter the previous streak mark one or two times and then streak the upper portion of the second quarter of the plate with broad strokes.
Incinerate and cool the loop between the quadrants
**Quadrant 3** — Rotate the plate, enter quadrant 2 one or two times and then streak with shorter more separated strokes from the top of the quadrant to the center.
Incinerate and cool the loop between the quadrants.
Quadrant 4 — Enter quadrant 3 and then streak with broad S-shaped motions through the center of the plate.
STREAK PLATE METHOD

ISOLATED COLONIES
POUR PLATE METHOD/ LOOP DILUTION

The sample is inoculated, also with a loop, into a series of cooled but still liquid agar tubes so as to dilute the number of cells in each successive tube in the series.

Steps in the loop dilution method:

1. Original sample.
2. Dilution of sample.
3. Further dilution of sample.

The appearance of plate:

A petri dish with multiple colonies indicating the dilution process.
Pour Plate Method/ Loop Dilution

Inoculated tubes are then plated out (poured) into sterile Petri dishes and are allowed to solidify (harden).

The number of cells per volume is so decreased that cells have ample space to form separate colonies in the second or third plate.

One difference between this and the streak plate method is that in this technique, some of the colonies will develop deep in the medium itself and not just on the surface.
STEPS IN LOOP DILUTION
ALSO CALLED A POUR PLATE OR SERIAL DILUTION

Steps in the loop dilution method

the appearance of plate
SPREAD PLATE METHOD

A small volume of liquid from a diluted sample is pipetted onto the surface of the medium and spread around evenly by a sterile spreading tool (sometimes called a “hockey stick”).

As with the streak plate, cells are spread over separate areas on the surface so that they can form individual colonies.
Spread plate
INCUBATION

a container of medium has been inoculated

it is incubated in a temperature-controlled chamber (incubator) to encourage microbial growth

the usual temperatures used in laboratory propagation fall between 20°C and 40°C. Incubators can also control the content of atmospheric gases such as oxygen and carbon dioxide that may be required for the growth of certain microbes.
During the incubation period (ranging from a few hours to several weeks), the microbe multiplies and produces a culture with macroscopically observable growth.

Microbial growth in a liquid medium materializes as cloudiness, sediment, a surface scum, or colored pigment. Growth on solid media may take the form of a spreading mat or separate colonies.
Isolation Requires Aseptic Technique

Aseptic Technique
Biotechnology Explorer™

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Isolation Requires Aseptic Technique

Aseptic technique is the process of:

• Preventing contamination of a culture with environmental microbes

• Preventing contamination of yourself or the environment with the organism in the culture

Remember everything is contaminated with a variety of environmental microbes.

Remember microbes are invisible, you must “see with your minds eye” during these procedures.
MICROORGANISMS USE CHEMICALS CALLED NUTRIENTS FOR GROWTH AND DEVELOPMENT. THEY NEED THESE NUTRIENTS TO BUILD MOLECULES AND CELLULAR STRUCTURES.

The most important nutrients are carbon, hydrogen, nitrogen, and oxygen.
MICROORGANISMS GET THEIR NUTRIENTS FROM SOURCES IN THEIR ENVIRONMENT

WHAT WILL HAPPEN IF THESE MICROORGANISMS OBTAIN THEIR NUTRIENTS BY LIVING ON OR IN OTHER ORGANISM

THEY CAN CAUSE DISEASE IN THAT ORGANISM BY INTERFERING WITH THEIR HOST’S NUTRITION, METABOLISM, AND, THUS DISRUPTING THEIR HOST’S HOMEOSTASIS, THE STEADY STATE OF AN ORGANISM.
**How They Feed Themselves**

**Organism**

**Autotrophs**
- Organisms that use carbon dioxide (CO$_2$) as their source of carbon
- Autotrophs make organic compounds from CO$_2$ and do not feed on organic compounds from other organisms.

**Heterotrophs**
- Organisms that obtain carbon from organic nutrients like proteins, carbohydrate, amino acids, and fatty acids
- Heterotrophic organisms acquire or feed on organic compounds from other organisms.
ORGANISM

USE CHEMICALS OR LIGHT AS A SOURCE OF ENERGY

CHROMOTROPHS

- Organisms that acquire energy from redox reactions involving inorganic and organic chemicals

PHOTOTROPHS

- Organisms that use light as their energy source
CHEMICAL REQUIREMENTS FOR MICROBIAL GROWTH

CARBON

Carbons one of the most important requirements for microbial growth. Carbon is the backbone of living matter.

OXYGEN

OBLIGATE AEROBES

- organisms that require oxygen
- Oxygen is essential for obligate aerobes because it serves as a final electron acceptor in the electron transport chain, which produces most of the ATP in these organisms.

FACULTATIVE ANAEROBES

- organisms can use oxygen when it is present, but can continue to grow by using fermentation or anaerobic respiration when oxygen is not available

OBLIGATE ANAEROBES

- bacteria cannot use molecular oxygen and can even be harmed by it
A culture medium is nutrient material prepared in the laboratory for the growth of microorganisms. Microorganisms that grow in size and number on a culture medium are referred to as a culture.
In order to use a culture medium, it must be sterile, meaning that it contains no living organisms. This is important because we only want microorganisms that we add to grow and reproduce, not others.
We must have the proper nutrients, pH, moisture, and oxygen levels (or no oxygen) for a specific microorganism to grow.

Many culture media are available for microbial growth.

Media are constantly being developed for the use of identification and isolation of bacteria in the research of food, water, and microbiology studies.
The most popular and widely used medium used in microbiology laboratories is the **solidifying agent** agar.

Agar is a complex polysaccharide derived from red algae.

Agar media are usually contained in test tubes or Petri dishes.

Petri dishes are named after their inventor, Julius Petri, who in 1887 first poured agar into glass dishes.
### TABLE 3.5  Three Categories of Media Classification

<table>
<thead>
<tr>
<th>Physical State (Medium’s Normal Consistency)</th>
<th>Chemical Composition (Type of Chemicals Medium Contains)</th>
<th>Functional Type (Purpose of Medium)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Liquid</td>
<td>1. Synthetic (chemically defined)</td>
<td>1. General purpose</td>
</tr>
<tr>
<td>2. Semisolid</td>
<td>2. Nonsynthetic (complex; not chemically defined)</td>
<td>2. Enriched</td>
</tr>
<tr>
<td>3. Solid (can be converted to liquid)</td>
<td></td>
<td>3. Selective</td>
</tr>
<tr>
<td>4. Solid (cannot be liquefied)</td>
<td></td>
<td>4. Differential</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5. Anaerobic growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6. Specimen transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7. Assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8. Enumeration</td>
</tr>
</tbody>
</table>
For a medium to support microbial growth, it must provide an energy source, as well as carbon, nitrogen, sulfur, phosphorous, and any other organic growth factors that the organism cannot make itself, source for the microorganisms to utilize.
A chemically defined medium is one whose exact chemical composition is known.

In complex media, the energy, carbon, nitrogen, and sulfur needed for microbial growth are provided by protein.

Different vitamins and organic growth factors can be provided by meat and yeast extracts. If a complex medium is in a liquid form it is called a nutrient broth. If agar is added, it is called a nutrient agar. Agar is not a nutrient; it is a solidifying agent.
Chemical and Physical Types of Culture Media

- Defined or synthetic
- Complex

<table>
<thead>
<tr>
<th>Table 7.4</th>
<th>Types of Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basis for Classification</td>
<td>Types</td>
</tr>
<tr>
<td>Chemical composition</td>
<td>Defined (synthetic), complex</td>
</tr>
<tr>
<td>Physical nature</td>
<td>Liquid, semisolid, solid</td>
</tr>
<tr>
<td>Function</td>
<td>Supportive (general purpose), enriched, selective, differential</td>
</tr>
</tbody>
</table>
### Defined or Synthetic Media

<table>
<thead>
<tr>
<th>Defined Medium for Cyanobacteria</th>
<th>Amount (g/liter)</th>
<th>Defined Medium for <em>Escherichia coli</em></th>
<th>Amount (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>1.5</td>
<td>Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>K₂HPO₄ · 3H₂O</td>
<td>0.04</td>
<td>Na₃HPO₄</td>
<td>16.4</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>0.075</td>
<td>KH₂PO₄</td>
<td>1.5</td>
</tr>
<tr>
<td>CaCl₂ · 2H₂O</td>
<td>0.036</td>
<td>(NH₄)₂SO₄</td>
<td>2.0</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.006</td>
<td>MgSO₄ · 7H₂O</td>
<td>200.0 mg</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.006</td>
<td>CaCl₂</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>EDTA (Na₂Mg salt)</td>
<td>0.001</td>
<td>FeSO₄ · 7H₂O</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.02</td>
<td>Final pH 6.8–7.0</td>
<td></td>
</tr>
<tr>
<td>Trace metal solution¹</td>
<td>1.0 ml/liter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final pH 7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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### Complex Media

<table>
<thead>
<tr>
<th>Nutrient Broth</th>
<th>Amount (g/liter)</th>
<th>Tryptic Soy Broth</th>
<th>Amount (g/liter)</th>
<th>MacConkey Agar</th>
<th>Amount (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone (gelatin hydrolysate)</td>
<td>5</td>
<td>Tryptone (pancreatic digest of casein)</td>
<td>17</td>
<td>Pancreatic digest of gelatin</td>
<td>17.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3</td>
<td>Peptone (soybean digest)</td>
<td>3</td>
<td>Pancreatic digest of casein</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
<td>2.5</td>
<td>Peptic digest of animal tissue</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sodium chloride</td>
<td>5</td>
<td>Lactose</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dipotassium phosphate</td>
<td>2.5</td>
<td>Bile salts</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>MacConkey Agar</td>
<td></td>
<td></td>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Neutral red</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Crystal violet</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Agar</td>
<td>13.5</td>
</tr>
</tbody>
</table>

*Note: Table 7.5 is a table showing examples of defined media for cyanobacteria and *Escherichia coli*. Table 7.6 is a table showing some common complex media, including nutrient broth, tryptic soy broth, and macConkey agar.*
Some Media Components

• Peptones
  • protein hydrolysates prepared by partial digestion of various protein sources

• Extracts
  • aqueous extracts, usually of beef or yeast

• Agar
  • sulfated polysaccharide used to solidify liquid media; most microorganisms cannot degrade it
Functional Types of Media

• **Supportive or general purpose media (e.g. TSA)**
  • support the growth of many microorganisms

• **Enriched media (e.g. blood agar)**
  • general purpose media supplemented by blood or other special nutrients

• **Selective**

• **Differential**
Selective Media

• favor the growth of some microorganisms and inhibit growth of others

• e.g., MacConkey and EMB agar
  • selects for gram-negative bacteria

• e.g., Mannitol Salt agar
  • selects for *Staphylococcus aureus*
Differential Media

• Distinguish between different groups of microorganisms based on their biological characteristics

• e.g., blood agar
  • hemolytic versus nonhemolytic bacteria

• e.g., MacConkey agar
  • lactose fermenters versus nonfermenters
<table>
<thead>
<tr>
<th>Medium</th>
<th>Functional Type</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood agar</td>
<td>Enriched and differential</td>
<td>Blood agar supports the growth of many fastidious bacteria. These can be differentiated based on their ability to produce hemolysins—proteins that lyse red blood cells. Hemolysis appears as a clear zone (β-hemolysis) or greenish halo around the colony (α-hemolysis) (e.g., <em>Streptococcus pyogenes</em>, a β-hemolytic streptococcus).</td>
</tr>
<tr>
<td>Eosin methylene blue (EMB) agar</td>
<td>Selective and differential</td>
<td>Two dyes, eosin Y and methylene blue, inhibit the growth of Gram-positive bacteria. They also react with acidic products released by certain Gram-negative bacteria when they use lactose or sucrose as carbon and energy sources. Colonies of Gram-negative bacteria that produce large amounts of acidic products have a green, metallic sheen (e.g., fecal bacteria such as <em>E. coli</em>).</td>
</tr>
<tr>
<td>MacConkey (MAC) agar</td>
<td>Selective and differential</td>
<td>The selective components in MAC are bile salts and crystal violet, which inhibit the growth of Gram-positive bacteria. The presence of lactose and neutral red, a pH indicator, allows the differentiation of Gram-negative bacteria based on the products released when they use lactose as a carbon and energy source. The colonies of those that release acidic products are red (e.g., <em>E. coli</em>).</td>
</tr>
<tr>
<td>Mannitol salt agar</td>
<td>Selective and differential</td>
<td>A concentration of 7.5% NaCl selects for the growth of staphylococci. Pathogenic staphylococci can be differentiated based on the release of acidic products when they use mannitol as a carbon and energy source. The acidic products cause a pH indicator (phenol red) in the medium to turn yellow (e.g., <em>Staphylococcus aureus</em>).</td>
</tr>
</tbody>
</table>
Strict Anaerobic Microbes

• all strict anaerobic microorganisms lack or have very low quantities of
  • superoxide dismutase
  • catalase

• these microbes cannot tolerate O$_2$
• anaerobes must be grown without O$_2$
Isolation of Pure Cultures

• Population of cells arising from a single cell developed by Robert Koch
• Allows for the study of single type of microorganism in mixed culture
• Spread plate, streak plate, and pour plate are techniques used to isolate pure cultures
The Streak Plate

- Involves technique of spreading a mixture of cells on an agar surface so that individual cells are well separated from each other
  - involves use of bacteriological loop
- Each cell can reproduce to form a separate colony (visible growth or cluster of microorganisms)

Note: This method only works if the spreading tool (usually an inoculating loop) is resterilized after each of steps 1–4.
The Spread Plate and Pour Plate

• Spread plate
  • small volume of diluted mixture containing approximately 30–300 cells is transferred
  • spread evenly over surface with a sterile bent rod

• Pour plate
  • sample is serially diluted
  • diluted samples are mixed with liquid agar
  • mixture of cells and agar are poured into sterile culture dishes

• Both may be used to determine the number of viable microorganisms in an original sample
A small amount of the sample is pipetted to the center of a solidified medium. The glass spreader is sterilized by dipping it into ethanol and briefly flaming it. The spreader is cooled and then used to spread the sample evenly over the surface of the medium.

The original sample is diluted several times. Some of the dilutions (often the most dilute) are mixed with warm agar and poured onto the plates. Isolated cells grow into colonies on the surface (appear round) and within the medium (appear lens-shaped). The isolated colonies can be counted or used to establish pure cultures.
Microbial Growth on Solid Surfaces

• Colony characteristics that develop when microorganisms are grown on agar surfaces aid in identification

• Microbial growth in biofilms is similar

• Differences in growth rate from edges to center is due to
  • oxygen, nutrients, and toxic products
  • cells may be dead in some areas
THANK YOU