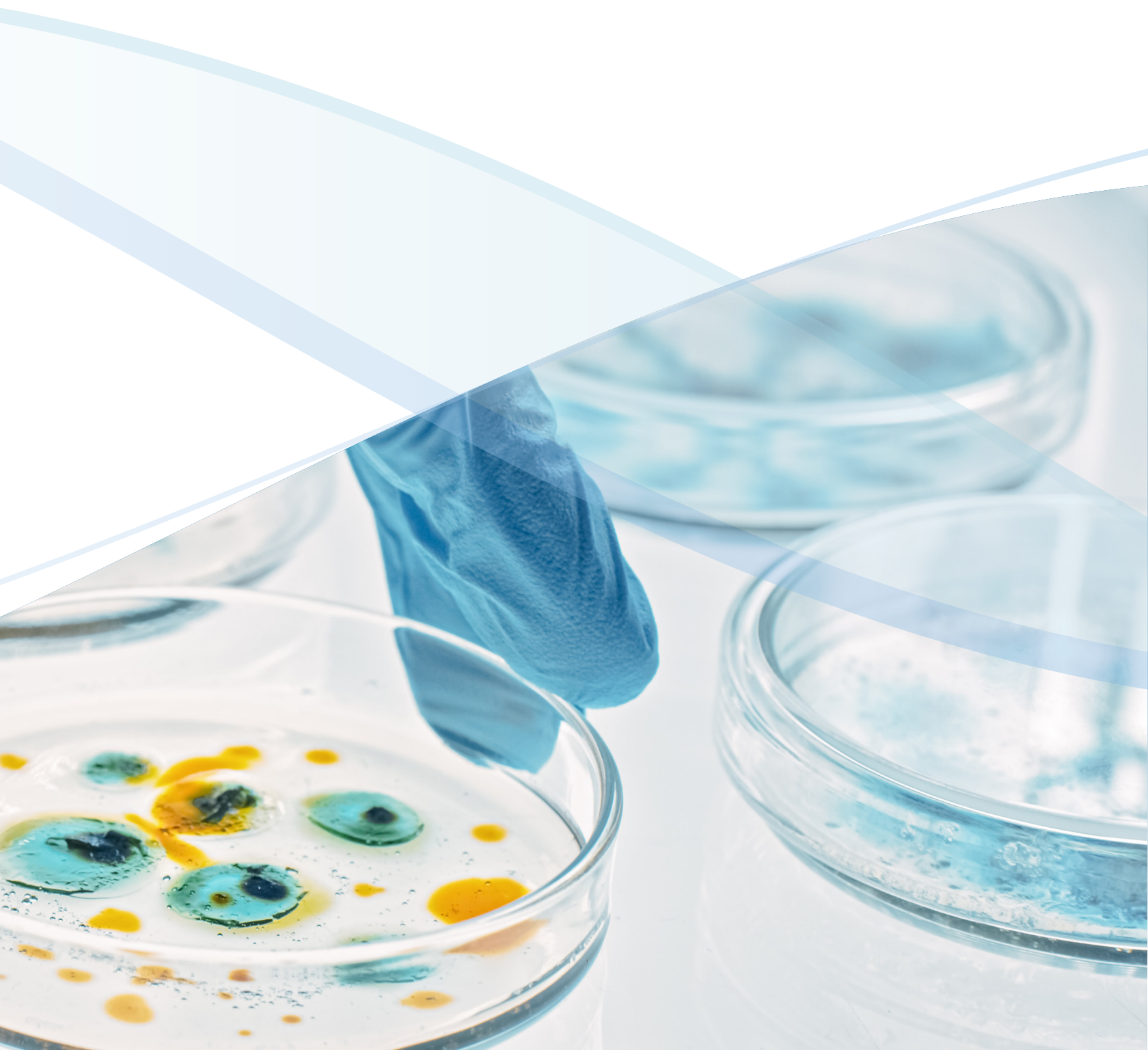
ATCC®

Introduction to Microbiology



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ASEPTIC TECHNIQUE

When working in a microbiology laboratory, aseptic technique is fundamental to the success and safety of an experiment. Generally, aseptic techniques are procedures or processes performed by scientists under sterile conditions to ensure that microbial contaminants do not harm colleagues and are not introduced into sterile solutions, supplies, or experimental cultures. To minimize the possibility of microbial contamination, the below rules and recommendations are encouraged. Additional information on risk assessment and precautions can be found in the Center for Disease Control (CDC) publication "Biosafety in Microbiological and Biomedical Laboratories, (BMBL)" at http://www.cdc.gov.[1]

PERSONAL PROTECTION AND CLEANLINESS

In a Microbiology laboratory, it is important to maintain the health and safety of all personnel. To avoid health risks, laboratory scientists should wear protective gear and be aware of any potential hazards nearby. Generally, protective gear serves two purposes; to protect the scientist from laboratory hazards and to shield the experiment from unintentional contamination. Below, we describe several mechanisms to maintain laboratory safety.

- Upon entering and exiting a lab, be sure to thoroughly wash your hands with anti-microbial soap.
- While in the lab, avoid touching your face or eyes. Further, do not handle make-up or contacts as they may become contaminated.
- Do not store or handle food or beverages in the laboratory.
- Only enter the lab wearing closed-foot shoes. Open-toed shoes should never be worn in a laboratory as it leaves you susceptible to potential cuts or infection from broken glassware or sharp equipment.
- While performing an experiment, wear a clean, fitted laboratory coat. For additional protection, use a closed-front laboratory coat. Laboratory coats should never leave the lab, and should be cleaned frequently. Additionally, ensure that the sleeves of your coat are fitted so that they will not catch fire near an open flame, nor become entangled.
- When working with microorganisms, wear fitted, disposable gloves. Flakes of dry skin harbor bacteria, which may provide a source of contamination; wearing gloves will mitigate the risk. Additionally, disposable gloves decrease the risk of infecting any hand wounds.
- Pull back long hair or use a hair cover. Long hair is notorious for attracting dust, a potential source of contamination. Additionally, long hair could be a health hazard when working near an open flame.
- Use protective eye-wear or a face shield when working with hazardous materials or cultures. If your eyes come in contact with microorganisms or harmful chemicals, wash your eyes for 15 minutes in an eye-wash and seek medical attention immediately.

HANDLING MICROBIAL CULTURES AND MEDIA

When working with any microbial strain, propagation success depends heavily on the prevention of cross-contamination by other microorganisms. Sources of contamination can include non-sterile supplies, media, reagents, unclean work surfaces and incubators, airborne particles, and unclean gloves. Below, we describe some tips for handling microbial cultures and media.

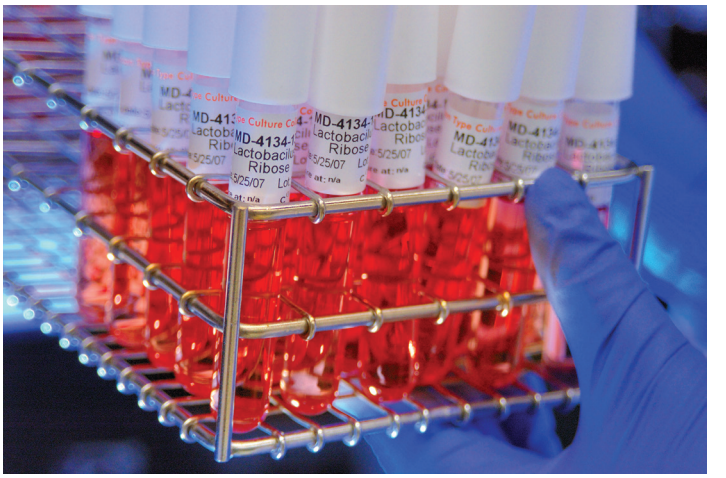
MAINTAIN A STERILE WORK AREA

- Before and after use, disinfect all work surfaces with 70% ethanol or an appropriate disinfectant. This is especially important after any spills.
- Maintain an uncluttered work space; all work surfaces should only contain equipment that is required for your experiment.
- Ensure that you have all necessary supplies before beginning an experiment. Being prepared will reduce the likelihood of careless contamination.
- Work may be performed in a thoroughly sterilized biosafety cabinet. Biosafety cabinets can be sterilized via ultraviolet light in conjunction with 70% ethanol or an appropriate disinfectant.
- Do not open windows or use fans that circulate outside air. If possible, work in laboratory settings that have air vents covered with filters. This will prevent the contamination of cultures by airborne particles.

- Frequently clean water baths used for thawing or warming media or solutions.
- Routinely sterilize incubators used for microbial propagation.

HANDLING MEDIA

- Before and after use, sterilize the outside container of all media and reagents with 70% ethanol. Also, do not leave containers of media open longer than necessary.
- Aliquot sterile solutions into smaller volumes whenever possible. If you are unsure of the sterility of your media, it is best to discard it immediately.
- Avoid pouring sterile liquids from one container to another, this increases the likelihood of media contamination. Rather, use sterile pipettes for the aseptic transfer of media.
- Never mouth pipette. This poses a health risk for personnel as well as increases the risk of contamination.
- Always use sterile glass or disposable plastic pipettes to work with liquid media. Use each pipette only once to avoid cross contamination.



An ATCC scientist carefully handling aliquoted media.

- Do not open sterile media, petri dishes, or pipette containers until you are ready to use them.

HANDLING MICROBIAL CULTURES

- Before working with media and microbial cultures, wipe your work area with 70% ethanol or an appropriate disinfectant.
- Ensure you are wearing appropriate protective clothing. This will protect you from the culture as well as reduce accidental culture contamination.
- Only use sterile glassware, equipment, media, and reagents. Check media for contamination by observing for turbidity.
- Handle only one microbial culture at a time. The risk of cross contamination or misidentification increases when more than one strain is handled at a time.
- When working with test tube cultures, hold cultures at an angle after you remove the lid to avoid airborne particles from falling into the culture. Sterilize the outside of the culture tube using a Bunsen burner flame.
- When working with plated cultures, hold the petri dish lid at an angle after you remove the lid to avoid airborne particles falling into the culture dish.
- When handling a microbial culture, work quickly and carefully in an environment that has minimal distractions. Do not leave the lid off your culture for extended periods of time.
- Never take cultures outside of the laboratory.
- Notify the laboratory supervisor immediately of any spills.

BIOLOGICAL SAFETY CABINETS

When working with hazardous or sterile materials, it is recommended that all associated procedures are performed in a biosafety cabinet. These apparatuses are enclosed, ventilated workspaces designed to protect laboratory personnel and materials from cross contamination during routine procedures. Generally, work spaces within the biosafety cabinet are protected through the use of a high-efficiency particulate air (HEPA) filtration method, which removes harmful microbes from the air.

An ATCC scientist working with a bacterial strain in a Class II biological safety cabinet.

BIOLOGICAL SAFETY CABINET CLASSES

Class I:

These biological safety cabinets are open-front negative pressure systems with HEPA filtration systems. Class I biosafety cabinets provide protection for laboratory personnel and the environment, but will not provide product protection. These cabinets are often used to enclose specific equipment or procedures that may generate potentially hazardous aerosols.

Class II:

These biological safety cabinets are open-front, ventilated, laminar-flow cabinets. These provide HEPA-filtered, recirculated airflow within the work space. Class II cabinets provide protection to laboratory personnel, the environment, and to products by drawing a curtain of sterile air over the products that are being handled. These cabinets are commonly used in microbiology laboratories working with potentially infectious agents (Biosafety Level 1 or 2) as they protect the contained materials from extraneous airborne contaminants.

Class III:

These biological safety cabinets are totally enclosed, ventilated systems with gas-tight construction. These cabinets are used through attached rubber gloves. Generally, the air supply is drawn into the cabinet through HEPA filters, and the exhaust air is filtered by two HEPA filters installed in a series. This biosafety cabinet system is commonly used with high-risk infectious agents (Biosafety Level 3 or 4) to prevent the escape of aerosols.

Below, we describe some tips on how to properly use a Class I or Class II biosafety cabinet when working with non-infectious materials.

KEEPING A BIOSAFETY CABINET SAFE

- Certify all biosafety cabinets upon installation
- Routinely test the quality of airflow
- Ensure the integrity of filters
- Biosafety cabinets should be approved by the resident Biosafety officer
- Ensure that all biosafety cabinets are routinely recertified

BIOSAFETY CABINET SANITATION

- Clean work surfaces with an appropriate disinfectant before and after use.
- If the biosafety cabinet is equipped with germicidal UV lights, decontaminate work surfaces before and after use by turning on the UV light for at least 15 minutes. Never use the UV light while the biosafety cabinet is in use.
- Routinely remove any biohazard waste from the biosafety cabinet.
- Using an appropriate disinfectant, wipe down the outer surface of all pipettes, pipette tip boxes, media, materials, etc., prior to placing them in the biosafety cabinet.
- Always wear a clean lab coat and sterile gloves when working in a biosafety cabinet.

PROPER USE OF A BIOSAFETY CABINET

- Turn on the biosafety cabinet 15 minutes prior to use.
- Only raise the biosafety cabinet sash to the recommended level, this will reduce disruption to the air flow as well as assist in the prevention of airborne contaminant entry.
- When using a biosafety cabinet, limit the amount of movement in the cabinet and do not remove your arms. Additionally, limit the access to the area around the biosafety cabinet. This will reduce disruption to the airflow.
- Do not use open flames within a biosafety cabinet. The resulting heat from the flame can disrupt the air flow provided by the biosafety cabinet, increasing the risk for contamination. Additionally, gas leaks associated with Bunsen burners or the use of an alcohol-based disinfectant near an open flame can result in fire or injury.

MORPHOLOGY

Microorganisms come in a variety of cellular and colony morphologies. These morphologies are often characteristic of a species and are frequently examined when identifying an unknown microorganism. In this section, we will describe some of the various cellular and colony morphologies of bacterial strains.

CELLULAR MORPHOLOGY

Bacterial species come in a number of cellular morphologies and aggregations. It is believed that these variations may have evolved in response to selective pressures related to nutrient uptake, cell division and segregation, attachment, motility, predation, and the advantages associated with cellular differentiation.[2-5] Below are some of the common morphologies, cellular aggregations, and flagellar arrangements associated with bacterial cells (See: FIGURE 1-2).

MORPHOLOGY

Cocci – Spherical shaped

Coccobacillus – Bacterial shape that is intermediate between cocci and bacilli

Bacillus – Rod shaped

Filamentous – Cells that resemble filaments. These structures are often branched

Helical – Twisted spiral-shaped form

Spirochete – Helically coiled, spiral-shaped cells

Vibrio – Curved rod or comma shape

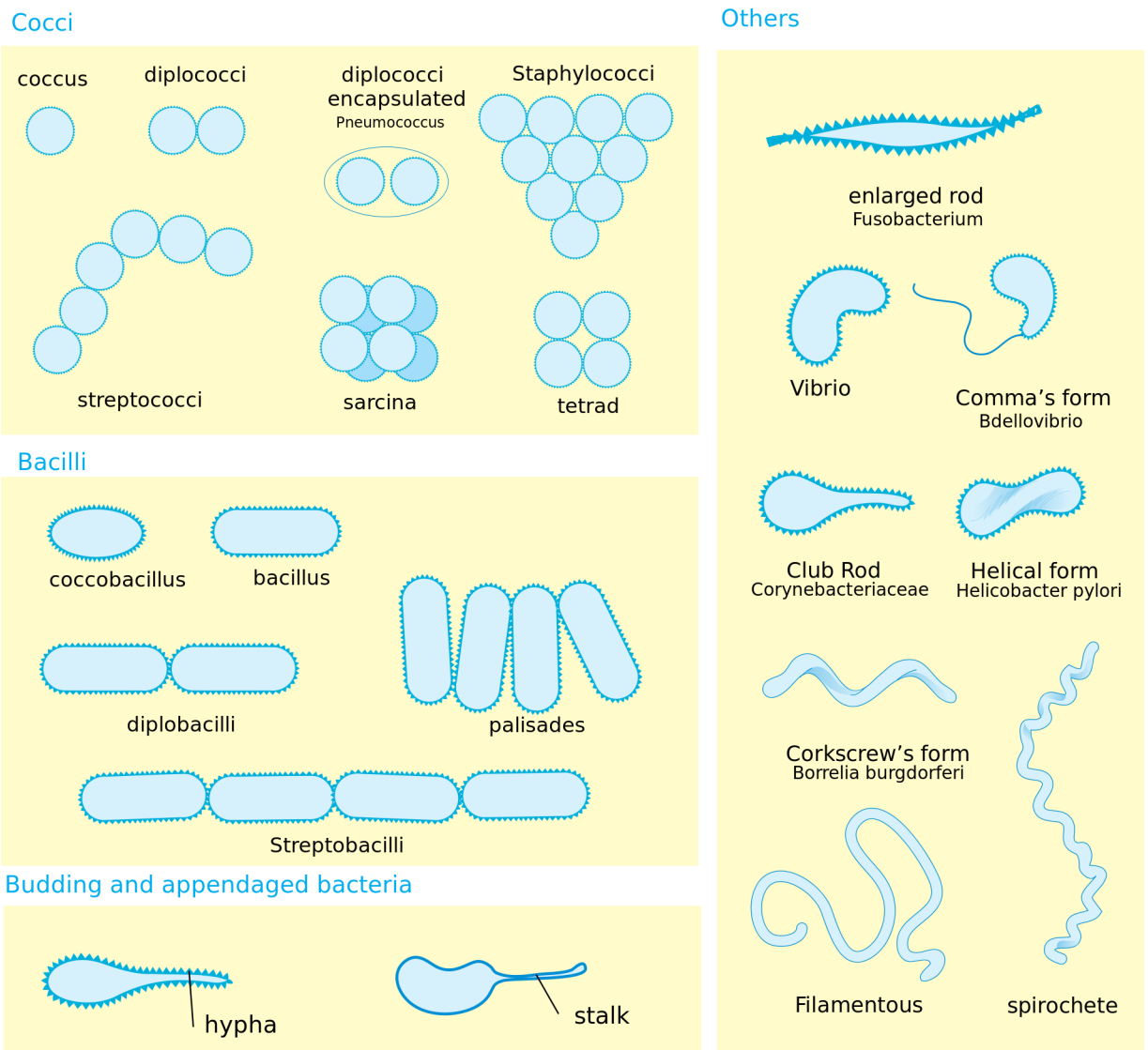


Figure 1: Cellular morphology and aggregation

CELLULAR AGGREGATIONS

Diplobacilli – Pairs of joined bacilli

Diplococci – Pairs of joined cocci

Diplococci Encapsulated – Pairs of cocci that are surrounded by a capsule

Palisades – Bacilli that group together at odd angles

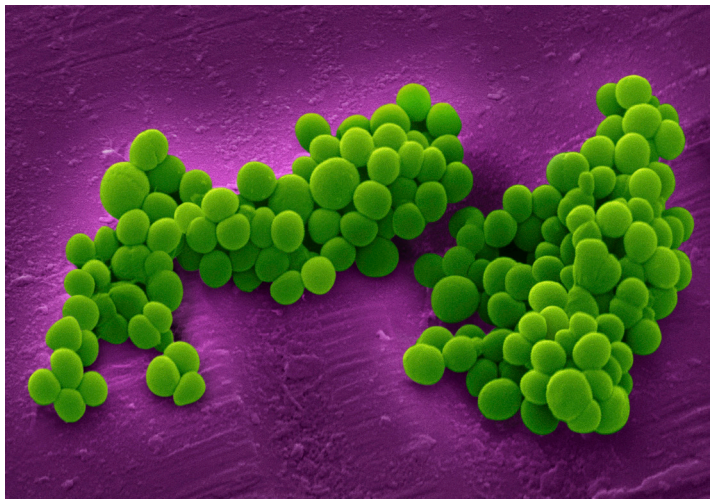
Sarcinae – Cocci in groups of eight, forming cubes

Staphylococci – Grape-like clusters of cocci

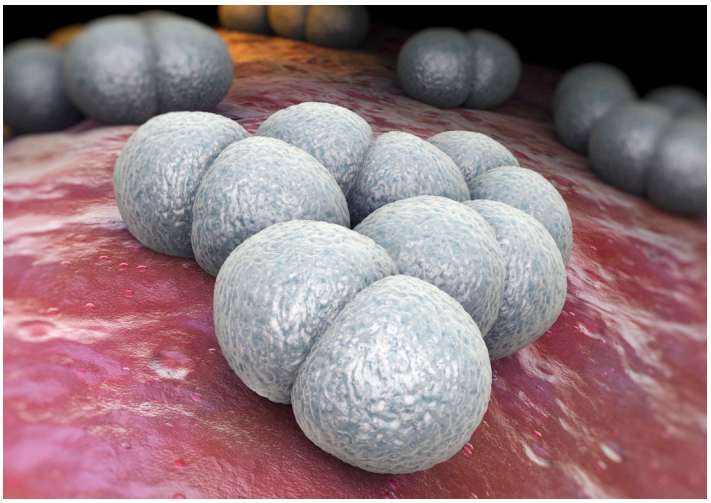
Streptobacilli – Rows or chains of bacilli

Streptococci – Rows or chains of cocci

Tetrad – Cocci that fail to separate following division, remaining in groups of four



Staphylococcus aureus - cocci bacteria with staphylococci arrangement.



Neisseria meningitidis - cocci bacteria with diplococci arrangement

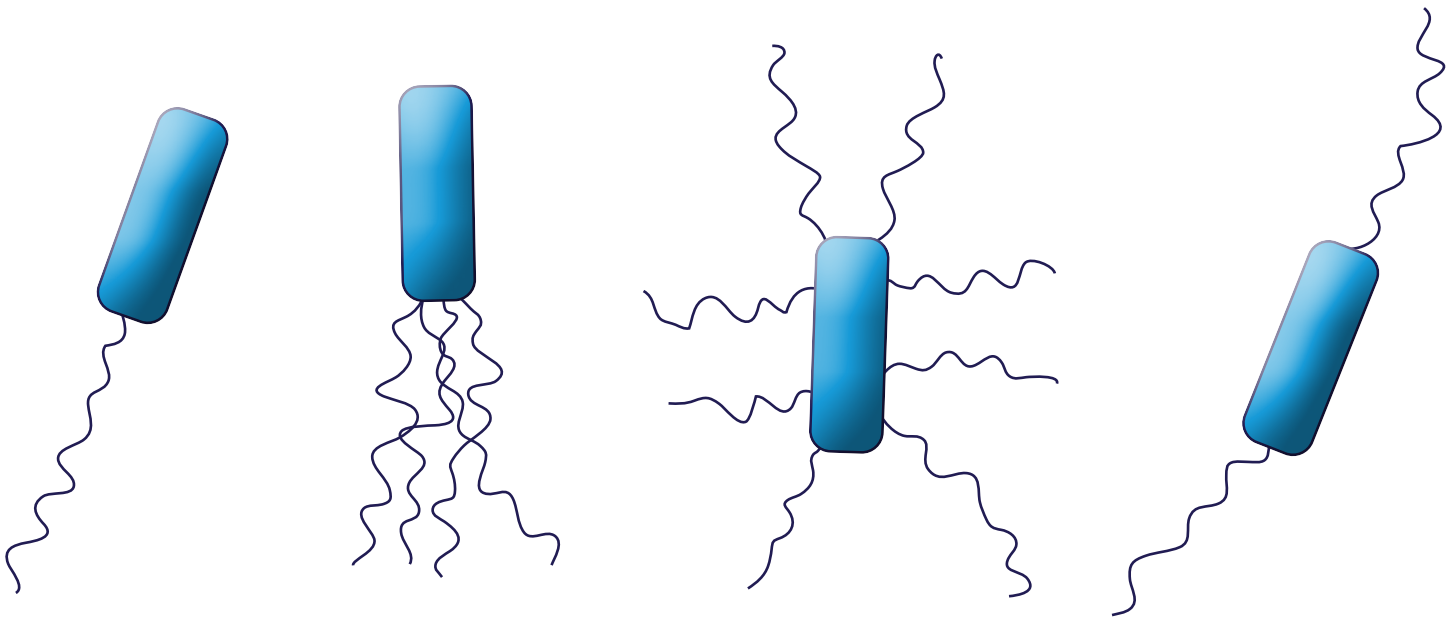
FLAGELLAR ARRANGEMENTS

Monotrichous – Single polar flagellum, often localized at one end of the cell

Lophotrichous – Multiple flagella located at the same spot on the bacterial cell surface

Peritrichous – Multiple flagella inserted around the periphery of the cell

Amphitrichous – A single flagellum localized on each of the two ends of the cell



Monotrichous (polar) Lophotrichous Peritrichous Amphitrichous

Figure 2: Flagella

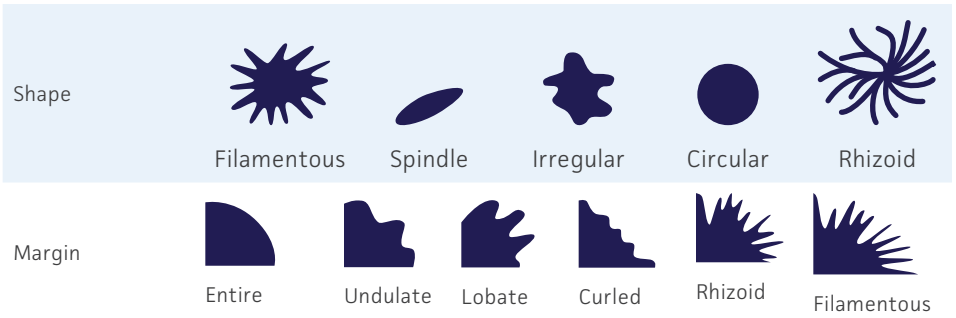
COLONY MORPHOLOGY

Colony morphology can significantly vary between species and is often used in the initial identification of a strain. Further, morphology can vary between strains within a species due to differential gene expression. Below are several terms commonly used to describe colony morphology (See: FIGURE 3).

Shape – This refers to the form of the colony, or its overall appearance. Common shapes include circular, rhizoid, irregular, filamentous, and spindle.

Margin – The margin refers to the edge of a colony, and may be an important characteristic in organism identification. Common margins include entire, undulate (resembling waves), lobate (lobed structure), curled, rhizoid, filamentous, or erose (irregularly notched).

Elevation – Elevation describes the side-view of the colony. The most common are flat, raised, convex (curved surface), pulvinate (cushion-shaped), and umbonate (having a knobby protuberance).



Apperance	Glistening or dull
Optical property	Transparent, translucent, or opaque.
Texture	Rough, smooth, mucoid, butyrous, or dry
Pigmentation	Nonpigmented (eg., cream, white) Pigmented (eg, yellow, blue, pink)

Figure 3: Colony Morphology

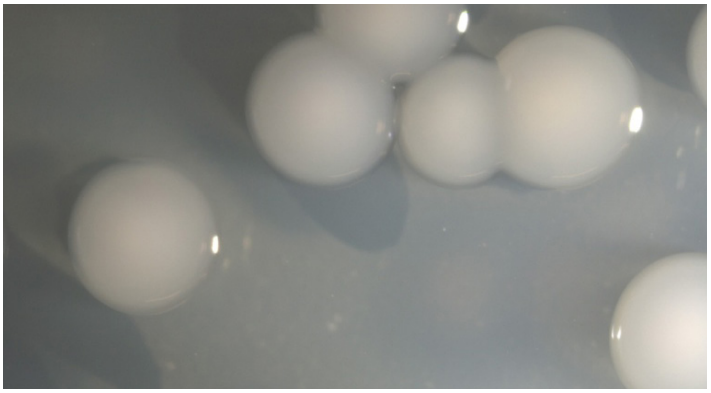
Size – The size of a colony is often a useful characteristic for identification, and can be measured. Sizes are often described as punctiform (shaped like a point), small, moderate, or large.

Appearance – The outwards aspects of the colony surface, often depicting if the colony is glistening (glossy, shiny) or dull (cloudy).

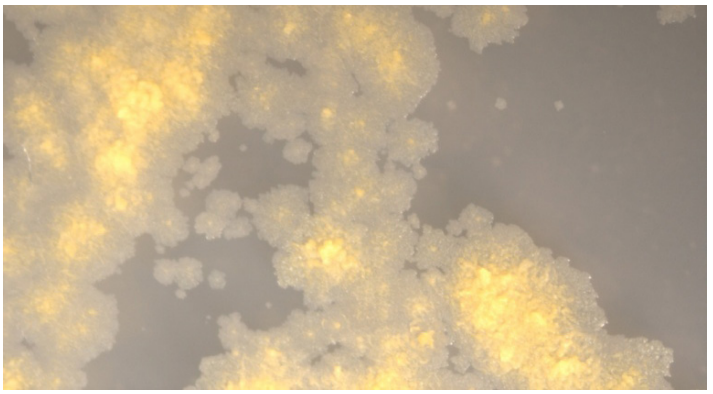
Optical Property – This describes the opacity of the colonies. Colonies are frequently described as opaque (impervious to light), translucent (lets light through diffusely), or transparent (allows light to pass through without disruption).

Texture – Describes the consistency of a colony. Rough colonies have a granular, flattened surface and are often associated with loss of virulence. Smooth colonies have a glistening, rounded surface. Other terms used to describe colony texture include mucoid (gummy, viscous), butyrous (buttery texture), or dry (brittle or powdery colonies).

Pigmentation – Some bacterial species produce pigments which can be either water soluble or soluble in fat. The production of pigments may vary depending on the environmental conditions or age of the colony.



Colonies are circular, entire, convex, large, smooth, glistening, cream-colored, and translucent.



Colonies are irregular, erose, flat, wrinkled, rough, dull, yellow, and opaque.

ENVIRONMENTAL MICROFLORA

Microorganisms are ubiquitous in the environment, surviving on locations such as human skin, dust particles, and inanimate surfaces. When working in a microbiology laboratory, it is important to use aseptic technique to ensure that these naturally occurring microbes do not contaminate your cultures as it can result in incorrect data and cost significant amounts of time and money. In the procedures below, we describe several common causes of contamination in the laboratory and provide procedures on how to examine each source.

HUMAN MICROFLORA

Human skin microflora are usually non-pathogenic organisms that colonize the skin surface either transiently or residentially. The overall extent of bacterial survival on the skin depends on a number of factors including exposure to a particular environment or substance, innate bactericidal activity in the skin, or the degree of adherence of bacteria to epithelial surfaces.[6] This exercise will demonstrate if washing or sanitizing your hands removes skin microflora.

MATERIALS

- Hand sanitizer
- Hand soap
- 2 sterile petri dishes with agar

PROCEDURE

1. Draw a line on the bottom of the agar plates, dividing each agar plate into two sections. Label each section 1, 2, 3, and 4.
2. Prior to washing hands, press three of your fingertips firmly onto section 1 of the agar.
3. Rinse your fingers in cold tap water for 20-30 seconds. Allow the excess water to drip off, and press three of your fingertips firmly onto section 2 of the agar.
4. Wash fingers with hand soap for 20-30 seconds. Rinse your fingers in cold tap water for 20-30 seconds. Allow the excess water to drip off, and press three of your fingertips firmly onto section 3 of the agar.
5. Sanitize fingers with hand sanitizer for 20-30 seconds. Press three of your fingertips firmly onto section 4 of the agar.
6. Incubate each plate agar-side-up for 48 hours at 30°C.
7. Observe the amount of growth in each section.

Thoroughly wash hands with soap. Photo courtesy of Amanda Mills and CDC.

INANIMATE SURFACES

Inanimate surfaces have often been described as a source of contamination. In fact, most gram-positive bacteria, such as *Enterococcus* spp. and *Staphylococcus aureus*, and gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli*, are capable of surviving months on dry surfaces.[7] This exercise will demonstrate the extent of surface contamination if no regular preventive surface disinfectant is used.

MATERIALS

- Small vial of sterile PBS
- Sterile cotton tipped applicator
- Sterile 1 mL pipette
- Pipette bulb
- Glass or metal streaking “hockey” stick
- 70% ethanol
- Bunsen burner
- Sterile petri dish with agar

PROCEDURE

Create a suspension after swabbing. Photo courtesy of Dr. Michael Rein and CDC.

1. Using aseptic technique, carefully open the vial of PBS and dip the head of the cotton tipped applicator into the PBS.
2. Using the moist cotton tipped applicator, swab several surfaces that you normally come in contact with during your laboratory experimentation. This could include your bench top, the sink, or your inoculation tools.
3. Dip the head of the cotton tipped applicator back into the PBS. Move the head of the applicator around within the PBS to help dislodge any bacteria that may have adhered. Replace the vial lid and properly dispose of the applicator.
4. Place a petri dish agar-side-down onto the bench top.
5. Select a sterile 1 mL pipette and gently attach it to the pipette bulb. Do not touch the tip of the pipette, this will contaminate it.
6. With your other hand, pick up the vial of PBS and remove the cap with the fourth and fifth fingers your opposite hand. Never lay a cap down on the lab bench, this will result in contamination. Hold the open tube at an angle so that dust does not fall into the tube and contaminate the culture.
7. Insert the sterile pipette tip into the vial and aspirate 0.1 mL of PBS.
8. Remove the pipette from the vial, replace the cap, and set it aside. Carefully hold the pipette, do not touch the tip or lay the pipette on the bench top.
9. With your free hand, pick up the lid to the petri dish and hold it at an angle over the plate. This will prevent dust from falling into your culture while you inoculate.
10. Slowly dispense the liquid from the pipette onto the center of the plate. Replace the petri dish lid, and properly dispose of the pipette.
11. Sterilize the hockey stick by submerging the short, flat portion of the hockey stick into the 70% ethanol. Pass the hockey stick quickly through the Bunsen burner flame to light it on fire. Do not leave the hockey stick in the flame. Once the flame has burned out, allow the hockey stick to cool for a few seconds.
12. With your free hand, lift up the lid of the petri dish. Place the sterile portion of your hockey stick onto the agar and move it around the plate, evenly spreading the PBS on the surface of the agar. Gently turn the plate while spreading the media to ensure the surface of the agar is evenly covered. Do not puncture the agar.
13. Continue to spread the culture media until it has dried onto surface of the plate. Replace the petri dish lid and re-sterilize the hockey stick.
14. Incubate the petri dish for 48 hours at 30°C.
15. Observe colony growth.

AIRBORNE CONTAMINATION

Airborne contamination frequently occurs from aerosolized microorganisms spread by coughing and sneezing, or by bacteria carried on the surface of microscopic dust particles. To avoid contamination by airborne particles, it is important to always use proper aseptic technique in the laboratory. This exercise will demonstrate the extent of contamination due to airborne and aerosolized microorganisms.

Example of an open air plate following incubation

MATERIALS

- Sterile petri dish with agar

PROCEDURE

1. Place an agar plate agar-side-down on your laboratory bench top.
2. Remove the lid from the plate and set it aside.
3. Leave the agar surface exposed to the air for 60 minutes.
4. Replace the lid.
5. Incubate the plate for 48 hours at 30°C
6. Observe colony growth.

CULTURING TECHNIQUES

A culture medium is a solution of nutrients that is required for microbial growth. Depending on their composition or use, culture media can be categorized into several groups; these include defined, complex, selective, and enrichment medium. In a **defined medium**, the exact chemical composition is known. These types of media are usually composed of pure biochemicals, and are often used to study the minimal nutrient requirement of a microorganism. In contrast, the exact chemical composition of a **complex medium** is not known. This latter medium type often contains reagents of a biological origin, such as yeast extract and peptone, where the exact chemical composition is unknown. Complex media usually provide a large range of growth factors that assist in the cultivation of unknown and fastidious bacterial species.[8]

Medium may also be formulated as selective or to enrich. A **selective medium** is formulated to inhibit the growth of certain bacterial species and/or promote the growth of a specific species. These media can consist of additional selective reagents, such as high salt concentration to select for halophiles, or can be used under selective growth conditions. An **enrichment medium** also allows for the growth of specific bacterial species; however, enrichment media are supplemented with a reagent that permits, rather than inhibits, the growth of a particular species.[8]

Generally, bacterial culture media are mixtures of proteins, salts, trace elements, amino acids, and carbohydrates. The presence and volume of these components can vary significantly among bacterial species depending on the macro- and micro-nutrient requirements of each strain. The manner of which bacterial strains are cultured also varies widely. Liquid media are often used for the growth and propagation of pure batch cultures, while solid agar-based media are used for the isolation of pure cultures. All culture media must be sterilized prior to its use. This is often accomplished by heating the media at high temperatures within an **autoclave**. This instrument provides 15 pounds per square inch of steam pressure, allowing for temperatures to reach and be maintained at 121°C. All media must be loosely capped prior to sterilization to equalize the pressure of the container and to prevent contamination upon removal from the autoclave. Below, we will describe how to inoculate various broth and agar cultures using aseptic technique.

BROTH CULTURE

A broth culture is a liquid nutritional medium used to culture live microorganisms in either a test tube or an Erlenmeyer flask. This form of culture allows for the rapid growth of many microorganisms, and is often used to prepare specimens for cryopreservation or to propagate large volumes of microbial cultures. Below we describe an aseptic protocol for the inoculation of a broth culture.

MATERIALS

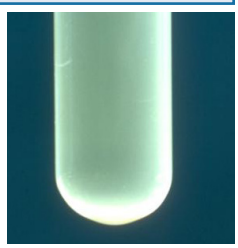
- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Sterile broth media

PROCEDURE

1. Flame a wire inoculating loop to sterilize it. The inoculating loop should be heated to a red glow using a Bunsen burner. Ensure that both the loop and the lower portion of the handle have been sterilized. Allow the loop to cool before obtaining a culture. If the loop is too hot, it will cause the cells to burst (See: NOTE 1).
2. With your other hand, pick up a culture tube and remove the cap with the fourth and fifth fingers of the hand that is holding the loop. Never lay a cap down on the lab bench, this will result in contamination. Always hold the open tube at an angle so that dust does not fall into the tube and contaminate the culture.
3. Pass the opening of the culture tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
4. Insert the sterile wire loop into the culture tube. If you have a culture growing on an agar slant, touch the inoculation loop onto the culture surface and gently gather a small amount of the microorganism. If you have a broth culture, the inoculating loop can be submerged within the culture.

NOTE 1

Sterile, disposable loops can be used to inoculate cultures. These loops are ready-to-use and do not require flame sterilization.



Actively growing broth culture of *Enterobacter cloacae*. Photo courtesy of Dr. J.J. Farmer and CDC

5 Flame the mouth of the culture tube again, replace the cap, and set it aside.
6 With your free hand, pick up a tube of sterile media and remove the cap with the fourth and fifth fingers of the hand that is holding the loop (See step 2).
7 Pass the opening of the media tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
8 Gently insert the inoculation loop into the media and move the loop back and forth several times to inoculate the media.
9 Remove the inoculation loop and flame the mouth of the test tube again, replacing the cap. Set the tube aside.
10 Sterilize the inoculating loop as described in step 1.
11 Incubate the broth culture under the appropriate growth conditions.

AGAR SLANT

Agar slants are a form of solid media generated from the addition of a gelling agent, such as agar, to a broth culture. To prepare an agar slant, agar is added to the broth culture then heated within an autoclave to dissolve the agar and sterilize the media. This medium will then solidify at 42°C. Agar slants provide a large surface area on which to grow microorganisms and are frequently used as a method to temporarily store actively growing cultures. Below we describe an aseptic protocol for the inoculation of an agar slant.

MATERIALS

- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Sterile agar slant

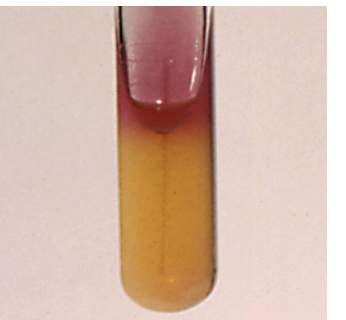
Agar slant containing an actively growing culture of *Chlorella* sp. Photo courtesy of the CDC

PROCEDURE

1 Flame a wire inoculating loop to sterilize it. The inoculating loop should be heated to a red glow using a Bunsen burner. Ensure that both the loop and the lower portion of the handle have been sterilized. Allow the loop to cool before obtaining a culture. If the loop is too hot, it will cause the cells to burst (See: NOTE 1).
2 With your other hand, pick up a culture tube and remove the cap with the fourth and fifth fingers of the hand that is holding the loop. Never lay a cap down on the lab bench, this will result in contamination. Always hold the open tube at an angle so that dust does not fall into the tube and contaminate the culture.
3 Pass the opening of the culture tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
4 Insert the sterile wire loop into the culture tube. If you have a culture growing on an agar slant, touch the inoculation loop onto the culture surface and gently gather a small amount of the microorganism. If you have a broth culture, the inoculating loop can be submerged within the culture.
5 Flame the mouth of the culture tube again, replace the cap, and set it aside.
6 With your free hand, pick up the sterile agar slant and remove the cap with the fourth and fifth fingers of the hand that is holding the loop (See step 2).
7 Pass the opening of the media tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
8 Gently insert the inoculation loop into the test tube and gently streak the microbial culture onto the surface of the agar slant. Do not puncture the agar.
9 Remove the inoculation loop and flame the mouth of the test tube again, replacing the cap. Set the tube aside.
10 Sterilize the inoculating loop as described in step 1.
11 Incubate the agar slant under the appropriate growth conditions.

AGAR STAB

The agar stab is an inoculation technique used when inoculating semi-solid medium for the analysis of motility or oxygen usage, or when inoculating certain types of solid medium. This particular method of inoculation allows one to culture a microorganism under limited oxygen exposure. Below, we describe an aseptic protocol for an agar stab.



Agar slant inoculated using the agar stab culturing technique. Photo courtesy of Dr. Edwards and CDC

MATERIALS

- Slant or broth culture
- Wire inoculation needle
- Bunsen burner
- Sterile agar slant or semi-solid agar

PROTOCOL

1. Flame a wire inoculating needle to sterilize it. The inoculating needle should be heated to a red glow using a Bunsen burner. Ensure that a large section of the needle has been sterilized. Allow the needle to cool before obtaining a culture. If the needle is too hot, it will cause the cells to burst.
2. With your other hand, pick up a culture tube and remove the cap with the fourth and fifth fingers of the hand that is holding the loop. Never lay a cap down on the lab bench, this will result in contamination. Always hold the open tube at an angle so that dust does not fall into the tube and contaminate the culture.
3. Pass the opening of the culture tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
4. Insert the sterile wire needle into the culture tube. If you have an agar slant, touch the sides of the inoculation needle onto the culture surface and gently gather a small amount of the culture. Do not stab the agar. If you have a broth culture, the inoculating needle can be submerged within the culture.
5. Flame the mouth of the culture tube again, replace the cap, and set it aside.
6. With your free hand, pick up the sterile agar slant and remove the cap with the fourth and fifth fingers of the hand that is holding the needle (See step 2).
7. Pass the opening of the media tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
8. Gently insert the inoculation needle into the test tube and push the needle through the agar until it is approximately 0.5 inches from the bottom of the tube.
9. Carefully remove the inoculation needle and flame the mouth of the test tube again, replacing the cap. Set the tube aside.
10. Sterilize the inoculating needle as described in step 1.
11. Incubate the agar slant under the appropriate growth conditions.

STREAK PLATE

Similar to the agar slant, streak plates are a form of solid media generated from the addition of a gelling agent, such as agar, to a broth culture. This medium is then added to a petri dish, rather than a test tube, and allowed to solidify. The term "streak plate" refers to how the culture is inoculated on the agar surface. This particular inoculation technique is used to isolate a pure colony from a single microbial species. Samples obtained from the resulting pure colony can then be grown on a new plate so that the organism can be identified, studied, or the colony morphology can be analyzed. For more information on the analysis of colony morphology, see the section entitled "Colony Morpholgy." Below we describe an aseptic protocol for the inoculation of a streak plate.

MATERIALS

- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Sterile petri dish with agar

PROCEDURE

1. Place a petri dish agar-side-up onto the bench top. Using a permanent marker, draw a "T" on your plate to separate your plate into 3 sections. Label each section (See: FIGURE 4).
2. Flame a wire inoculating loop to sterilize it. The inoculating loop should be heated to a red glow using a Bunsen burner. Ensure that both the loop and the lower portion of the handle have been sterilized. Allow the loop to cool before obtaining a culture. If the loop is too hot, it will cause the cells to burst (See: NOTE 1).
3. With your other hand, pick up a culture tube and remove the cap with the fourth and fifth fingers of the hand that is holding the loop. Never lay a cap down on the lab bench, this will result in contamination. Always hold the open tube at an angle so that dust does not fall into the tube and contaminate the culture.
4. Pass the opening of the culture tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
5. Insert the sterile wire loop into the culture tube. If you have an agar slant, touch the inoculation loop onto the culture surface and gently gather a small amount of the microorganism. If you have a broth culture, the inoculating loop can be submerged within the culture.
6. Flame the mouth of the culture tube again, replace the cap, and set it aside.
7. With your free hand, pick up the agar-half of the petri dish, leaving the lid face up on your bench.
8. Streak out the culture onto section 1 of your plate (See: FIGURE 5). Return the petri dish to its lid.
9. Sterilize the inoculating loop as described in step 1.
10. Once cool, pick up the agar-half of the petri dish and pass the inoculation loop 1-3 times through section 1, dragging the culture through section 2 multiple times. Be sure your streaks do not overlap (See: FIGURE 5). Return the petri dish to its lid.

11 Sterilize the inoculating loop as described in step 1.

12 Once cool, pick up the agar-half of the petri dish and pass the inoculation loop 1-3 times through section 2, dragging the culture through section 3 multiple times. Be sure your streaks do not overlap (See: FIGURE 5). Return the petri dish to its lid.

13 Sterilize the inoculating loop as described in step 1.

14 Incubate the petri dish under the appropriate growth conditions. Be sure to incubate the plate agar-side-up, this will prevent condensation from dropping into the culture. With appropriate streak plate technique, you will obtain isolated colonies in section 3 of your plate.

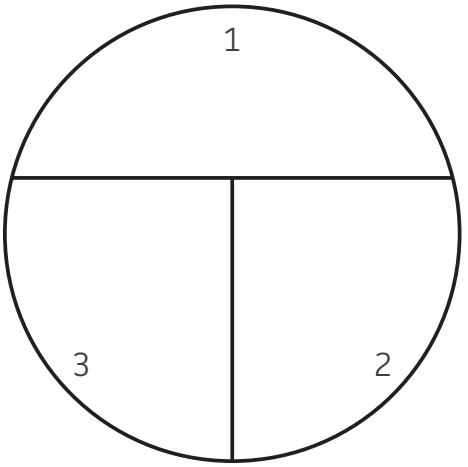


Figure 4: Streak plate set-up

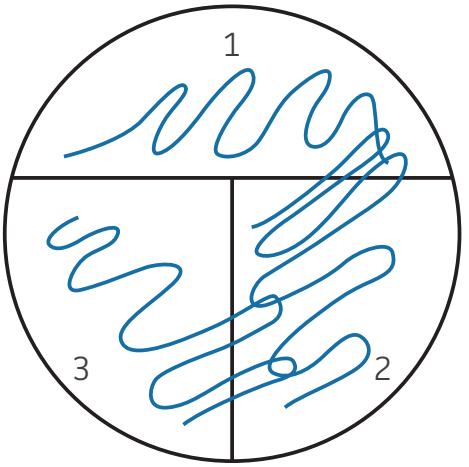
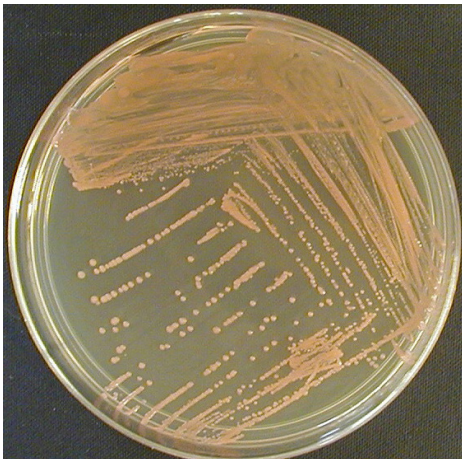


Figure 5: How to inoculate a streak plate



Example of a streak plate exhibiting isolated colonies following incubation

SPREAD PLATE

Similar to the streak plate technique, a spread plate is another form of inoculation that used to isolate pure colonies from a single microbial species. This particular technique is primarily used to quantify the number of microorganisms within an actively growing broth culture. For more information on the enumeration of a microorganism, view the section entitled "Bacterial Enumeration". Below we describe an aseptic protocol for the inoculation of a spread plate.

MATERIALS

- Broth culture
- Sterile 1 mL pipette
- Pipette bulb
- Glass or metal streaking "hockey" stick
- 70% ethanol
- Bunsen burner
- Sterile petri dish with agar

PROCEDURE

1 Place a petri dish agar-side-down onto the bench top.

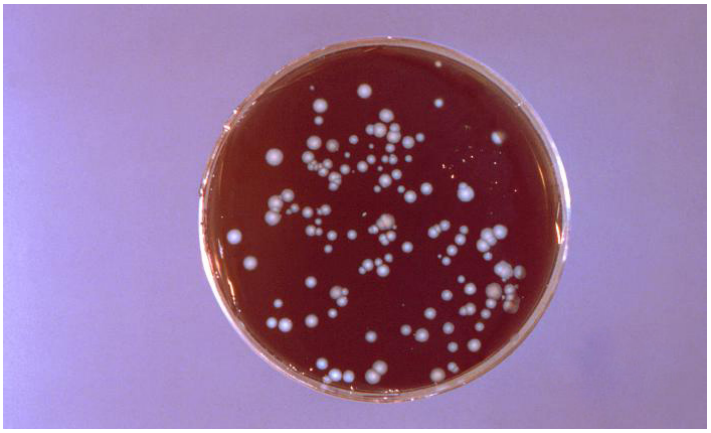
2 Select a sterile 1 mL pipette and gently attach it to the pipette bulb. Do not touch the tip of the pipette, this will contaminate it.

3 With your other hand, pick up the culture tube and remove the cap with the fourth and fifth fingers of your opposite hand. Never lay a cap down on the lab bench, this will result in contamination. Always hold the open tube at an angle so that dust does not fall into the tube and contaminate the culture.

4 Pass the opening of the culture tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.

5 Insert the sterile pipette tip into the culture tube and aspirate 0.1 mL of growing culture.

6 Remove the pipette from the culture tube, flame the mouth of the culture tube again, replace the cap, and set it aside. Carefully hold the pipette, do not touch the tip or lay the pipette on the bench top.



Example of a spread plate exhibiting isolated colonies of Legionella pneumophilia following incubation. Photo courtesy of Dr. Jim Feeley and CDC.

7 With your free hand, pick up the lid to the petri dish and hold it at an angle over the plate. This will prevent dust from falling into your culture while you inoculate.

8 Slowly dispense the culture media from the pipette onto the center of the plate. Replace the petri dish lid, and properly dispose of the pipette.

9 Sterilize the hockey stick by submerging the short, flat portion of the hockey stick into the 70% ethanol. Pass the hockey stick quickly through the Bunsen burner flame to light it on fire. Do not leave the hockey stick in the flame. Once the flame has burned out, allow the hockey stick to cool for a few seconds.

10 With your free hand, lift up the lid of the petri dish. Place the sterile portion of your hockey stick onto the agar to dissipate the heat. Then spread the inoculum over the plate, evenly spreading the culture media on the surface of the agar. Gently turn the plate while spreading the media to ensure the surface of the agar is evenly covered. Do not puncture the agar (See: NOTE 2).

11 Continue to spread the culture media until it has dried onto surface of the plate. Replace the petri dish lid and re-sterilize the hockey stick.

12 Incubate the petri dish under the appropriate growth conditions. Be sure to incubate the plate agar-side-up, this will prevent condensation from dropping into the culture. With appropriate spread plate technique, you will obtain isolated colonies on your plate.

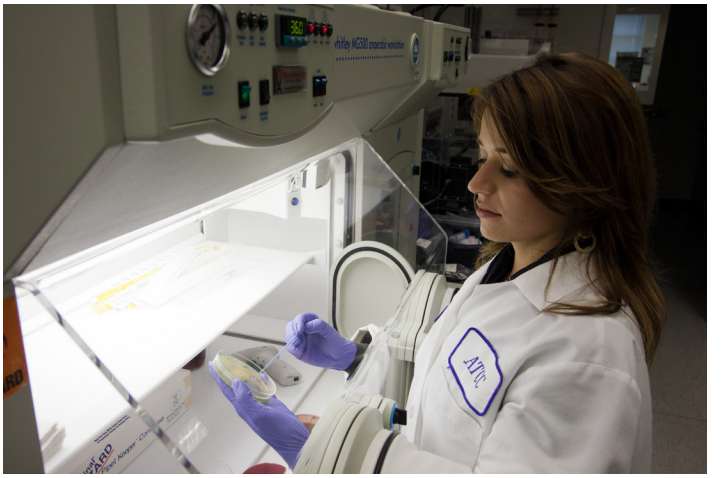
NOTE 2

Pre-drying the plates will aid in spreading the culture media evenly and quickly.

CULTURING CONDITIONS

Because bacteria can grow and thrive in a variety of environments, optimal growth temperatures may vary significantly between species. In general, most pathogenic or commensal bacterial strains grow well at body temperature (37°C). In contrast, many environmental strains thrive at lower temperatures, often within a range of 25°C to 30°C.

Bacterial species can be categorized based on their growth temperature; these include **psychrophiles** (0°C to 20°C), **mesophiles** (25°C to 40°C), and **thermophiles** (45°C to 122°C). Though bacterial strains require optimal temperatures for growth and reproduction, most strains can withstand considerable drops in temperature and survive several days at 4°C. At these lower temperatures, bacterial growth and metabolism are significantly diminished.



In addition to varying requirements for optimal growth temperatures, bacteria also differ in their use of oxygen for respiration. **Aerobic** organisms, such as *Bacillus* species, use oxygen as a terminal electron acceptor during respiration. Similarly, **microaerophiles**, such as *Helicobacter* pylori, also require the use of oxygen, but at lower levels than naturally occurring in the environment. In contrast, **anaerobic** organisms use electron acceptors such as nitrate or sulfate, among other inorganic acceptors. These inorganic compounds, however, have a lower reduction potential than oxygen thus resulting in less efficient respiration.

The use of oxygen and inorganic compounds by anaerobic organisms can differ greatly between species. **Obligate anaerobes**, such as *Clostridium* species, can only survive and reproduce in the absence of oxygen; these organisms are often killed by the presence of oxygen. Similarly, **aerotolerant anaerobes**, such as *Lactobacillus* species, cannot use oxygen during respiration; however, unlike strict anaerobes, these microorganisms can tolerate oxygen for short periods of time. Lastly, **facultative anaerobes**, such as *Escherichia coli* and *Staphylococcus* species, are able to survive in both the presence and absence of oxygen. If given the choice, these organisms prefer the use of oxygen during respiration as it has the greatest reduction potential as compared to other electron acceptors.

When working with anaerobic cultures, it is important to avoid unnecessary exposure to oxygen. Anaerobic conditions may be obtained for either transfer or incubation by the methods listed below. Unless specifically mentioned, the standard anaerobic gas mixture is 80% N_2, 10% CO_2, and 10% H_2.

Anaerobic conditions for transfer may be obtained by either of the following:

1. Use of an anaerobic gas chamber.
2. Placement of test tubes under a gassing cannula system hooked to anaerobic gas.

During incubation, anaerobic conditions may be maintained by any of the following:

1. Loose screw-caps on test tubes in an anaerobic chamber.
2. Loose screw-caps on test tubes in an activated anaerobic GasPak™ jar.
3. Use of sterile butyl rubber stoppers on test tubes so that an anaerobic gas headspace is retained.

Below, we describe a general procedure to analyze the temperature and oxygen requirements of a microorganism. The oxygen requirements of an organism can also be analyzed using thioglycollate medium, which is described in the section entitled “Differential Tests”.

Materials

- Broth culture
- 6 sterile agar plates per culture
- 3 sterile pre-reduced agar plates per culture
- Wire inoculation loop
- Bunsen burner

Procedure

1. Inoculate the six sterile agar plates using the streak plate culturing technique.
2. Under anaerobic conditions, inoculate the three pre-reduced agar plates using the streak plate culturing technique.

3 Incubate the plates agar-side-up at either 25°C, 30°C, or 37°C under the following conditions:
   a Aerobic – incubate plates in a standard incubator.
   b Microaerophilic – incubate plates in a candle jar. This can be any jar large enough to hold petri dishes and a candle. Place your plate and a lit candle into the jar and seal the lid. The candle flame will consume most of the oxygen in the jar, producing elevated levels of carbon dioxide.
   c Anaerobic – incubate pre-reduced plates in a sealed anaerobic jar, such as the GasPak™ anaerobic system (Becton Dickinson). This system consists of a polycarbonate jar, a lid with a gasket to prevent air flow, an indicator strip for the detection of oxygen, and a ready-to-use chemical sachet that creates an anaerobic atmosphere.
4 Observe the cultures for signs of growth.

LIGHT MICROSCOPY

The simple brightfield light microscope is one of the most well-known and well-used research tools in microbiology. This type of microscope directly projects light from an incandescent source into the lens system to produce a field of view with a bright background. Microorganisms are visualized in this light path using various stains that absorb the light differentially.[9,10]

In microbiology, the overall value of a brightfield microscope depends on two factors: how well the equipment can magnify a sample and its ability to distinguish objects as single entities. These factors are obtained through the combinatorial use of a built in illuminator, an adjustable condenser with diaphragm control, a mechanical stage, various objective lenses, and a binocular eyepiece. In this section, we will describe the structure and proper use of a light microscope.

ANATOMY OF A LIGHT MICROSCOPE

While most light microscopes are similar in construction, there can be differences in the general use of the microscope depending on the manufacturer. Below are some of the common features found on a brightfield light microscope (See: FIGURE 6).

Arm – The part of the microscope that connects the head to the base (See: NOTE 3).

Base – The bottom support of the microscope (See: NOTE 3).

Brightness Control – A knob that is used to control the intensity of the light source.

Condenser – A lens mounted in or below the mechanical stage whose purpose is to focus or condense light onto the sample. The condenser lens will increase illumination and resolution power.

Course Adjustment – The rough focus knob on the microscope used to move the objective lenses toward or away from the sample (See Fine Adjustment).

Diaphragm – A rotating disk used to adjust the amount of light that passes through the condenser lens through the use of holes that vary in diameter.

Diopter Adjustment – An ocular adjustment knob that can modify the focus in each eyepiece to compensate for the difference in vision between your eyes.

Fine Adjustment – A knob used for fine tuning the focus on a specimen.

Head – The upper portion of the microscope that contains the oculars and prisms.

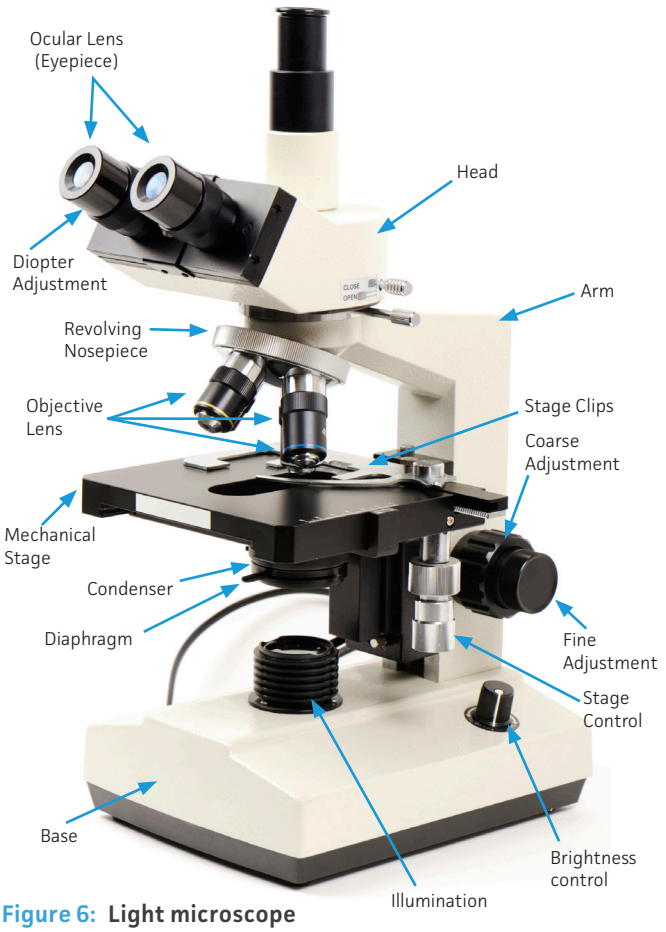


Figure 6: Light microscope

Illumination – The light source that is mounted beneath the mechanical stage. Tungsten, Fluorescent, or Halogen lights are commonly used here.

Mechanical Stage – The platform used to hold a slide. It can be moved both vertically and horizontally to adjust the position of the slide beneath the objective lens.

Objective Lens – Lenses that are used to magnify an object. The shortest objective lens is the scanning objective (4X), which is used to determine what portion of the slide you want to magnify. The other lenses, in increasing length and magnification power, include the low power lens (10X), the high dry lens (40X), and the oil immersion lens (100X). The oil immersion lens has the highest resolving power, and is designed to work with a drop of oil placed between it and the slide.

> **NOTE 3**
> When carrying the microscope, grab the arm with one hand and place your other hand beneath the base.

Ocular Lens – The ocular, or eyepiece, is the lens found atop the microscope that is looked through. Most ocular lenses have either a 10X or 15X magnification level.

Revolving Nosepiece – An apparatus that holds the objectives in place. It can be turned clockwise and counterclockwise to adjust which objective is in use.

Stage Clips – Clips on the mechanical stage used to hold the slide in place.

Stage Control – A knob used to control the horizontal movement of the mechanical stage.

PROPER USE OF A LIGHT MICROSCOPE

Microscopes are expensive, highly valuable tools. To ensure the quality and value of your microscope, we have provided a generalized protocol on the proper use of a microscope and a brief list of tips on its handling and care.

MATERIALS

-
- Microscope
- Prepared slide
- Lens cleaner
- Lens paper
- Dust jacket

Procedure

1. **Clean the microscope** – Use lens paper to remove dust from all the lenses, including the condenser and light source. Dried oil can be removed using a lens cleaner solution.
2. **Prepare the microscope** – Plug in the microscope and turn on the light source. Rotate the revolving nosepiece so that the scanning objective (4X) clicks into place.
3. **Mount the specimen on the stage** – Obtain a prepared slide. Lower the stage with the course adjustment knob so that the slide can be placed onto the stage. Secure the slide to the mechanical stage using the stage clips. Ensure that your specimen is centered over the light source coming through the condenser.
4. **Adjust the oculars** – Look through the oculars and adjust appropriately to ensure that each ocular lens is in focus and are spaced to suit your vision.
5. **Adjust the lighting** – Check the condenser height, if the field of view appears grainy it may indicate that the condenser is too high. Look through the oculars and adjust the diaphragm and brightness level so that the field of view has a comfortable level of brightness. Too much light on a lower magnification power will cause the image to bleach out, making it difficult to locate the specimen.
6. **Focus the specimen** – Using the course adjustment knob, slowly raise the mechanical stage until the specimen is almost in focus. Use the fine adjustment knob to fine tune your focus. If you have difficulty finding the specimen, move the slide back and forth while you are focusing.
7. **Magnify the sample** – Move the slide so that the part of the specimen you wish to concentrate on is in the center field. Rotate the nosepiece until the low power (10X) objective clicks into position. As the microscope is parfocal, the specimen should still be in focus. Open the diaphragm slightly to admit more light. Repeat these steps with the high dry (40X) objective.
8. **Prepare the oil immersion lens** – Rotate the nosepiece so that the specimen is between the high dry (40X) objective lens and the oil immersion (100X) objective lens. Place a small drop of immersion oil on top of the slide where the light passes through the specimen. Click the oil immersion lens into place. The lens should be immersed in the oil. Open the diaphragm all the way to allow for maximum light to pass through the specimen. Carefully use the fine focus adjustment knob to allow your image to go into focus (See: NOTE 4).

   NOTE 4

   If you need to return to a lower magnification to focus, remove the oil from the slide first. Getting oil on the other objectives will ruin the lens.
9. **Clean the microscope** – After completing your microscopic work, always clean the oil from the oil immersion lens and mechanical stage using an appropriate lens cleaner and lens paper. Before storing the microscope, rotate the scanning objective lens into place, turn off the light source, unplug the microscope, and lower the stage. To avoid the accumulation of dust, cover your microscope with a dust jacket during storage.

CARE OF THE MICROSCOPE

- Always handle the microscope with two hands. With one hand, firmly grasp the arm. With your other hand, place your hand below the base of the microscope.
- Hold the plug when unplugging the microscope. Never unplug the microscope by pulling on the cable.
- Microscope bulbs can be expensive and have a limited shelf life. Turn the illuminator off when the microscope is not in use.
- Always make sure the stage and lenses are clean before and after using the microscope. Only use good quality lens tissue and lens cleaner when cleaning an optical surface.
- Cover the instrument with a dust jacket when not in use.
- Always use the course and fine adjustment knobs smoothly to prevent damage of the objective lens.

OBSERVING MICROORGANISMS

Microorganisms are commonly examined microscopically either through a wet mount or by the staining of heat-inactivated smears. Below, we describe how to analyze cellular morphology and provide several techniques and procedures commonly used to examine and distinguish microorganisms.

PREPARING A SMEAR

Although viewing live microorganisms is ideal, most microscopic examinations require the staining of heat-fixed smears. To prepare the slide, a small amount of a cell suspension is spread onto a slide and allowed to air-dry. After the liquid has completely dried, the slide is gently heated to allow the microorganism in the smear to stick to the slide, preventing it from washing off during the staining process. Below is a general procedure on how to prepare a heat-fixed smear.

MATERIALS

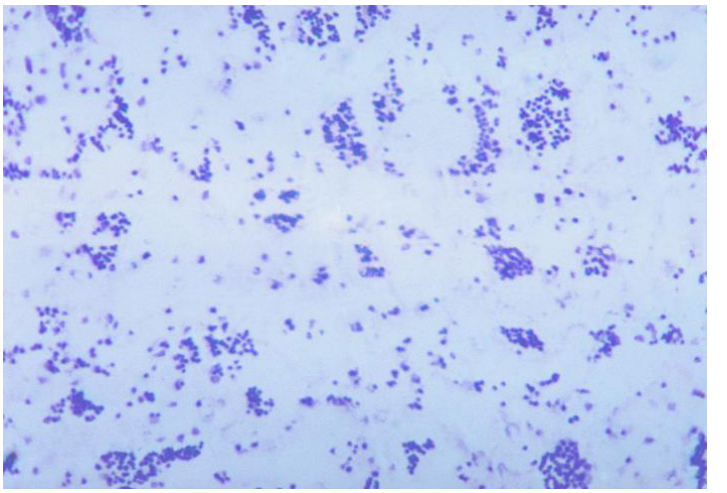
- Slant or broth cultures of bacteria
- Clean glass slides
- Wire inoculation loop
- Bunsen burner
- Clean water
- Clothespin

Procedure

1. Flame a wire inoculating loop to sterilize it. The inoculating loop should be heated to a red glow using a Bunsen burner. Ensure that both the loop and the lower portion of the handle have been sterilized. Allow the loop to cool before obtaining a culture. If the loop is too hot, it will cause the cells to burst (See: NOTE 1).
2. With your other hand, pick up a culture tube and remove the cap with the fourth and fifth fingers of the hand that is holding the loop. **Never lay a cap down on the lab bench, this will result in contamination**. Always hold the open tube at an angle so that dust does not fall into the tube and contaminate the culture.
3. Pass the opening of the test tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
4. Insert the sterile wire loop into the culture tube. If you have an agar slant, touch the inoculation loop onto the culture surface and gently gather a small amount of the culture. If you have a broth culture, the inoculating loop can be submerged within the culture.
5. Flame the mouth of the culture tube again, replace the cap, and set it aside.
6. Cultures gathered from an agar slant can be mixed with a small drop of water on the slide, creating a smear. Cultures gathered from broth can be directly smeared on a clean slide, no water is necessary.
7. Flame the wire inoculating loop to sterilize it before setting it down. Heat the loop thoroughly and gently to remove all traces of the microbial culture.
8. Allow the smear to air-dry. Once the smear is fully dried, securely clamp the slide with the clothespin and heat fix the organisms by passing the slide through the burner flame two to three times. Excess heat will distort the cells.

SIMPLE STAIN

A simple stain involves the use of a single dye and is frequently used to determine cellular shape, size, and arrangement. The most common dyes used in this stain include methylene blue, crystal-violet, and safranin. Each of these dyes is a basic stain that harbors a positive charge, allowing it to bind electrostatically to negatively charged molecules such as proteins, nucleic acids, and polysaccharides. Since the surface of most bacterial cells is negatively charged, basic dyes will readily adhere to the cell surface.



Simple stain of *Francisella tularensis* using methylene blue. Photo courtesy of Dr. PB Smith and CDC

MATERIALS

- Slant or broth cultures
- Clean glass slides
- Wire inoculation loop
- Bunsen burner
- Clothespin
- Staining tray
- Wash bottle
- Basic dye

PROCEDURE

1. Prepare a bacterial smear as described in the section entitled "Preparing a Smear".
2. Place the heat-fixed slide onto a staining rack and saturate the smear with a basic dye for approximately one minute. You can use methylene blue, crystal-violet, or safranin for this stain.

3 Gently wash off the stain with water.
4 Carefully blot the slide with a dry paper towel.
5 Observe the slide using light microscopy, using proper microscope technique. You will need the oil immersion lens to view the bacteria. With proper staining technique, all cells will appear blue if methylene blue is used, purple if crystal violet is used, or red if safranin is used.

GRAM STAIN

The Gram stain is a differential stain used to identify gram-positive and gram-negative strains. Cells are distinguished from each other by differences in cell wall structure, which is colorimetrically indicated by how the cell takes up and retains the stain.

The Gram stain procedure first involves staining the cells with crystal-violet, which is a purple dye that is taken up by both gram-positive and gram-negative cells. This dye is then fixed in the cell using an iodine mordant. In gram-positive cells, the dye complex will be maintained in the cell even after decolorization with a 95% ethanol solution. This likely occurs due to the composition of the cell wall structure, which is made up of a thick single layer of peptidoglycan that comprises 60-90% of the cell wall. Further, decolorization of gram-positive cells will result in the dehydration of the thick cell wall, which causes the pores within the cell wall to close, thus trapping the dye within the cell.

In contrast, following decolorization with ethanol, gram-negative cells become colorless. Generally, the cell wall structure of gram-negative cells is comprised of 10-20% peptidoglycan. Further, gram-negative cells have an outer membrane that is not present in gram-positive cells; this layer is made up of polysaccharides, lipids, and proteins. Upon exposure to the ethanol decolorization solution, the outer membrane dissolves, allowing the crystal-violet dye complex to leach out of the cells, rendering the cells colorless. To view gram-negative bacteria, safranin is used to counterstain the cells. If the Gram stain is performed correctly, gram-positive cells will appear purple and gram-negative cells will appear red.

MATERIALS

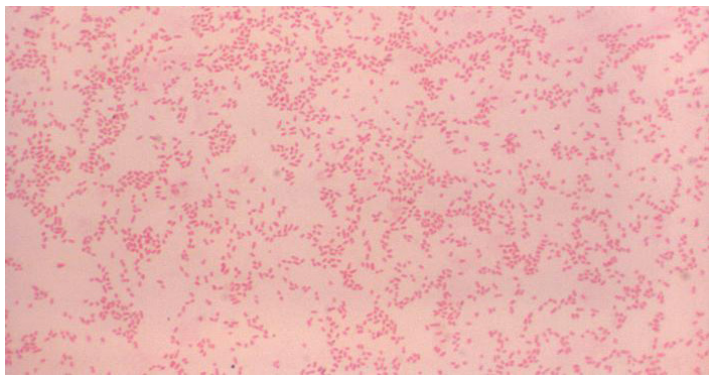
- Slant or broth cultures
- Clean glass slides
- Wire inoculation loop
- Bunsen burner
- Clothespins
- Staining tray
- Wash bottle
- Crystal Violet
- Gram's Iodine Solution
- 95% Ethanol
- Safranin

PROCEDURE

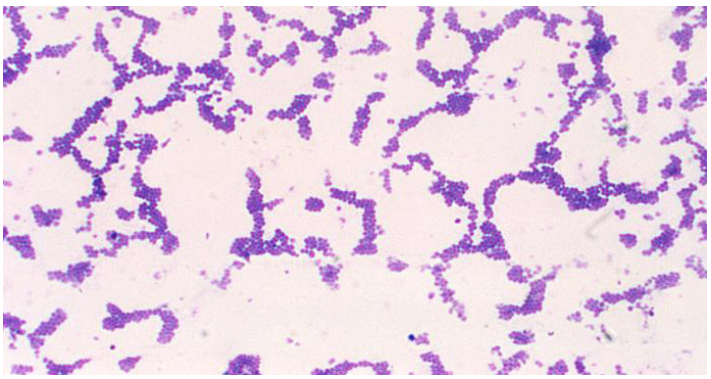
1 Prepare a bacterial smear as described in the section entitled "Preparing a Smear".
2 Saturate the smear with crystal violet for 1 minute.
3 Gently rinse the slide with water. Drain.
4 Saturate the smear with Gram's Iodine Solution for 1 minute.
5 Gently rinse the slide with water. Drain.
6 Decolorize with 95% ethanol solution for 3-5 seconds. Immediately rinse the slide with a gentle stream of water (See: NOTE 5).
7 Counterstain with safranin for one minute.
8 Gently rinse the slide with water. Blot the slide dry with a paper towel.
9 Observe the slide using light microscopy, using proper microscope technique. You will need the oil immersion lens to view the bacteria. With proper staining technique, gram-positive bacteria will stain purple and gram-negative bacteria will stain pink.

NOTE 5

Do not leave the decoloring solution on too long. This will result in the decolorization of gram-positive cells.



Gram stain of *Brucella canis* (gram-negative). Photo courtesy of Dr. WA Clark and CDC



Gram stain of *Staphylococcus aureus* (gram-positive). Photo courtesy of Dr. Richard Facklam and CDC

ACID FAST STAIN

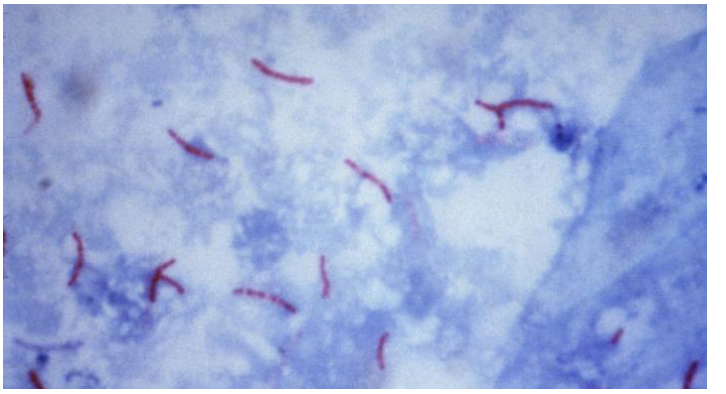
The acid fast stain is a differential stain used to identify acid fast organisms, including mycobacteria, actinomycetes, and nocardia. Acid fast organisms are characterized by their wax-like cell walls that consist of mycolic acids, fatty acids, complex lipids, and waxes. As this type of cell wall is nearly impermeable, these organisms are commonly stained with heated carbolfuchsin dye, which is a lipid-soluble stain that contains phenol to aid in cell wall penetration. The smear is then decolorized with an acid alcohol, which strips the stain from all non-acid fast cells, leaving the acid-fast organisms stained red. The decolorized non-acid-fast cells are then counterstained with a contrasting basic dye, such as methylene blue. If the acid fast stain is performed correctly, acid fast bacteria will appear red and non-acid-fast bacteria will appear blue.

MATERIALS

- Slant or broth cultures
- Clean glass slides
- Wire inoculation loop
- Bunsen burner
- Clothespin
- Staining tray
- Wash bottle
- Carbolfuchsin
- Acid Alcohol
- Methylene Blue

Procedure

1. Prepare a bacterial smear as described in the section entitled "Preparing a Smear".
2. Cover the slide with a paper towel. Saturate the paper with carbolfuchsin.
3. Heat the slide gently over the Bunsen burner for 5 minutes. Be sure that you keep the paper towel saturated with carbolfuchsin during heating. Following heating, allow the slide to cool to room temperature.
4. Remove the paper towel from the slide, and rinse the slide gently with water. Dispose of the used paper towel appropriately.
5. Decolorize the smear with acid alcohol for 20-30 seconds or until the solution runs clear.
6. Gently rinse the slide with water. Drain.
7. Counterstain the smear with methylene blue for one minute.
8. Gently rinse the slide with water. Drain, and carefully blot the slide dry.
9. Observe the slide using light microscopy, using proper microscope technique. You will need the oil immersion lens to view the bacteria. With proper staining technique, acid fast bacteria will stain red and non-acid-fast bacteria will stain blue.



Acid-fast stain revealing *Mycobacterium tuberculosis*. Photo courtesy of Dr. George P. Kubica and CDC

ENDOSPORE STAIN

The endospore stain is a differential stain used to identify the presence of endospores. Endospores are defensive structures that enable some bacterial species to survive in hostile conditions, including heat, desiccation, variations in pH, and chemicals. These metabolically-inactive structures are formed within vegetative cells and are release upon cell death. Upon exposure to favorable conditions, endospores will germinate into a vegetative cell.

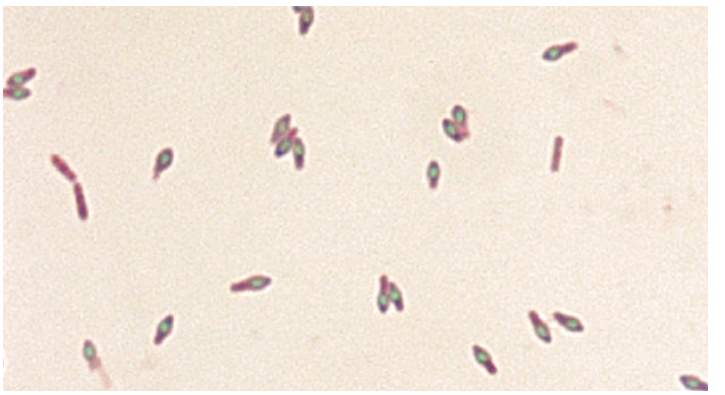
Because of their tough keratin coats, spores are impervious to most staining techniques. To visualize endospores, malachite green dye must be forced into the spore using heat. This dye is then rinsed away from vegetative cells to allow cells to be counterstained with safranin. If the endospore stain is performed correctly, endospores will appear green and vegetative cells will appear red.

MATERIALS

- Slant or broth culture
- Clean glass slides
- Wire inoculation loop
- Bunsen burner
- Clothespin
- Staining tray
- Wash bottle
- Malachite Green
- Safranin

Procedure

1. Prepare a bacterial smear as described in the section entitled "Preparing a Smear".
2. Place a small piece of paper towel over the smear. Saturate the slide with malachite green.
3. Within a fume hood, gently steam the slide over a Bunsen burner flame for 5 minutes. Add more stain if it begins to dry out.



Endospore stain of *Clostridium* botulinum. Photo courtesy of Larry Stauffer and CDC

4 Cool the slide to room temperature and remove the paper towel. Gently rinse the slide with water.
5 Counterstain with safranin for 2 minutes.
6 Gently rinse the slide. Drain and blot dry.
7 Observe the slide using light microscopy, using proper microscope technique. You will need the oil immersion lens to view the bacteria. With proper staining technique, spores will stain green and vegetative cells will stain red.

CAPSULE STAIN

Capsules are formed by several pathogenic bacterial species, such as *Klebsiella pneumoniae* and *Streptococcus pneumoniae*, as a means to escape phagocytosis by macrophages or polymorphonuclear phagocytes. The bacterial capsule primarily consists of polysaccharides, but can be composed of other materials such as polypeptides. As this structure is water-soluble, it can be difficult to stain because most dyes cannot adhere to the capsule. The best way to visualize a bacterial capsule is to stain the background using an acid stain, such as India ink, and to stain the cell with a basic stain, such as crystal violet. This staining technique will result in a black background with the capsule appearing as a clear halo surrounding a purple cell.

MATERIALS

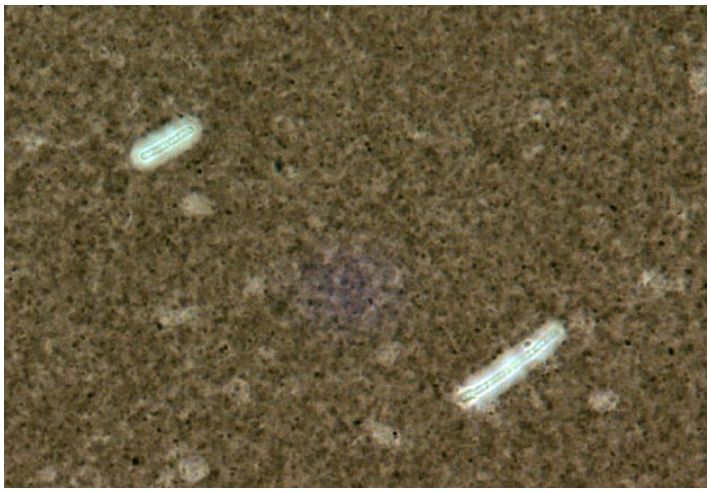
- Slant culture
- Clean glass slides
- Wire inoculation loop
- Bunsen burner
- Staining tray
- Wash bottle
- India ink
- Crystal violet

PROCEDURE

1 Place a single drop of India ink onto a clean slide.
2 Flame a wire inoculating loop to sterilize it. The inoculating loop should be heated to a red glow using a Bunsen burner. Ensure that both the loop and the lower portion of the handle have been sterilized. Allow the loop to cool before obtaining a culture. If the loop is too hot, it will cause the cells to burst (See: NOTE 1).
3 With your other hand, pick up a culture tube and remove the cap with the fourth and fifth fingers of the hand that is holding the loop. Never lay a cap down on the lab bench, this will result in contamination. Always hold the open tube at an angle so that dust does not fall into the tube and contaminate the culture (See: NOTE 6).
4 Pass the opening of the test tube through the Bunsen burner flame. This will kill any microorganisms on the lip of the tube.
5 Insert the sterile wire loop into the culture tube. Touch the inoculation loop onto the culture surface and gently gather a small amount of the culture. Flame the mouth of the culture tube again, replace the cap, and set it aside.
6 Mix the culture into the drop of India ink. Make sure there are no large clumps of the organism, and try to avoid spreading the drop.
7 Flame the wire inoculating loop to sterilize it before setting it down. Heat the loop thoroughly and gently to remove all traces of the microbial culture.
8 Place the end of another clean slide at an angle to the end of the slide containing the India ink and organism. Spread out the drop into a fine film using the capillary action of the dye and slide.
9 Allow the film to air dry. Do not dry the slide using heat, this will cause the capsule to melt.
10 Saturate the slide with crystal violet for 1 minute.
11 Gently rinse the slide with water.
12 Allow the slide to air dry once more. Do not dry the slide using heat, this will cause the capsule to melt.
13 Observe the slide using light microscopy, using proper microscope technique. You will need the oil immersion lens to view the bacteria and capsule. With proper staining technique, you will see a dark background with the capsule appearing as a clear halo surrounding the cell.

NOTE 6

When performing the capsule stain, use a culture that is several days old. Fresh cultures may not have developed capsules yet. Further, though the capsule may be present at the initial isolation of the strain, they tend to disappear upon subculturing or preservation.



Capsule stain of *Bacillus* anthracis. Photo courtesy of Larry Stauffer and CDC.

FLAGELLA STAIN

Many bacterial species are motile, often using flagella to move toward nutrients or colonize new environments. As flagella are very thin, they can be difficult to see using most staining techniques. To analyze the number and location of flagella, a special staining technique that increases the diameter of the flagellum must be used. This procedure often requires the use of a dye, such as Leifson flagella stain or Ryu flagella stain, which will precipitate on a flagellum.

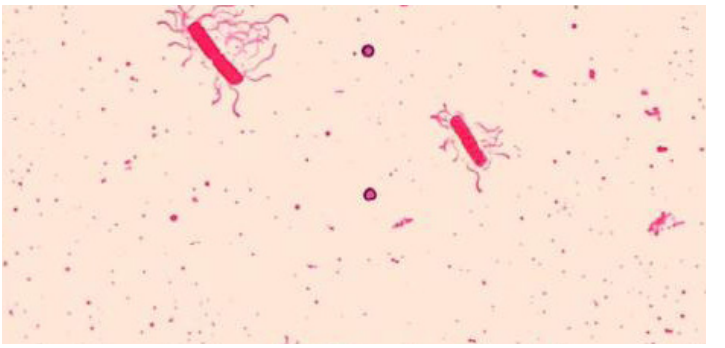
The number and location of flagella can vary significantly between species. For more information, refer to the section entitled "Cellular Morphology".

MATERIALS

- Slant or broth cultures
- Clean glass slides
- Wire inoculation loop
- Bunsen burner
- Staining tray
- Wash bottle
- Leifson Flagella Stain

PROCEDURE

1. Prepare a bacterial smear as described in the section entitled "Preparing a Smear". DO NOT heat-fix the culture; this will destroy the flagella structure.
2. Place the slide on the staining tray and saturate the smear with Leifson flagella stain. Incubate the sample with the dye for 7-15 minutes.
3. As soon as a golden film develops on the dye surface, gently rinse the slide with water.
4. Allow the slide to air dry.
5. Observe the slide using light microscopy, using proper microscope technique. You will need the oil immersion lens to view the bacteria and capsule. With proper staining technique, both cells and flagella will stain red.



Leifson flagella stain of *Bacillus cereus* (Peritrichous). Photo courtesy of Dr. William A. Clark and CDC

WET MOUNT

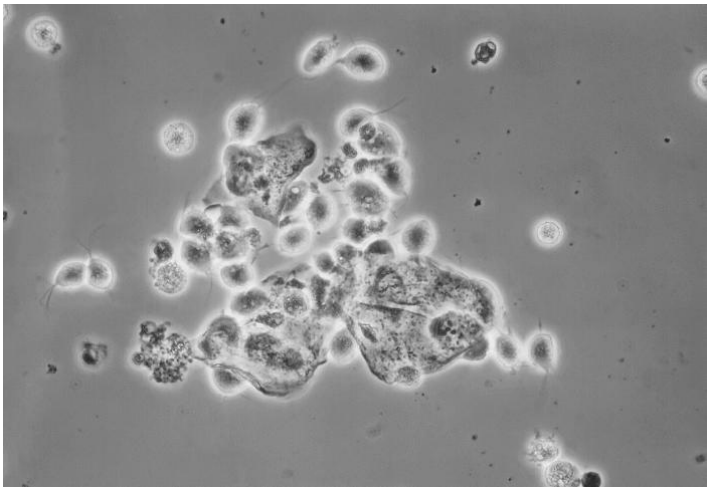
A wet mount is commonly used to examine microorganisms that grow in water or liquid media as a means to observe motility, behavior, and morphology. To prepare a wet mount, a small aliquot from a liquid culture is placed between a slide and coverslip. The sample is then analyzed microscopically.

MATERIALS

- Pond water or broth culture of a microorganism
- Clean glass slides
- Clean coverslips
- Eye dropper

PROCEDURE

1. Using an eye dropper, suck up a few drops of your liquid specimen and place one drop of the sample onto the middle of a clean slide.
2. Carefully pick up a coverslip by grasping the outer edges. Be careful when handling coverslips, they are very fragile and can break easily.
3. Gently cover the sample with the coverslip. To avoid trapping air bubbles, set one edge of the coverslip on the slide at a 45° angle, and let the rest of the coverslip drop. Ensure that the edges of the coverslip match up with the edges of the slide. Do not press down on the coverslip.
4. Observe the slide using light microscopy, using proper microscope technique. The 40X objective should be used to observe the specimen.



Wet mount preparation revealing the presence of the protozoan *Trichomonas vaginalis*. Photo courtesy of Joe Miller and CDC

BACTERIAL ENUMERATION

Bacterial cell counts are necessary in order to establish or monitor bacterial growth rates, determine population doubling time, or to set up new cultures with known cell counts. Bacterial cultures can be titered by determining the viable cell count, which is the number of live colony forming units per milliliter (CFU/mL), or by analyzing total cell count by measuring the optical density at a wavelength of 600 nm (OD_{600}) using spectrophotometry. It must be noted that the growth rate and culturing requirements of bacteria can vary drastically between species, thus making it difficult to quantify a bacterial titer. For more information on how to culture a particular strain, refer to Bergey's Manual of Systematic Bacteriology 2nd Edition.[11] Below, we provide two general procedures on how to determine the bacterial cell count.

VIABLE COUNT

To enumerate CFU/mL within a bacterial suspension at a given time-point, an aliquot is removed from an actively growing solution and diluted in an appropriate broth medium. The extent of the dilution will depend on the growth rate and phase of the strain. In order to accurately determine viable cell count, a dilutions series should be prepared and plated. To determine a dilution, use the following formula:

$$\text{Dilution} = \frac{\text{Volume of Sample}}{\text{Volume of Sample + Volume of Diluent}}$$

For example, if 1.0 mL of a sample is mixed with 9.0 mL of diluent, the dilution is 1/(1+9), or 1/10. This is a one to ten dilution as one volume is diluted to a total of volume of ten. The dilution can also be written as 1:10, or exponentially as 10^{-1}.

Following the preparation of a dilution series, each dilution is aseptically plated using the spread plate technique onto several plates consisting of an appropriate agar-based medium, and then grown under optimal conditions. At this point in the procedure, it must be noted that the volume of culture plated should also be factored into the final calculated dilution. For example, if 0.1 mL of culture is plated, it is considered to be a 1:10 dilution. This is because the final cell count is calculated as the number of CFU per milliliter, and 0.1 mL is one-tenth of a milliliter (See: FIGURE 7).

Following a suitable growth period, number of colonies that have grown on each plate can be enumerated. For best results, only use plates harboring colony counts between a range of 25-250 colonies as this will provide an accurate representation of the bacterial titer. To determine the CFU/mL, use the formula below:

$$\text{CFU/mL} = \frac{\text{Average Colony Count}}{\text{Dilution Factor}}$$

Specifically, calculate the average of the number of colonies from one dilution series then divide by the final dilution on the plate. For example, if you have three counts of 40, 37, and 43 from a set of plates at final of dilution of 10-7, the bacterial titer would be 4.0 x 108 CFU/mL.

To create a growth curve using this enumeration technique, at least 2-3 different dilutions should be made from samples taken at incremental time points. This information can be plotted as CFU/mL over time.

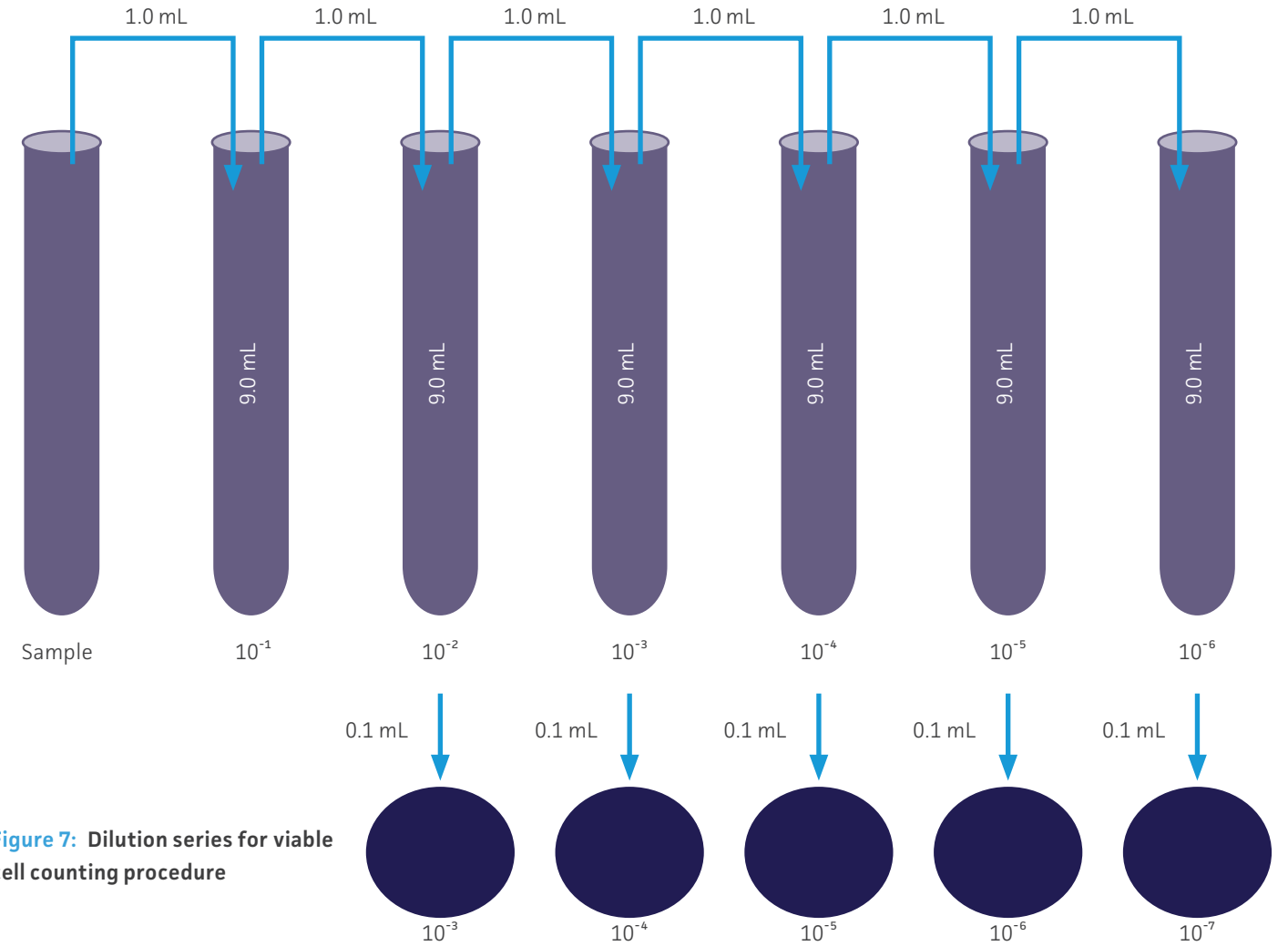


Figure 7: Dilution series for viable cell counting procedure

MATERIALS

- Broth culture
- 6 Dilution blanks
- Sterile 1 mL pipettes
- Pipette bulb
- Glass or metal streaking "hockey" stick
- 70% Ethanol
- Bunsen burner
- 10 Sterile petri dishes with agar
- Vortex

PROCEDURE

1. Label your dilution tubes and agar plates as indicated in FIGURE 7. Ensure that you label two plates for each dilution.
2. Gently mix the culture suspension by carefully vortexing the mixture (See: NOTE 7).
3. Aseptically remove 1.0 mL of the culture and pipet it into the 10-1 dilution blank. Properly discard the pipette. Mix the 10-1 dilution by gentle vortexing.
4. With a fresh pipette, remove 1.0 mL from the 10-1 dilution blank and transfer it to the 10-2 dilution tube. Mix well.
5. Using a fresh pipette between each tube, continue the serial dilution through 10-6.
6. Once your dilution series is prepared, return to the 10-2 dilution tube. Gently vortex the sample. Then, with a fresh pipette, transfer 0.1 mL to each of two agar petri dishes. Using the spread plate culturing technique, aseptically plate the media.
7. Continue to plate the dilution series.
8. Incubate each plate agar-side-up at an appropriate temperature and growth period.
9. Choose plates with 25-250 colonies and count the number of colonies present on the plate. Discard the plates after the colonies have been counted.
10. Use the colony counts to determine the viable cell count.

NOTE 7

When performing a dilution series, always vortex the sample between dilutions. This will evenly distribute the bacteria throughout the culture.

SPECTROPHOTOMETRY

A spectrophotometer is also used to titer bacterial suspensions though determining the optical density, or absorbance, of a sample. The concentration of a bacterial culture is measured by projecting a beam of light, at a single wavelength of 600 nm, through the suspension within a transparent cuvette. When the beam of light passes through the sample, some light is absorbed while remaining light is quantified by a photometer. Generally, the more concentrated a solution is, the more light will be absorbed, and the higher the optical density measurement will be. To ensure that the OD_{600} reading generated by the spectrophotometer is the optical density of the bacterial suspension and not that of the medium, blank the machine before use with a medium-only control.

To create a growth curve using this enumeration technique, measure samples taken at incremental time points. As the culture becomes more turbid, the sample should be diluted to obtain a more accurate reading. This information can be plotted as the optical density over time.

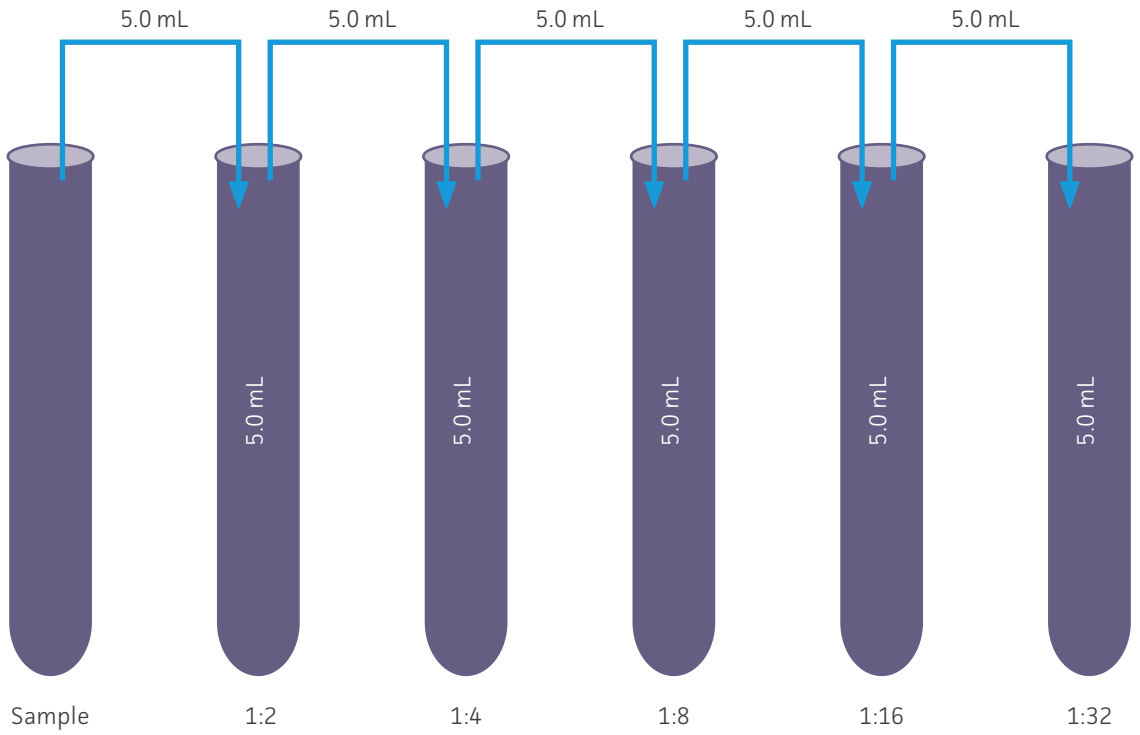


Figure 8: Dilution series for spectrophotometry procedure

MATERIALS

- Broth culture
- Sterile broth
- 5 Dilution blanks
- Sterile pipettes
- Pipette bulb
- Vortex
- Spectrophotometer
- Disposable cuvettes
- Kimwipes

PROCEDURE

1. Turn on your spectrophotometer. Set the wavelength to 600 nm.
2. Prepare a dilution series of two-fold dilutions in sterile broth as depicted in FIGURE 8.
3. Fill a disposable cuvette with sterile media. This is the blank. Wipe the cuvette with a kimwipe to remove dust. Insert the curvette into the spectrophotometer holder and ensure that cuvette is placed correctly in the spectrophotometer. Placement of the cuvette will vary depending on the type of equipment used.
4. Adjust the spectrophotometer to read the blank. This setting should read the absorbance of the blank as zero. Remove the blank and properly dispose of the disposable cuvette.
5. Read the absorbance from each of the dilutions. Ensure you use a fresh disposable cuvette for each dilution.
6. Record the OD_{600} reading for each dilution.

DIFFERENTIAL TESTS

There are numerous ways to identify an unknown bacterium. In many laboratories, unknown strains are examined using genotypic and phenotypic analyses. Bacterial identity is frequently analyzed genotypically through 16S rRNA gene sequencing. These sequences are commonly used to analyze bacterial phylogeny and taxonomy as they are highly conserved and universal among prokaryotic species. In general, the 16S rRNA gene sequence is composed of both variable and conserved regions, the comparison of these sequences can allow for differentiation at the genus level (>90% simi*lari*ty).[12,13]

During phenotypic analysis, strains are initially examined for characteristic colony and cellular morphology; this is observed via colonial growth and staining, respectively. Additionally, samples are tested for culture purity on blood agar plates. Generally, the presence of more than one colony type is often, but not always, indicative of a mixed culture. In addition to purity and morphology, strains are also examined for consistency in sugar metabolism, antibiotic susceptibility, and/or broad-spectrum biochemical reactivity.

To confirm the bacterial species identity based on metabolic requirements and enzyme production, differential media, API® strip tests, or Vitek® cards can be used. The use of differential media can be time consuming and expensive, and is often only used in teaching laboratories; these assays will be further discussed within this section. In many professional labs, API strip tests and Vitek cards are used as they are easy to use and employ over 20 different biochemical tests in reaction cells to quickly assess the growth and viability of the bacterial strain.[14] The API strip test characterizes bacterial identity through a manual, micro-method that generates a seven-digit code based upon the reaction of the individual biochemical assays. This generated code is the identifier number linked to a specific bacterial species. In contrast, the Vitek system provides an automated, computer-based method of species identification via the measurement of light attenuation associated with each biochemical reaction.[14,15]

API strip test

Below, we provide various procedures for differential tests commonly used in teaching laboratories for the identification of an unknown bacterium. For additional assistance, refer to Bergey's Manual of Determinative Bacteriology, which is the primary reference on bacterial classification.[16]

BLOOD AGAR

Some bacterial species produce and excrete hemolysins that degrade hemoglobin and lyse red blood cells by either hydrolyzing or forming pores in phospholipid bilayers. To analyze the hemolytic capability of an organism, bacterial strains are commonly examined on blood agar plates. These plates, which contain various nutrients and 5% sheep blood, can also be used to propagate fastidious bacteria and examine colony purity and morphology.

There are three types of hemolysis reactions: alpha, beta, and gamma. In alpha-hemolysis, hemolysins partially degrade red blood cells. This reaction produces a green discoloration of the medium underneath or surrounding the colony due to the release of biliverdin and other heme compounds from the damaged red blood cells. In beta-hemolysis, hemolysins completely destroy red blood cells, resulting in a clearing around the colony. In gamma-hemolysis, an organism is incapable of inducing hemolysis and the agar remains unchanged.

MATERIALS

- Slant or broth cultures
- Wire inoculation loop
- Bunsen burner
- Sterile petri dishes with blood agar

PROCEDURE

1. Using the streak plate culturing technique, aseptically inoculate the blood agar plates.
2. Incubate the plates at 37°C for 24-48 hours.
3. Observe plates for signs of hemolysis.

CATALASE TEST

Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide to oxygen and water. This enzyme is produced by most organisms that are frequently exposed to oxygen, and is used to protect the cell from oxidative damage caused by reactive oxygen species.

The presence of catalase can be easily examined by adding a small volume of hydrogen peroxide to a microbial sample, then observing the reaction. A positive result is indicated by the evolution of oxygen gas, which is visualized by the formation of bubbles.

$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$$

MATERIALS

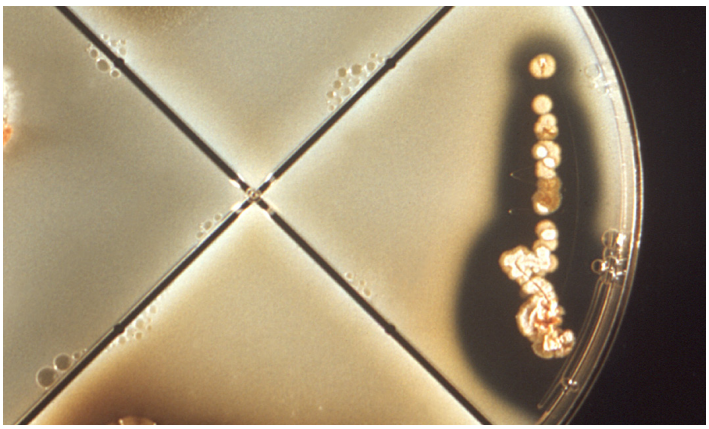
- Slant cultures
- Wire inoculation loop
- Bunsen burner
- Clean microscope slide
- Hydrogen peroxide (H_2O_2)

PROCEDURE

1. Using aseptic technique, place a small amount of microbial culture onto a clean microscope slide. Spread the culture to create a thin film.
2. Add a few drops of hydrogen peroxide to the culture.
3. Observe the reaction. A positive result is the rapid evolution of oxygen, which is evidenced by the formation of bubbles. A negative result will not have the formation of bubbles.

CASEASE TEST

Casease is a proteolytic enzyme produced by certain bacterial species for the hydrolysis of casein, which is a protein commonly found in mammalian milk. This enzyme allows microorganisms to breakdown casein into smaller molecules for use in various anabolic processes. The production of casease is frequently analyzed using skim milk plates. If casease is produced by an organism growing on a skim milk plate, the casein within the medium will be broken down, changing the color of the agar from opaque white to transparent.



Casease-positive test. Photo courtesy of Dr. David Berd and CDC.

MATERIALS

- Slant or broth cultures
- Wire inoculation loop
- Bunsen burner
- Sterile petri dishes with skim milk agar

PROCEDURE

1. Using the streak plate culturing technique, aseptically inoculate the skim milk agar plates.
2. Incubate the plates at an appropriate temperature and incubation period.
3. Observe plates. If there is a clear halo around the colony, it indicates that the organism produce casease.

CITRATE TEST

Simmon's Citrate Agar is a defined medium used to test for the ability of a microorganism to use citrate as the sole carbon source. This medium contains sodium citrate, ammonium dihydrogen phosphate, various nutrients, and the pH indicator bromothymol blue. Use of citrate increases the pH of the medium to above 7.5, which causes the bromothymol blue indicator to change in color to blue. Thus, if citrate is used as the sole carbon source, the medium will turn blue. If the medium remains green, the organism is citrate negative.

MATERIALS

- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Simmon's citrate agar slants

PROCEDURE

1. Using the agar slant culturing technique, inoculate a Simmon's citrate agar slant with a growing bacterial culture.
2. Incubate the agar slant at an appropriate temperature and incubation period.
3. Observe agar slants for color change. If slants remain green, the organism is citrate negative. If the slants are now blue, the organism is citrate positive.

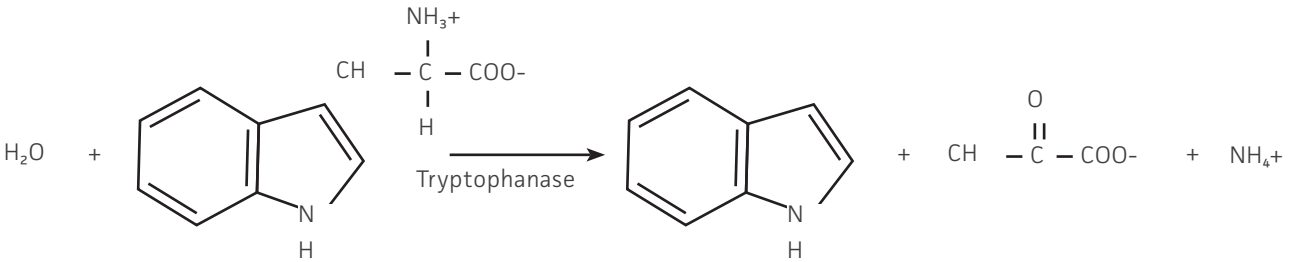
INDOLE PRODUCTION

The indole test is used to determine if a bacterial species can convert the amino acid tryptophan into indole. This can be tested using SIM medium, which is a semi-solid differential medium that tests three parameters including sulfur reduction, indole production, and motility. This medium is composed of various nutrients, iron, sodium thiosulfate, and peptone, which contains tryptophan.

When performing this assay, the sulfur and motility test results should be analyzed prior to the indole test. If an organism is able to reduce the sulfur to hydrogen sulfide, a black precipitate will form. This precipitate is generated when the hydrogen sulfide combines with iron to form ferric sulfide. Thus, if there is any blackening in the media, it indicates that the organism is capable of reducing sulfur.

Motility can be analyzed by observing the turbidity of the semi-solid agar. If the agar appears turbid, it indicates that the microorganism was able to move away from the agar stab; this is a positive test for motility. If an organism is not motile, it will remain in the stab.

Some microorganisms have the ability to hydrolyze tryptophan using an enzyme called tryptophanase. The byproducts of this reaction include indole, pyruvate, and ammonium. To analyze a culture for the production of indole, Kovac's reagent is added to the SIM medium. This reagent contains hydrochloric acid, p-dimethylaminobenzaldehyde (DMABA), and n-amyl alcohol. If indole is produced, it reacts with DMABA to form a red quinoidal compound. Thus, if a red ring forms on the surface of the agar, it is indicative of a positive indole reaction.



MATERIALS

- Slant or broth culture
- Wire inoculation needle
- Bunsen burner
- SIMS agar
- Kovac's reagent

PROCEDURE

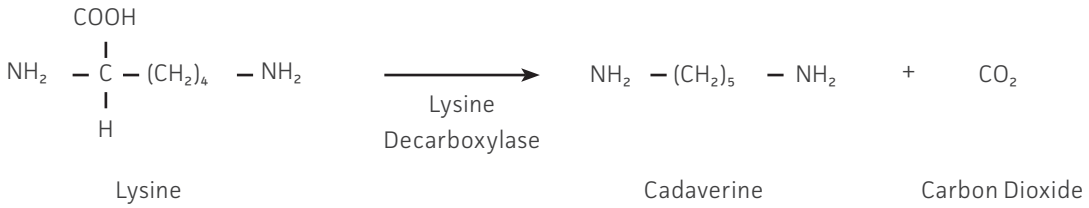
1. Using the agar stab culturing technique, inoculate the SIMS agar with a growing bacterial culture.
2. Incubate the agar slant at an appropriate temperature and incubation period.
3. Observe agar for the production of a black precipitate. The presence of the precipitate indicates a positive result for sulfur reduction.
4. Observe the agar for turbidity. The presence of turbidity moving away from the agar stab indicates a positive result for motility.
5. Add several drops of Kovac's reagent to the culture. The formation of a red ring on the surface of the agar indicates a positive indole test.

LYSINE DECARBOXYLASE TEST

Decarboxylases are enzymes that remove the carboxyl group from a specific amino acid. To identify organisms that produce the lysine decarboxylase enzyme, the lysine decarboxylase test is used. For this assay, organisms are inoculated into a decarboxylase broth that contains nutrients, dextrose, pyridoxal (enzyme cofactor), a pH indicator, and lysine. Following an appropriate incubation period, the medium is analyzed for changes in color. The pH indicator used for this assay is bromcresol purple, which turns yellow at an acidic pH and purple at an alkaline pH.

If an organism is able to ferment the dextrose within the medium, acidic byproducts are produced. These byproducts will lower the pH of the medium, altering the color of the pH indicator to yellow. If the organism is able to decarboxylate the lysine present in the medium, this will produce alkaline products. These alkaline products will increase the pH of the medium, altering the color of the pH indicator to purple. It is possible to have an organism that is able to both ferment dextrose and decarboxylate lysine. In this case, the medium will initially turn yellow from dextrose fermentation, and will eventually turn purple as the alkaline products from decarboxylation accumulate.

Overall, if the inoculated medium remains the same color or turns yellow, it is considered to be a negative result for the production of lysine decarboxylase. If the medium turns purple, the organism is able to produce lysine decarboxylase.

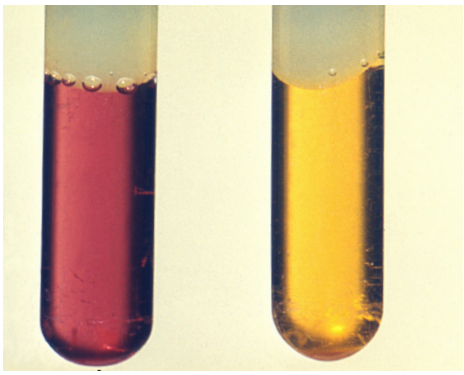


MATERIALS

- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Lysine decarboxylase broth
- Sterile mineral oil

PROCEDURE

1. Using aseptic technique, inoculate the lysine decarboxylase broth with a growing bacterial culture.
2. Add a small overlay of sterile mineral oil to the tube. The addition of oil helps diminish exposure to oxygen, which promotes fermentation.
3. Incubate the broth at an appropriate temperature and incubation period.
4. Observe the culture. If the culture has not changed in color, it indicates that the organism does not produce lysine decarboxylase and is unable to ferment dextrose. If the culture is yellow, it indicates that the organism does not produce lysine decarboxylase and is able to ferment dextrose. If the culture is purple, it indicates that the organism produces lysine decarboxylase.



Decarboxylase test results. Tube 1: Organism is able to produce decarboxylase; Tube 2: Organism ferments dextrose but does not produces decarboxylase. Photo courtesy of Dr. R. Weaver and CDC.

MACCONKEY AGAR

MacConkey agar is a differential, selective medium used to differentiate between members of the *Enterobacteriaceae* family based on lactose fermentation. This medium consists of bile salts, nutrients, peptone, lactose, neutral red dye, and crystal violet dye. The presence of the crystal violet dye and bile salts inhibits the growth of gram-positive bacteria. The neutral red dye is used in this medium as a pH indicator. In acidic conditions the dye will appear red, and in alkaline conditions the dye will appear colorless.

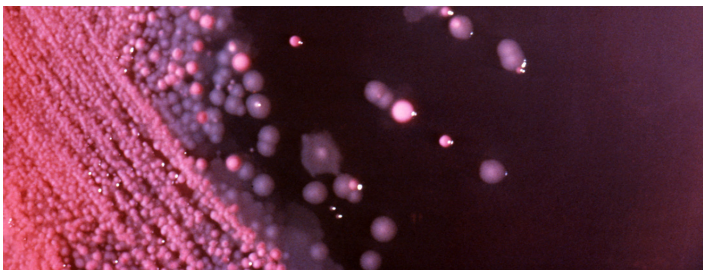
Organisms that are capable of fermenting the lactose within the medium will produce acidic byproducts, which will cause the bacterial growth to appear pink because of the neutral dye changing color. Bile salts may precipitate in surrounding area, causing the medium near the colony to appear hazy. Organisms that are not capable of fermenting lactose will use peptone instead. This will result in the production of alkaline byproducts, which will cause colonies to appear white or colorless.

MATERIALS

- Slant or broth cultures
- Wire inoculation loop
- Bunsen burner
- Sterile petri dishes with MacConkey agar

PROCEDURE

1. Using the streak plate culturing technique, aseptically inoculate the MacConkey agar plates.
2. Incubate the plates at an appropriate temperature and incubation period.
3. Observe plates for signs of growth. If growth is present, it indicates that the strain is gram-negative.
4. If growth is present, observe the color of the colonies. If the culture appears pink or red, it indicates that the organism was able to ferment lactose. If the culture appears white or clear, it indicates that the organism was unable to ferment lactose.



MacConkey agar exhibiting an organism that can ferment lactose. Photo courtesy of Dr. Gilda Jones and CDC.

MANNITOL SALT AGAR

Mannitol salt agar is a selective and differential medium that contains a high concentration of salt (7.5%), mannitol, and a pH indicator. The high salt concentration is selective for gram-positive bacteria within the *Staphylococcus* genus as these organisms can tolerate high saline levels. Organisms from other genera may have the ability to survive on this medium; however, growth of these strains will be minimal.

The mannitol within the medium helps to differentiate between *Staphylococcus* species. If an organism is able to ferment mannitol, an acidic byproduct is formed that will cause the pH indicator to change from red to yellow. This particular aspect of the medium is useful in differentiating between pathogenic and non-pathogenic strains as most pathogenic strains can ferment this sugar whereas most non-pathogenic strains cannot.

MATERIALS

- Slant or broth cultures
- Wire inoculation loop
- Bunsen burner
- Sterile petri dishes with mannitol salt agar

PROCEDURE

1. Using the streak plate culturing technique, aseptically inoculate the mannitol salt agar plates.
2. Incubate the plates at an appropriate temperature and incubation period.
3. Observe plates for signs of growth. If growth is present, it indicates that the strain can tolerate high salt concentrations and may be a member of the *Staphylococcus* genus.
4. If growth is present, observe agar for a change in color from red to yellow. If the medium is yellow around the bacterial growth, it indicates that the strain is able to ferment mannitol.

METHYL RED TEST

The Methyl Red test is used to differentiate between members of the *Enterobacteriaceae* family based on their pattern of glucose metabolism. If an organism uses the mixed acid fermentation pathway and produces stable acidic end-products, it is considered to be positive for the Methyl Red test.

Following growth in a broth consisting of peptone, buffers, and glucose, the production of acidic end-products can be detected by the addition of the methyl red. This pH indicator is red in acidic conditions under pH 4.4, and yellow at a pH above 6.2. Thus, if acidic products are present in the medium, the medium will become more acidic, causing the methyl red indicator to remain red when added. In contrast, if the organism does not use the mixed acid fermentation pathway, the medium will remain at a neutral pH, causing the methyl red indicator to change from red to yellow.

Glucose $\longrightarrow$ Pyruvate $\longrightarrow$ Lactic acid, acetic acid, formic acid, etc.

MATERIALS

- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Methyl Red broth
- Methyl Red pH indicator

PROCEDURE

1. Using aseptic technique, inoculate the methyl red broth with a growing bacterial culture.
2. Incubate the broth at an appropriate temperature and incubation period.
3. Add several drops of the methyl red pH indicator.
4. Observe the culture. If the methyl red pH indicator remains red, the organism is considered to be methyl red positive. If the methyl red pH indicator changes in color to yellow, the organism is considered to be methyl red negative.

MOTILITY

To observe the motility of a microorganism, a wet mount or semi-solid medium can be used. When using semi-solid agar, organisms are inoculated using the agar stab culturing technique. If the organism can move away from the stab, it indicates that the organism is capable of motility.

MATERIALS

- Slant or broth culture
- Wire inoculation needle
- Bunsen burner
- Motility agar

PROCEDURE

1. Using the agar stab culturing technique, inoculate the motility agar with a growing bacterial culture.
2. Incubate the agar slant at an appropriate temperature and incubation period.
3. Observe the culture. If the medium appears turbid around the stab, it indicates that the organism is capable of motility. If the organism is only growing within the stab, it indicates that the organism is non-motile (See: NOTE 8).

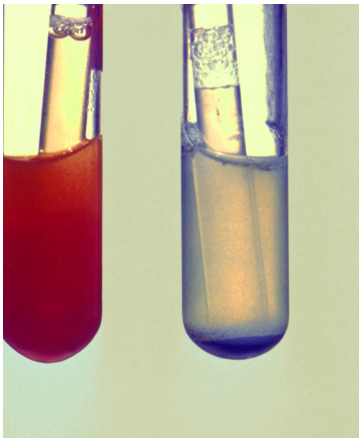
NOTE 8

This assay should not be used to determine the motility of an obligate aerobe. These organisms cannot survive without oxygen, and may be unable to grow within the agar stab.

NITRATE REDUCTION TEST

The nitrate reduction test is used to determine if an organism can reduce nitrate (NO_3-) to nitrite (NO_2-) or nitrogen gas (N_2). To detect the formation of nitrite, sulfanilic acid and α-naphthylamine are added to the culture. If nitrite is present, it will react with the sulfanilic acid to produce diazotized sulfanilic acid. This compound then reacts with the α-naphthylamine to form a red colored compound. Thus, if the medium turns red after the addition of sulfanilic acid and α-naphthylamine, it indicates that the bacterium reduced the nitrate to nitrite.

If the addition of sulfanilic acid and α-naphthylamine does change the color of the medium to red, it can indicate that either the bacterium is unable to reduce nitrate or that nitrate was reduced to nitrogen gas. To analyze this, a small amount of powdered zinc is added to the medium. Zinc acts as a reducing agent, so the addition of zinc to the medium will reduce any remaining nitrate to nitrite. If the medium changes in color to red, it indicates that there was nitrate present in the tube, thus indicating that the bacterium is unable to reduce nitrate. If the color of the medium remains unchanged, it indicates that there is no nitrate remaining in the broth as it was converted to nitrogen gas.



Nitrate reduction test performed using Durham fermentation tubes. Tube 1: Positive nitrogen reduction test with some nitrogen gas formation. Tube 2: Nitrate completely reduced to nitrogen gas. Photo courtesy of Dr. R. Weaver and CDC.

MATERIALS

- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Nitrate broth
- Sulfanilic acid
- α-naphthylamine
- Powdered zinc

PROCEDURE

1. Using aseptic technique, inoculate the nitrate broth with a growing bacterial culture.
2. Incubate the broth at an appropriate temperature and incubation period.
3. Add a dropperful of sulfanilic acid and α-naphthylamine (See: NOTE 9).
4. Observe the culture. If the medium becomes red, it indicates that the bacterium reduced the nitrate to nitrite.
5. If the medium did not change color, add a small amount of powdered zinc to your culture (See: NOTE 9).
6. Observe the culture. If the medium changes in color to red, it indicates that the bacterium was unable to reduce nitrate. If the medium color remains unchanged, it indicates that the bacterium reduced nitrate to nitrogen gas.

NOTE 9

Sulfanilic acid, α-naphthylamine, and zinc are very hazardous. When adding each of these chemicals to your culture, work in a well-ventilated fume hood.

OXIDASE TEST

The oxidase test is used to determine if a microorganism produces cytochrome oxidase. As cytochrome oxidase is used in the electron transport chain to transfer electrons from a donor molecule to oxygen, the presence of this enzyme is indicative of aerobic respiration. In the oxidase test, N,N,N',N'-tetramethyl phenylenediamine dihydrochloride is used as a final electron acceptor in place of oxygen. When this artificial acceptor receives electrons from cytochrome oxidase, it will change in color from clear to dark blue or purple.

MATERIALS

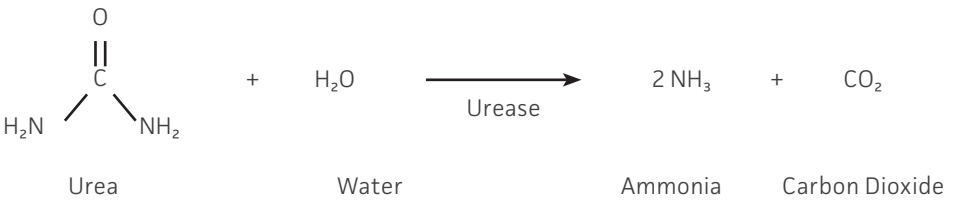
- Slant cultures
- Sterile cotton swab
- N,N,N',N'-tetramethyl phenylenediamine dihydrochloride

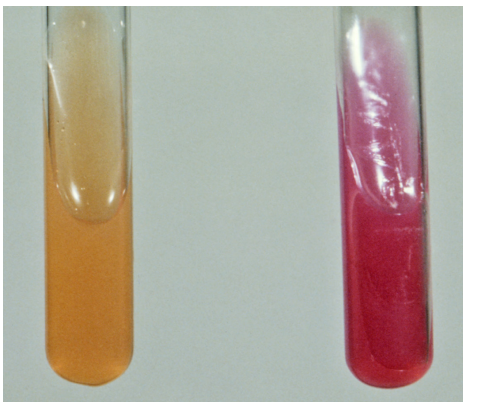
PROCEDURE

1. Using aseptic technique, obtain a small amount of bacteria from an agar slant culture.
2. Add 1-2 drops of N,N,N',N'-tetramethyl phenylenediamine dihydrochloride to the culture. The reaction will occur immediately or within 10-30 seconds.
3. Observe the reaction. A positive reaction is indicated by a color change to dark blue or purple. A negative test will result in the absence of color.

UREASE TEST

The urease test is a differential assay used to identify organisms that have the ability to produce and excrete the enzyme urease. This enzyme is used by microorganisms to hydrolyze urea to ammonia and carbon dioxide. In this assay, organisms are inoculated into a medium that contains urea, buffers, nutrients, and the phenol red pH indicator. In acidic conditions the pH indicator will turn yellow, in alkaline conditions the pH indicator will turn pink. If an organism produces urease, ammonia will be produced. The production of ammonia will cause the pH of the medium to become alkaline, resulting in the pH indicator changing to pink.





Urease test results. Tube 1: Organism is negative for urease production; Tube 2: Organism is positive for urease production. Photo courtesy of CDC.

MATERIALS

- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Urease broth

PROCEDURE

1. Using aseptic technique, inoculate the urease broth with a growing bacterial culture.
2. Incubate the broth at an appropriate temperature and incubation period.
3. Observe the culture. If the culture is pink, it indicates that the organism is able to produce urease. If the culture is yellow, the organism does not produce urease.

THIOGLYCOLLATE MEDIUM

Thioglycollate medium is a differential medium designed to test the oxygen requirements of bacteria. This medium is composed of nutrients, sodium thioglycollate, thioglycollic acid, L-cystine, an indicator dye, and 0.05% agar. The sodium thioglycollate, thioglycollic acid, and L-cystine reduce the oxygen in the medium to water, thus slowing down the diffusion of oxygen throughout the medium. This allows bacteria to occupy specific layers of the medium based on their oxygen requirements. The addition of indicator dyes, such as methylene blue or resazurin, provides a visual indication of the presence of oxygen. In the presence of oxygen, methylene blue will appear blue and reszaurin will appear pink. In the absence of oxygen, both dyes will appear clear.

In this medium, obligate aerobes will only grow in the top oxygen-rich layer, obligate anaerobes will only grow in the lower layers of the medium, microaerophilic organisms will grow in a small layer just below the oxygen-rich layer, facultative anaerobes will grow throughout the media with a higher concentration near the oxygen-rich layer, and aerotolerant organisms will grow evenly throughout the media (See: FIGURE 9).

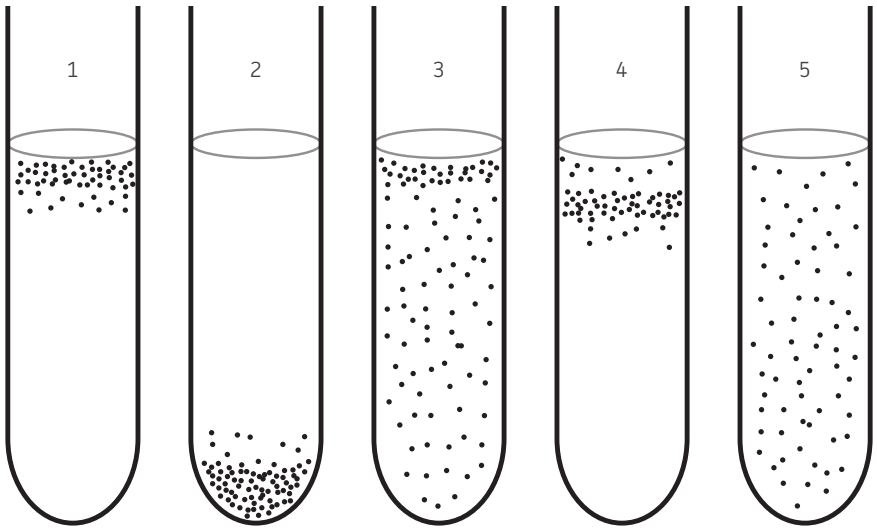


Figure 9: Expected growth of microorganisms in thioglycollate medium. Tube 1: Obligate aerobe; Tube 2: Obligate anaerobe; Tube 3: Facultative anaerobe; Tube 4: Microaerophile; Tube 5: Aerotolerant.

MATERIALS

- Slant or broth culture
- Wire inoculation needle
- Bunsen burner
- Thioglycollate medium

PROCEDURE

1. Using aseptic technique, inoculate the thioglycollate medium using the agar stab culturing technique.
2. Incubate the broth at an appropriate temperature and incubation period.
3. Observe the culture. Use FIGURE 9 as a guide in determining the aerotolerance of the organism.

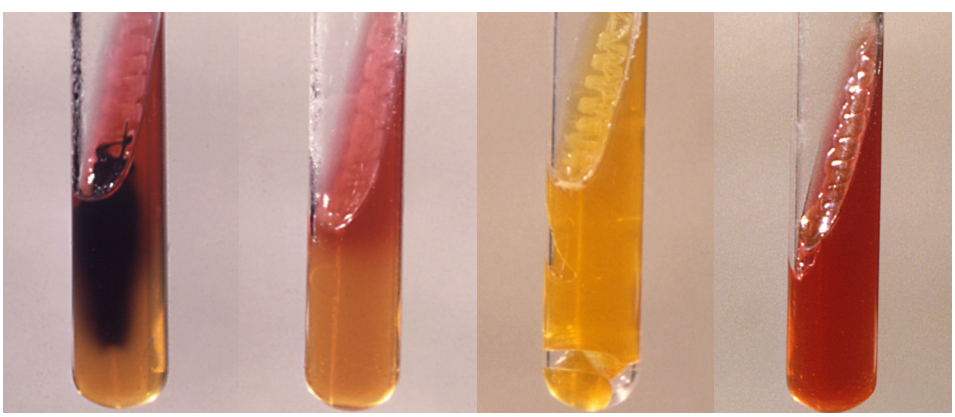
TRIPLE SUGAR IRON

Triple sugar iron agar is a differential medium that is used to differentiate between enteric bacteria based on their ability to reduce sulfur and carbohydrates. This medium is composed of glucose, lactose, sucrose, ferrous sulfate, peptone, and the phenol red pH indicator. If an organism can ferment each of the three sugars present in the medium, it will produce acidic byproducts that will cause the pH indicator to change from red to yellow throughout the medium.

If an organism can only ferment glucose, the small amount of glucose in the medium will be used within the early stages of growth, changing the medium to yellow. Since this substrate is present in very small concentrations, the small amount of acid byproducts produced on the slant surface will oxidize quickly, changing the media along the slant back to red. The catabolism of peptone in the medium, which produces alkaline byproducts, will also contribute to the change in color. The butt of the agar will remain yellow as the acid reaction is maintained due to reduced oxygen content.

If an organism is capable of reducing sulfur, the hydrogen sulfide gas produced will react with the iron in the medium to form ferrous sulfide. This will appear in the medium as a black precipitate. This precipitate is commonly observed in the butt of the medium.

If gas is produced during the fermentation reaction, it can be visualized by either fissures in the agar or as gas bubbles between the agar and the wall of the test tube.



Examples of triple sugar iron agar results. Tube 1: Glucose fermentation only, hydrogen sulfide produced (K/A, H2S); Tube 2: Glucose fermentation only (K/A); Tube 3: Glucose, lactose and/or sucrose fermented, gas produced (A/A, G); Tube 4: No fermentation, peptone catabolized (K/K). Photo courtesy of M.M. Galton and CDC.

Result (Slant/Butt)	Symbol	Interpretation
Red/Yellow	K/A	Glucose fermentation only, peptone catabolized
Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red/Red	K/K	No fermentation, peptone catabolized
Yellow/Yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation, gas produced
Red/Yellow with bubbles	K/A,G	Glucose fermentation only, gas produced
Red/Yellow with bubbles and black precipitate	K/A,G, H_2S	Glucose fermentation only, gas produced, H_2S produced
Yellow/Yellow with bubbles and black precipitate	A/A, G, H_2S	Glucose and lactose and/or sucrose fermentation, gas produced, H_2S produced
Red/Yellow with black precipitate	K/A, H_2S	Glucose fermentation only, H_2S produced
Yellow/Yellow with black precipitate	A/A, H_2S	Glucose and lactose and/or sucrose fermentation, H_2S produced

A= acid production; **K**= alkaline reaction; **G**= gas production; **H_2S**= Sulfur reduction

MATERIALS

- Slant or broth culture
- Wire inoculation needle
- Bunsen burner
- Triple sugar iron agar

PROCEDURE

1. Using both the agar stab and agar slant culturing techniques, aseptically inoculate the triple sugar iron agar with a growing bacterial culture. You will first inoculate the agar using the agar stab culturing technique; gently push the needle through the agar until you reach the butt of the agar. Carefully remove the needle from the agar, and then streak the needle back and forth along the surface of the slant.
2. Incubate the agar slant at an appropriate temperature and incubation period.
3. Observe the culture for sugar fermentation, production of hydrogen sulfide, and gas production. Use the table to help you describe and record your results.

VOGES-PROSKAUER TEST

Similar to the Methyl Red test, the Voges-Proskauer test is used to differentiate between members of the *Enterobacteriaceae* family based on their pattern of glucose metabolism. If an organism is capable of breaking down glucose to acetoin, it is considered positive for the Voges-Proskauer test. Following growth in a broth consisting of peptone, buffers, and glucose, the production of acetoin can be detected by the addition of α-naphthol and potassium hydroxide. If acetoin is present, it will react with the added reagents to form a red color. If a copper color appears following the addition of alpha-naphthol and potassium hydroxide, the culture is negative for the production of acetoin.

Glucose ⟶ Pyruvate ⟶ Acetoin

MATERIALS

- Slant or broth culture
- Wire inoculation loop
- Bunsen burner
- Voges-Proskauer broth
- α-naphthol
- Potassium hydroxide (KOH)

PROCEDURE

1. Using aseptic technique, inoculate the Voges-Proskauer broth with a growing bacterial culture.
2. Incubate the broth at an appropriate temperature and incubation period.
3. Add several drops of the α-naphthol (See: NOTE 10).
4. Add several drops of the potassium hydroxide (KOH).
5. Observe the culture. A change in medium color to red indicates the presence of acetoin, and is considered a positive Voges-Proskauer test. The formation of a copper color indicates a negative test result.

NOTE 10

Always add α-naphthol before adding potassium hydroxide. A reversal in the order of these reagents may result in either a false-negative or weak-positive result.

REFERENCES

1 United States Department of Health and Human Serves, C. f. D. C., and National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories 5edn, (U.S. Government Printing Office, HHS Publication No. (CDC) 21-1112, 2009).

2 Young, K. D. Bacterial Morphology: Why have different shapes? Curr Opin Microbiol 10, 596-600 (2008).

3 Young, K. D. The Selective Value of Bacterial Shape. Microbiol Mol Biol Rev 70, 660-703 (2006).

4 Tamames, J., Gonzalez-Moreno, M., Mingorance, J., Valencia, A. & Vicente, M. Bringing gene order into bacterial shape. Trends in genetics: TIG 17, 124-126 (2001).

5 Cooper, S. & Denny, M. A conjecture on the relationship of bacterial shape to motility in rod-shaped bacteria. FEMS Microbiology Letters 148, 227-231 (1997).

6 Davis, C. in Medical Microbiology (ed S. Baron) Ch. 6, (University of Texas Medical Branch at Galveston, 1996).

7 Kramer, A., Schwebke, I. & Kampf, G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC infectious diseases 6, 130, doi:10.1186/1471-2334-6-130 (2006).

8 Todar, K. Todar's Online Textbook of Bacteriology, <http://www.textbookofbacteriology.net/> (2008-2012).

9 Bradbury, S. & Bracegirdle, B. Introduction to Light Microscopy. (Springer-Verlag, 1998).

10 Levine, S. & Johnstone, L. The Microscope Book. (Sterling Publishing Co., 1996).

11 Bergey's Manual of Systematic Bacteriology. 2 edn, Vol. 1-5 (2012).

12 Clarridge, J. E., 3rd. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 17, 840-862, table of contents, doi:10.1128/CMR.17.4.840-862.2004 (2004).

13 Janda, J. M. & Abbott, S. L. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal of clinical microbiology 45, 2761-2764, doi:10.1128/JCM.01228-07 (2007).

14 Janda, J. M. & Abbott, S. L. Bacterial identification for publication: when is enough enough? Journal of clinical microbiology 40, 1887-1891 (2002).

15 Shetty, N., Hill, G. & Ridgway, G. L. The Vitek analyser for routine bacterial identification and susceptibility testing: protocols, problems, and pitfalls. Journal of clinical pathology 51, 316-323 (1998).

16 Bergey, D. Bergey's Manual of Determinative Bacteriology. 8 edn, (Williams & Wilkins, 1974).

QUICK STRAIN GUIDE

Table 1: Teacher's Tool Box

ATCC® No.	Organism	Designation	BSL
19659-MINI-PACK™	*Bacillus spizizenii*	PRD 66	1
13124™	*Clostridium perfringens*	NCTC 8237	2
13048-MINI-PACK™	*Enterobacter aerogenes*	NCDC 819-56	1
25922-MINI-PACK™	*Escherichia coli*	FDA strain Seattle 1946	1
700603-MINI-PACK™	*Klebsiella pneumoniae* subsp. *pneumoniae*	K6	2
14468™	*Mycobacterium smegmatis*	W-113	1
35659-MINI-PACK™	*Proteus mirabilis*	LRA 08 01 73	2
9027-MINI-PACK™	*Pseudomonas paraeruginosa*	R. Hugh 813	2
14028-MINI-PACK™	*Salmonella enterica* subsp. *enterica* serovar Typhimurium	CDC 6516-60	2
43862™	*Serratia marcescens*	LRA 04.03.73	1
BAA-1026-MINI-PACK™	*Staphylococcus aureus*	Vitek 8753	2
12228-MINI-PACK™	*Staphylococcus epidermidis*	FDA strain PCl 1200	1
49619-MINI-PACK™	*Streptococcus pneumoniae*	262	2
19615-MINI-PACK™	*Streptococcus pyogenes*	Bruno	2

Table 2: Controls for Morphological Analysis

Assay	Organism
Gram Positive	*Staphylococcus epidermidis* *Streptococcus pyogenes* *Streptococcus pneumoniae* *Bacillus subtilis*
Gram Negative	*Pseudomonas aeruginosa* *Escherichia coli* *Klebsiella pneumoniae* *Enterobacter aerogenes* *Proteus mirabilis* *Serratia marcescens* *Salmonella enterica*
Acid Fast	*Mycobacterium smegmatis*
Flagella	*Pseudomonas aeruginosa* *Escherichia coli* *Salmonella enterica*
Capsule	*Streptococcus pneumoniae*
Endospore	*Bacillus subtilis* *Clostridium perfringens*
Pigment Production	*Pseudomonas aeruginosa* (blue) *Serratia marcescens* (pink)

Table 3: Controls for Differential Tests

Assay	Positive Control	Negative Control
Blood Agar	*Streptococcus pyogenes* (Beta) *Streptococcus pneumoniae* (Alpha)	*Staphylococcus epidermidis* (Gamma)
Catalase Test	*Staphylococcus epidermidis* *Escherichia coli* *Serratia marcescens* *Bacillus subtilis*	*Streptococcus pyogenes*
Casease Test	*Pseudomonas aeruginosa** *Bacillus subtilis*	*Escherichia coli**
Citrate	*Klebsiella pneumoniae* *Proteus mirabilis* *Serratia marcescens*	*Escherichia coli*
Indole Production	*Escherichia coli*	*Klebsiella pneumoniae* *Serratia marcescens* *Salmonella enterica*
Lysine Decarboxylase	*Serratia marcescens*	*Proteus mirabilis*
MacConkey Agar	*Klebsiella pneumoniae* *Pseudomonas aeruginosa* (Does not ferment lactose)	*Staphylococcus aureus* (No growth)
Mannitol Salt Agar	*Staphylococcus aureus* (Ferments mannitol) *Staphylococcus epidermidis* (Does not ferment mannitol)	*Serratia marcescens**
Methyl Red	*Escherichia coli**	*Enterobacter aerogenes*
Motility	*Pseudomonas aeruginosa* *Escherichia coli* *Proteus mirabilis*	*Staphylococcus epidermidis*
Nitrate Reduction Test	*Pseudomonas aeruginosa* (To N_2) *Escherichia coli** (To Nitrite) *Staphylococcus epidermidis* (To Nitrite)	*Streptococcus pneumoniae**
Oxidase	*Pseudomonas aeruginosa*	*Escherichia coli* *Serratia marcescens* *Salmonella enterica*
Thioglycollate Medium	*Mycobacterium smegmatis** (Aerobe) *Escherichia coli** (Facultative anaerobe) *Streptococcus pyogenes** (Microaerophile)	*Clostridium perfringens** (Anaerobe)
Triple Sugar Iron	*Escherichia coli** (A/A) *Salmonella enterica* (K/A, H_2S)	*Pseudomonas aeruginosa* (K/K)
Urease	*Proteus mirabilis*	*Escherichia coli*
Voges Proskauer	*Enterobacter aerogenes* *Serratia marcescens*	*Escherichia coli* *Proteus mirabilis*

*Strain not tested by ATCC

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