GLOBAL EDITION

Microbiology

A Laboratory Manual

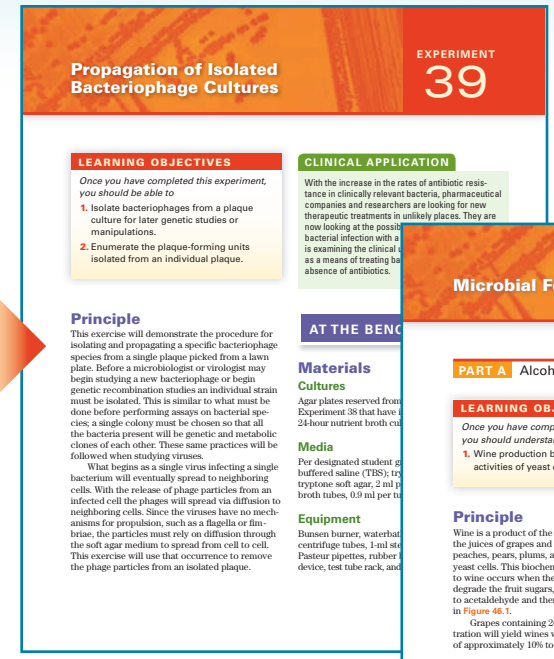
ELEVENTH EDITION

Cappuccino • Welsh

Pearson

A flexible approach to the **modern** microbiology lab

NEW! "Propagation of Isolated Bacteriophage Cultures" experiment has been added to the **Eleventh Edition**. This experiment (39) guides students to isolate bacteriophages for genetic manipulation, an important technique in current clinical research as a possible way to treat antibiotic-resistant bacterial infections.



EXPERIMENT 39

Propagation of Isolated Bacteriophage Cultures

LEARNING OBJECTIVES

Once you have completed this experiment, you should be able to

1. Isolate bacteriophages from a plaque culture for later genetic studies or manipulations.
2. Enumerate the plaque-forming units isolated from an individual plaque.

CLINICAL APPLICATION

With the increase in the rates of antibiotic resistance in clinically relevant bacteria, pharmaceutical companies and researchers are looking for new therapeutic treatments in unlikely places. They are now looking at the possibl... bacterial infection with a ... is examining the clinical ... as a means of treating ba... absence of antibiotics.

Principle

This exercise will demonstrate the procedure for isolating and propagating a specific bacteriophage species from a single plaque picked from a lawn plate. Before a microbiologist or virologist may begin studying a new bacteriophage or begin genetic recombination studies an individual strain must be isolated. This is similar to what must be done before performing assays on bacterial species; a single colony must be chosen so that all the bacteria present will be genetic and metabolic clones of each other. These same practices will be followed when studying viruses.

What begins as a single virus infecting a single bacterium will eventually spread to neighboring cells. With the release of phage particles from an infected cell the phages will spread via diffusion to neighboring cells. Since the viruses have no mechanisms for propulsion, such as a flagella or fimbriae, the particles must rely on diffusion through the soft agar medium to spread from cell to cell. This exercise will use that occurrence to remove the phage particles from an isolated plaque.

AT THE BENC...

Materials

Cultures

Agar plates reserved from ... Experiment 38 that have ... 24-hour nutrient broth cu...

Media

Per designated student g... buffered saline (TBS); try... tryptone soft agar, 2 ml p... broth tubes, 0.9 ml per tu...

Equipment

Bunsen burner, waterbat... centrifuge tubes, 1-ml ste... Pasteur pipettes, rubber ... device, test tube rack, and...

EXPERIMENT 46

Microbial Fermentation

PART A Alcohol Fermentation

LEARNING OBJECTIVE

Once you have completed this experiment, you should understand

1. Wine production by the fermentative activities of yeast cells.

Principle

Wine is a product of the natural fermentation of the juices of grapes and other fruits, including peaches, pears, plums, and apples, by the action of yeast cells. This biochemical conversion of juice to wine occurs when the yeast cells enzymatically degrade the fruit sugars, fructose and glucose, first to acetaldehyde and then to alcohol, as illustrated in Figure 46.1.

Grapes containing 20% to 30% sugar concentration will yield wines with an alcohol content of approximately 10% to 15%. Also present in grapes are acids and minerals whose concentrations are increased in the finished product and that are responsible for the characteristic tastes and bouquets of different wines. For red wine, the crushed grapes must be fermented with their skins to allow extraction of their color into the juice. White wine is produced from the juice of white grapes.

The commercial production of wine is a long and exacting process. First, the grapes are crushed or pressed to express the juice, which is called **must**. Potassium metabisulfite is added to the must to retard the growth of acetic acid bacteria, molds, and wild yeast that are endogenous to grapes in the vineyard. A wine-producing strain of yeast, *Saccharomyces cerevisiae* var. *ellipsoideus*, is used to inoculate the must, which is then incubated for 3 to 5 days under aerobic conditions at 21°C to 32°C. This is followed by an anaerobic incubation period. The wine is then aged for 1 year to 5 years in aging tanks or wooden barrels. During this time, the wine is clarified of any turbidity, thereby producing volatile esters that are responsible for characteristic flavors. The clarified product is then filtered, pasteurized at 60°C for 30 minutes, and bottled.

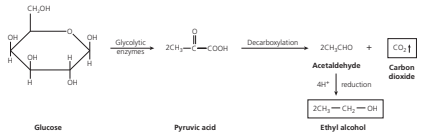


Figure 46.1 Biochemical pathway for alcohol production

REVISED EXPERIMENTS include options for alternate media, making the experiments affordable and accessible to all sizes of lab programs. Experiment 46 now includes both wine and lactic acid fermentation, looking at the production of wine and yogurt.

AT THE BENCH

Materials

Cultures

48- to 72-hour nutrient broth cultures (50 ml per 250-ml Erlenmeyer flask) of *Staphylococcus aureus* BSL-2 *and Bacillus cereus*; 72- to 96-hour Sabouraud broth cultures (50 ml per 250-ml Erlenmeyer flask) of *Aspergillus niger* and *Saccharomyces cerevisiae*.

Media

Per designated student group (pairs or groups of four): five nutrient agar plates, five Sabouraud agar plates, and one 10-ml tube of nutrient broth.

Equipment

Microincinerator or Bunsen burner, 800-ml beaker (waterbath), tripod and wire gauze screen with heat-resistant pad, thermometer, sterile test tubes, glassware marking pencil, and inoculating loop.

Procedure Lab One

1. Label the covers of each of the nutrient agar and Sabouraud agar plates, indicating the experimental heat temperatures to be used: 25°C (control), 40°C, 60°C, 80°C, and 100°C.
2. Score the underside of all plates with a glassware marking pencil into two sections. On the nutrient agar plates, label one section *S. aureus* BSL-2 and the other *B. cereus*. On the Sabouraud agar plates, label one section *A. niger* and the second *S. cerevisiae*.
3. Using aseptic technique, inoculate the nutrient agar and Sabouraud agar plates labeled 25°C by making a single-line loop inoculation of each test organism in its respective section of the plate.
4. Using a sterile pipette and mechanical pipetter, transfer 10 ml of each culture to four sterile test tubes labeled with the name of the organism and the temperature (40°C, 60°C, 80°C, and 100°C).
5. Set up the waterbath as illustrated in Figure 40.2, inserting the thermometer in an uncapped tube of nutrient broth.

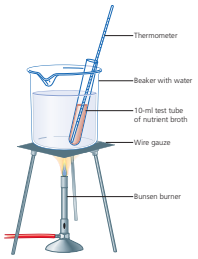


Figure 40.2 Waterbath for moist heat experiment

6. Slowly heat the water to 40°C; check the thermometer frequently to ensure that it does not exceed the desired temperature. Place the four cultures of the experimental organisms into the beaker and maintain the temperature at 40°C for 10 minutes. Remove the cultures and aseptically inoculate each organism in its appropri... ate section on the ...
7. Raise the waterbat... repeat Step 6 for th... plates labeled 60°C...
8. Raise the waterbat... repeat Step 6 for th... plates labeled 80°C...
9. Raise the waterbat... repeat Step 6 for th... plates labeled 100°...
10. Incubate the nutrie... inverted position fo... and the Sabouraud ... 5 days at 25°C in a ...

Procedure L...

1. Observe all plates f... the test organisms ...
2. Record your result... the Lab Report.

NEW! BioSafety Levels (BSLs) alert students to appropriate safety techniques. The organisms within this manual are mostly BSL-1 organisms, with any BSL-2 organisms now marked within the text. The Eleventh Edition also reflects the most up to date safety protocols from governing bodies such as the EPA, ASM, and AOAC, better preparing students for professional lab work.

TIPS FOR SUCCESS

- Gram stain your unknown culture first and then determine which tests would be useful in identifying your bacteria. For example, the oxidase test and the citrate test would be of no use in identifying a Gram positive cocci bacteria.
- Since many of the tests utilize agars that are similar in appearance, be sure to label all tubes and plates to ensure that results are collected for the correct test.

NEW! Tips for Success appear throughout the experiments and draw attention to common mistakes and stumbling blocks in the lab. Each tip explains why specific techniques are necessary to yield accurate results and helps guide students on how to perform crucial procedural steps correctly.

Pearson Mastering Microbiology **prepares students** for the modern microbiology lab

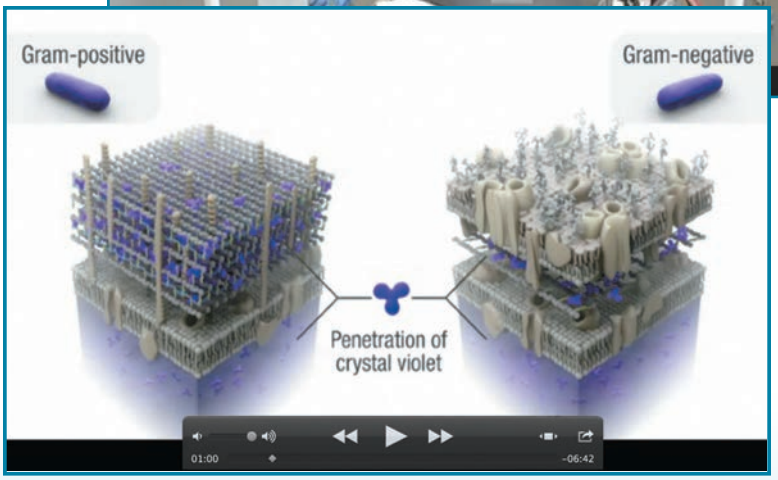
Pearson Mastering Microbiology®

The items mentioned here are available in the Study Area of various Pearson Mastering Microbiology courses.

Pre-Lab Quizzes can be assigned for each of the 76 experiments in *Microbiology: A Laboratory Manual, Eleventh Edition*. Each quiz consists of 10 multiple-choice questions with personalized wrong answer feedback.

MicroLab Tutors help instructors and students get the most out of lab time and make the connection between microbiology concepts, lab techniques, and real-world applications.

These tutorials combine live-action video and molecular animation paired with assessment and answer-specific feedback to help students to interpret and analyze lab results.



MicroLab Tutor Coaching Activities include the following topics:

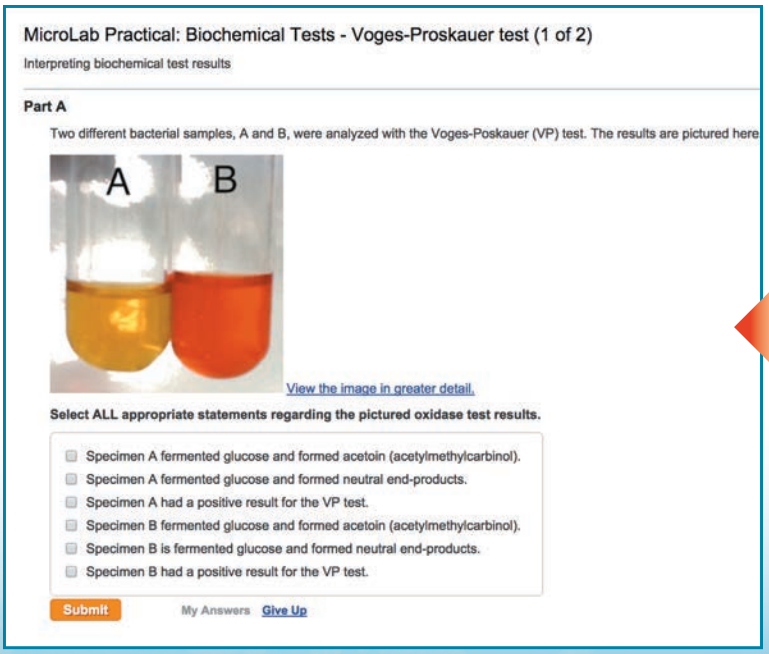
- Use and Application of the Acid-Fast Stain
- Multitest Systems—API 20E
- Aseptic Transfer of Bacteria
- ELISA
- Gram Stain
- Use and Application of Microscopy
- Polymerase Chain Reaction (PCR)
- Safety in the Microbiology Laboratory
- Quantifying Bacteria with Serial Dilutions and Pour Plates
- Smear Preparation and Fixation
- Streak Plate Technique
- Survey of Protozoa
- Identification of Unknown Bacteria

Pearson Mastering Microbiology®

Lab Technique Videos give students an opportunity to see techniques performed correctly and quiz themselves on lab procedures both before and after lab time. Lab Technique videos can be assigned as pre-lab quizzes in MasteringMicrobiology and include coaching and feedback.

Lab Technique Videos include:

- **NEW!** The Scientific Method
- **NEW!** How to Write a Lab Report
- Acid-fast Staining
- Amylase Production
- Carbohydrate Catabolism
- Compound Microscope
- Differential and Selective Media
- Disk-diffusion Assay
- ELISA
- Gram Stain
- Hydrogen Sulfide Production
- Litmus Milk Reactions
- Negative Staining
- Respiration
- Serial Dilutions
- Simple Staining
- Smear Preparation
- Structural Stains
- Safety in the Microbiology Laboratory

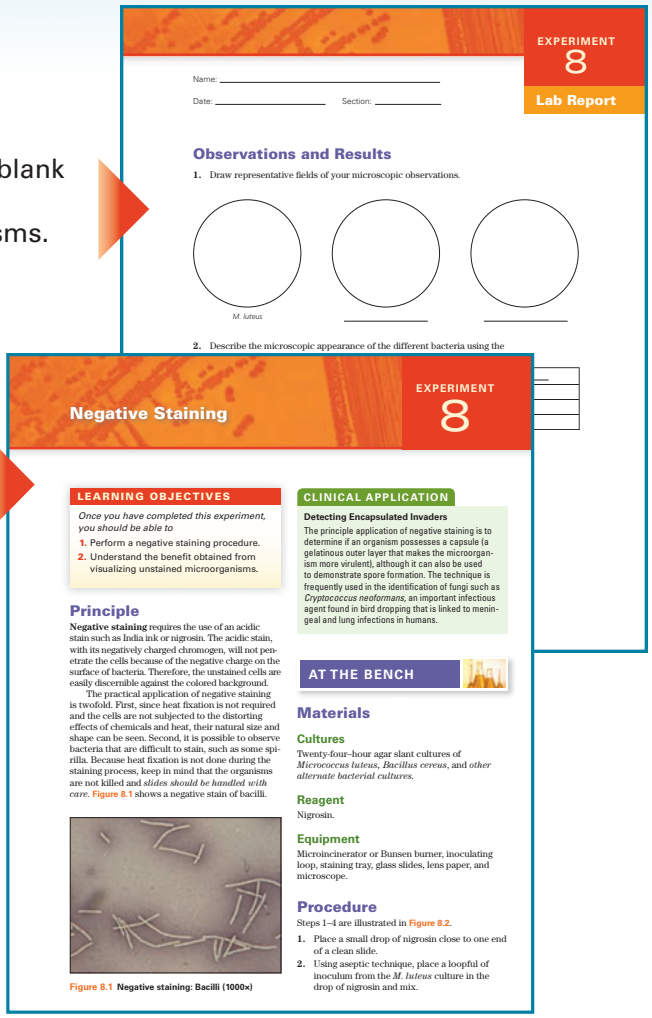


MicroLab Practical Activities assess students' observation skills and give them extra practice to analyze important lab tests, procedures, and results.

Instructors: Tailor this lab manual to **perfectly fit** your course!

NEW! Easy-to-adapt Lab Reports include blank spaces for individual course customization. Instructors can select their preferred organisms.

NEW! Revised Experiments include options for alternate media, reduced volumes, and fewer bacteria, making the experiments affordable and accessible to any-sized lab program.


EXPERIMENT 8

Lab Report

Name: _____

Date: _____ Section: _____

Observations and Results

1. Draw representative fields of your microscopic observations.

M. luteus

2. Describe the microscopic appearance of the different bacteria using the

EXPERIMENT 8

Negative Staining

LEARNING OBJECTIVES

Once you have completed this experiment, you should be able to

1. Perform a negative staining procedure.
2. Understand the benefit obtained from visualizing unstained microorganisms.

Principle

Negative staining requires the use of an acidic stain such as India ink or nigrosin. The acidic stain, with its negatively charged chromogen, will not penetrate the cells because of the negative charge on the surface of bacteria. Therefore, the unstained cells are easily discernible against the colored background.

The practical application of negative staining is twofold. First, since heat fixation is not required and the cells are not subjected to the distorting effects of chemicals and heat, their natural size and shape can be seen. Second, it is possible to observe bacteria that are difficult to stain, such as some spirilla. Because heat fixation is not done during the staining process, keep in mind that the organisms are not killed and *slides should be handled with care.* Figure 8.1 shows a negative stain of bacilli.

Figure 8.1 **Negative staining: Bacilli (1000×)**

CLINICAL APPLICATION

Detecting Encapsulated Invaders

The principle application of negative staining is to determine if an organism possesses a capsule (a gelatinous outer layer that makes the microorganism more virulent), although it can also be used to demonstrate spore formation. The technique is frequently used in the identification of fungi such as *Cryptococcus neoformans*, an important infectious agent found in bird dropping that is linked to meningeal and lung infections in humans.

AT THE BENCH

Materials

Cultures

Twenty-four–hour agar slant cultures of *Micrococcus luteus*, *Bacillus cereus*, and *other alternate bacterial cultures.*

Reagent

Nigrosin.

Equipment

Microincinerator or Bunsen burner, inoculating loop, staining tray, glass slides, lens paper, and microscope.

Procedure

Steps 1–4 are illustrated in Figure 8.2.

1. Place a small drop of nigrosin close to one end of a clean slide.
2. Using aseptic technique, place a loopful of inoculum from the *M. luteus* culture in the drop of nigrosin and mix.

REVISED! Instructor's Guide for *Microbiology: A Laboratory Manual*
by James G. Cappuccino, Chad T. Welsh
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Updated to reflect changes in the lab manual, this guide is a valuable teaching aid for instructors and provides:

- NEW! Recommended readings for each experiment
- Detailed lists of required materials
- Tables for calculating the amount of media and equipment needed for your class
- Procedural points to emphasize
- Suggestions for optional procedural additions or modifications
- Helpful tips for preparing or implementing each experiment
- Answers to the Review Questions in the lab manual
- Information on laboratory safety protocol for instructional and technical staff

MICROBIOLOGY

A LABORATORY MANUAL

ELEVENTH EDITION

GLOBAL EDITION

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SUNY Rockland Community College

Chad Welsh

Lindenwood University

Pearson

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Contents

Preface

Microbiology is a dynamic science. It is constantly evolving as more information is added to the continuum of knowledge, and as microbiological techniques are rapidly modified and refined. The eleventh edition of *Microbiology: A Laboratory Manual* continues to provide a blend of traditional methodologies with more contemporary procedures to meet the pedagogical needs of all students studying microbiology. As in previous editions, this laboratory manual provides a wide variety of critically selected and tested experiments suitable for undergraduate students in allied health programs, as well as elementary and advanced general microbiology courses.

Our Approach

This laboratory manual is designed to guide students in the development of manipulative skills and techniques essential for understanding the biochemical structure and function of a single cell. Its main goal is to encourage students to apply these laboratory skills in the vocational field of applied microbiology and allied health or to further pursue the study of life at the molecular level.

In this manual, comprehensive introductory material is given at the beginning of each major area of study, and specific explanations and detailed directions precede each experiment. This approach augments, enhances, and reinforces course lectures, enabling students to comprehend more readily the concepts and purposes of each experiment. This also provides a review aid if the laboratory and lecture sections are not taught concurrently. The manual should also reduce the time required for explanations at the beginning of each laboratory session and thus make more time available for performing the experiments. Finally, care has been taken to design experimental procedures so that the supplies, equipment, and instrumentation commonly found in undergraduate institutions will suffice for their successful execution.

Organization

This manual consists of 72 experiments arranged into 16 parts. The experiments progress from those that are basic and introductory, requiring minimal manipulations, to those that are more complex, requiring more sophisticated skills. The format of each experiment is intended to facilitate presentation of the material by the instructor and to maximize the learning experience. To this end, each experiment is designed with the following components.

Learning Objectives

This introductory section defines the specific principles and/or techniques to be mastered.

Principle

This is an in-depth discussion of the microbiological concept or technique and the specific experimental procedure.

Clinical Application

Clinical or medical applications that appear within each experiment help students connect what they are learning in lecture with what they are doing in the lab. For students who intend to have careers as nurses or in other allied health fields, Clinical Applications explain the relevance of each lab technique to their career plans.

At the Bench

This section signals the beginning of the experiment, and includes the materials, notes of caution, and procedural instructions—all of the things students will need to know at the bench, during the course of the experiment.

Materials

This comprehensive list helps students and instructors prepare for each laboratory session. Materials appear under one of the following headings:

Cultures These are the selected test organisms that have been chosen to demonstrate effectively the experimental principle or technique under study. The choice is also based on their ease of cultivation and maintenance in stock culture. A complete listing of the experimental cultures and prepared slides is presented in Appendix 6.

Media These are the specific media and their quantities per designated student group. Appendix 3 lists the composition and method of preparation of all the media used in this manual.

Reagents These include biological stains as well as test reagents. The chemical composition and preparation of the reagents are presented in Appendices 4 and 5.

Equipment Listed under this heading are the supplies and instrumentation that are needed during the laboratory session. The suggested equipment was selected to minimize expense while reflecting current laboratory technique.

Procedure

This section provides explicit instructions, augmented by diagrams, that aid in the execution and interpretation of the experiment.


A caution icon has been placed in experiments that may use potentially pathogenic materials. The instructor may wish to perform some of these experiments as demonstrations.

Lab Report

These sheets, located at the end of each experiment, facilitate interpretation of data and subsequent review by the instructor. The Observations and Results portion of the report provides tables for recording observations and results, and helps the students draw conclusions from and interpret their data. The Review Questions aid the instructor in determining the student's ability to understand the experimental concepts and techniques. Questions that call for more critical thinking are indicated by the brain icon.

New to the Eleventh Edition

For this eleventh edition, the primary aim was to build upon and enrich the student experience. The changes described below are intended to impart the relevance of microbiological lab techniques to published standard protocols, and to enhance student understanding in the validity of each of the microbiological procedures as they apply laboratories in both the educational and industrial setting.

New Tips for Success

The eleventh edition includes new Tips for Success that will help students avoid common mistakes while they learn laboratory techniques. These tips alert students to potential issues or mistakes that other students have encountered while doing the same experiments. Warning students about potential issues before they begin experiments will reduce the number of procedural mistakes and maximize the number of successful experiments.

New Experiment 39: Propagation of Isolated Bacteriophage Cultures

This experiment builds on previous experiments by utilizing procedures for the cultivation and enumeration of coliphages isolated from individual plaques produced in Experiment 38. These techniques when combined should allow students to isolate, cultivate, and further propagate bacteriophages from commercial or environmental sources.

New Experiment 46: Microbial Fermentation

The previous version of this experiment examined alcohol fermentation by yeast cells. The current experiment has been expanded to include lactic acid fermentation. Students will produce yogurt in an experimental setting, examining changes in culture pH and liquid consistency over time as a means to study bacterial lactic acid production during anaerobic metabolism.

Information Concerning Governing Bodies

Where appropriate, information concerning governing bodies, such as the Environmental Protection Agency (EPA), the Clinical and

Laboratory Standards Institute (CLSI), and AOAC International, has been included in the introductory material for some experiments. By drawing attention to governing bodies beyond the American Society for Microbiology (ASM) that have published laboratory standards, students will be introduced to the various industry standards that regulate microbiology laboratories.

Updates and Revisions

Throughout the manual, updates and revisions have been made to background information, terminology, equipment, and procedural techniques, including the following:

- Added a new procedure for a streak plate method in Experiment 2.
- Updated protocols in many experiments, including Experiment 20, to utilize microvolume procedures.
- Added an alternate protocol in Experiment 12 that uses the McFarland Standards to culture preparations for each lab.
- Protocols for the utilization of plate readers in measuring turbidity and bacterial growth have been added to Experiment 12 and other experiments.
- Results tables have been updated for many experiments (e.g., Experiments 20 and 21) to allow instructors to modify or customize the lab to include organisms of interest not listed in the protocol.
- Added biosafety level (BSL) markers throughout the text. Organisms are clearly labeled with biosafety level 2 if they require additional precautions (BSL-2).

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I would like to express my sincere gratitude to Dr. James Cappuccino for the opportunity to work with him on this laboratory manual that has been his work for the past 20+ years. My hope is that with this eleventh edition we will begin a long and rewarding collaboration.

I also wish to extend my appreciation to the staff at Pearson who helped me through the creation of this manual. Specifically I would like to thank Kelsey Churchman, Senior Acquisitions Editor, Chriscelle Palaganas, Program Manager, and Arielle Grant, Project Manager.

Chad Welsh

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Laboratory Safety

General Rules and Regulations

A rewarding laboratory experience demands strict adherence to prescribed rules for personal and environmental safety. The former reflects concern for your personal safety in terms of avoiding laboratory accidents. The latter requires that you maintain a scrupulously clean laboratory setting to prevent contamination of experimental procedures by microorganisms from exogenous sources.

Because most microbiological laboratory procedures require the use of living organisms, an integral part of all laboratory sessions is the use of aseptic techniques. Although the virulence of microorganisms used in the academic laboratory environment has been greatly diminished because of their long-term maintenance on artificial media, all microorganisms should be treated as potential pathogens (organisms capable of producing disease). Thus, microbiology students must develop aseptic techniques (free of contaminating organisms) in the preparation of pure cultures that are essential in the industrial and clinical marketplaces.

The following basic steps should be observed at all times to reduce the ever-present microbial flora of the laboratory environment.

1. Upon entering the laboratory, place coats, books, and other paraphernalia in specified locations—never on bench tops.
2. Keep doors and windows closed during the laboratory session to prevent contamination from air currents.
3. At the beginning and termination of each laboratory session, wipe bench tops with a disinfectant solution provided by the instructor.
4. Do not place contaminated instruments, such as inoculating loops, needles, and pipettes, on bench tops. Loops and needles should be sterilized by incineration, and pipettes should be disposed of in designated receptacles.
5. On completion of the laboratory session, place all cultures and materials in the disposal area as designated by the instructor.
6. Rapid and efficient manipulation of fungal cultures is required to prevent the dissemination of their reproductive spores in the laboratory environment.

To prevent accidental injury and infection of yourself and others, observe the following regulations:

1. Wash your hands with liquid detergent, rinse with 95% ethyl alcohol, and dry them with paper towels upon entering and prior to leaving the laboratory.
2. Always use the appropriate safety equipment as determined by your instructor:
   - **a.** A laboratory coat or apron may be necessary while working in the laboratory. Lab coats protect clothing from contamination or accidental discoloration by staining solutions.
   - **b.** You may be required to wear gloves while performing the lab exercises. Gloves shield your hands from contamination by microorganisms. They also prevent the hands from coming in direct contact with stains and other reagents.
   - **c.** Masks and safety goggles may be required to prevent materials from coming in contact with your eyes.
3. Wear a paper cap or tie back long hair to minimize its exposure to open flames.
4. Wear closed shoes at all times in the laboratory setting.
5. Never apply cosmetics or insert contact lenses in the laboratory.
6. Do not smoke, eat, or drink in the laboratory. These activities are absolutely prohibited.
7. Carry cultures in a test-tube rack when moving around the laboratory. Likewise, keep cultures in a test-tube rack on the bench tops when not in use. This serves a dual purpose: to prevent accidents and to avoid contamination of yourself and the environment.

8. Never remove media, equipment, or especially, microbial cultures from the laboratory. Doing so is absolutely prohibited.
9. Immediately cover spilled cultures or broken culture tubes with paper towels and then saturate them with disinfectant solution. After 15 minutes of reaction time, remove the towels and dispose of them in a manner indicated by the instructor.
10. Report accidental cuts or burns to the instructor immediately.
11. Never pipette by mouth any broth cultures or chemical reagents. Doing so is strictly prohibited. Pipetting is to be carried out with the aid of a mechanical pipetting device only.
12. Do not lick labels. Use only self-stick labels for the identification of experimental cultures.
13. Speak quietly and avoid unnecessary movement around the laboratory to prevent distractions that may cause accidents.

The following specific precautions must be observed when handling body fluids of unknown origin due to the possible transmission of human immunodeficiency virus (HIV) and hepatitis B virus in these test specimens.

1. Wear disposable gloves during the manipulation of test materials such as blood, serum, and other body fluids.
2. Immediately wash hands if contact with any of these fluids occurs and also on removal of the gloves.
3. Wear masks, safety goggles, and laboratory coats if an aerosol might be formed or splattering of these fluids is likely to occur.
4. Decontaminate spilled body fluids with a 1:10 dilution of household bleach, covered with paper toweling, and allowed to react for 10 minutes before removal.
5. Place test specimens and supplies in contact with these fluids into a container of disinfectant prior to autoclaving.

I have read the above laboratory safety rules and regulations and agree to abide by them.

Name: _____ Date: _____

Laboratory Protocol

Student Preparation for Laboratory Sessions

The efficient performance of laboratory exercises mandates that you attend each session fully prepared to execute the required procedures. Read the assigned experimental protocols to effectively plan and organize the related activities. This will allow you to maximize use of laboratory time.

Preparation of Experimental Materials

Microscope Slides: Meticulously clean slides are essential for microscopic work. Commercially precleaned slides should be used for each microscopic slide preparation. However, wipe these slides with dry lens paper to remove dust and finger marks prior to their use. With a glassware marking pencil, label one end of each slide with the abbreviated name of the organism to be viewed.

Labeling of Culture Vessels: Generally, microbiological experiments require the use of a number of different test organisms and a variety of culture media. To ensure the successful completion of experiments, organize all experimental cultures and sterile media at the start of each experiment. Label culture vessels with non–water-soluble glassware markers and/or self-stick labels prior to their inoculation. The labeling on each of the experimental vessels should include the name of the test organism, the name of the medium, the dilution of sample (if any), your name or initials, and the date. Place labeling directly below the cap of the culture tube. When labeling Petri dish cultures, only the name of the organism(s) should be written on the bottom of the plate, close to its periphery, to prevent obscuring observation of the results. The additional information for the identification of the culture should be written on the cover of the Petri dish.

Inoculation Procedures

Aseptic techniques for the transfer or isolation of microorganisms, using the necessary transfer instruments, are described fully in the experiments in Part 1 of the manual. Technical skill will be acquired through repetitive practice.

Inoculating Loops and Needles: It is imperative that you incinerate the entire wire to ensure absolute sterilization. The shaft should also be briefly passed through the flame to remove any dust or possible contaminants. To avoid killing the cells and splattering the culture, cool the inoculating wire by tapping the inner surface of the culture tube or the Petri dish cover prior to obtaining the inoculum, or touch the edge of the medium in the plate.

When performing an aseptic transfer of microorganisms, a minute amount of inoculum is required. If an agar culture is used, touch only a single area of growth with the inoculating wire to obtain the inoculum. Never drag the loop or needle over the entire surface, and take care not to dig into the solid medium. If a broth medium is used, first tap the bottom of the tube against the palm of your hand to suspend the microorganisms. Caution: Do not tap the culture vigorously as this may cause spills or excessive foaming of the culture, which may denature the proteins in the medium.

Pipettes: Use only sterile, disposable pipettes or glass pipettes sterilized in a canister. The practice of pipetting by mouth has been discontinued to eliminate the possibility of autoinfection by accidentally imbibing the culture or infectious body fluids. Instead, use a mechanical pipetting device to obtain and deliver the material to be inoculated.

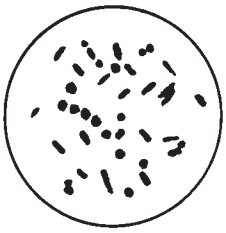
Incubation Procedure

Microorganisms exhibit a wide temperature range for growth. However, for most used in this manual, optimum growth occurs at 37°C over a period of 18 to 24 hours. Unless otherwise indicated in specific exercises, incubate all cultures under the conditions cited above. Place culture tubes in a rack for incubation. Petri dishes may be stacked; however, they must always be incubated in an inverted position (top down) to prevent water condensation from dropping onto the surface of the culture medium. This excess moisture could allow the spread of the microorganisms on the surface of the culture medium, producing confluent rather than discrete microbial growth.

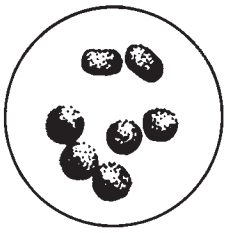
Procedure for Recording Observations and Results

The accurate accumulation of experimental data is essential for the critical interpretation of the observations upon which the final results will be based. To achieve this end, it is imperative that you complete all the preparatory readings that are necessary for your understanding of the basic principles underlying each experiment. Meticulously record all the observed data in the Lab Report of each experiment.

In the experiments that require drawings to illustrate microbial morphology, it will be advantageous to depict shapes, arrangements, and cellular structures enlarged to 5 to 10 times their actual microscopic size, as indicated by the following illustrations. For this purpose a number 2 pencil is preferable. Stippling may be used to depict different aspects of cell structure (e.g., endospores or differences in staining density).



Microscopic drawing



Enlarged drawing

Review Questions

The review questions are designed to evaluate the student's understanding of the principles and the interpretations of observations in each experiment. Completion of these questions will also serve to reinforce many of the concepts that are discussed in the lectures. At times, this will require the use of ancillary sources such as textbooks, microbiological reviews, or abstracts. The designated critical-thinking questions are designed to stimulate further refinement of cognitive skills.

Procedure for Termination of Laboratory Sessions

1. Return all equipment, supplies, and chemical reagents to their original locations.
2. Neatly place all capped test tube cultures and closed Petri dishes in a designated collection area in the laboratory for subsequent autoclaving.
3. Place contaminated materials, such as swabs, disposable pipettes, and paper towels, in a biohazard receptacle prior to autoclaving.
4. Carefully place hazardous biochemicals, such as potential carcinogens, into a sealed container and store in a fume hood prior to their disposal according to the institutional policy.
5. Wipe down table tops with recommended disinfectant.
6. Wash hands before leaving the laboratory.

PART 1

Basic Laboratory Techniques for Isolation, Cultivation, and Cultural Characterization of Microorganisms

LEARNING OBJECTIVES

Once you have completed the experiments in this section, you should be familiar with

1. The types of laboratory equipment and culture media needed to develop and maintain pure cultures.
2. The types of microbial flora that live on the skin and the effect of hand washing on them.
3. The concept of aseptic technique and the procedures necessary for successful subculturing of microorganisms.
4. Streak-plate and spread-plate inoculation of microorganisms in a mixed microbial population for subsequent pure culture isolation.
5. Cultural and morphological characteristics of microorganisms grown in pure culture.

Introduction

Microorganisms are ubiquitous. They are found in soil, air, water, food, sewage, and on body surfaces. In short, every area of our environment is replete with them. The microbiologist separates these mixed populations into individual species for study. A culture containing a single unadulterated species of cells is called a **pure culture**. To isolate and study microorganisms in pure culture, the microbiologist requires basic laboratory apparatus and the application of specific techniques, as illustrated in Figure P1.1.

Media

The survival and continued growth of microorganisms depend on an adequate supply of nutrients and a favorable growth environment. For survival, most microbes must use soluble low-molecular-weight substances that are frequently derived from the enzymatic degradation of complex nutrients. A solution containing these nutrients is a **culture medium**. Basically, all culture media are liquid, semisolid, or solid. A liquid medium lacks a solidifying agent and is called a **broth medium**. A broth medium is useful for the cultivation of high numbers of bacterial cells in a small volume of medium, which is particularly helpful when an assay requires a high number of healthy bacterial cells. A broth medium supplemented with a solidifying agent called **agar** results in a solid or semisolid medium. Agar, an extract of seaweed, is a complex carbohydrate composed mainly of galactose, and is without nutritional value. Agar serves as an excellent solidifying agent because it liquefies at 100°C and solidifies at 40°C. Because of these properties, organisms, especially pathogens, can be cultivated at temperatures of 37.5°C or slightly higher without fear of the medium liquefying. A completely solid medium requires an agar concentration of 1.5% to 1.8%. A concentration of less than 1% agar results in a **semisolid medium**. A semisolid medium is useful for testing a cell's ability to grow within the agar at lower oxygen levels and

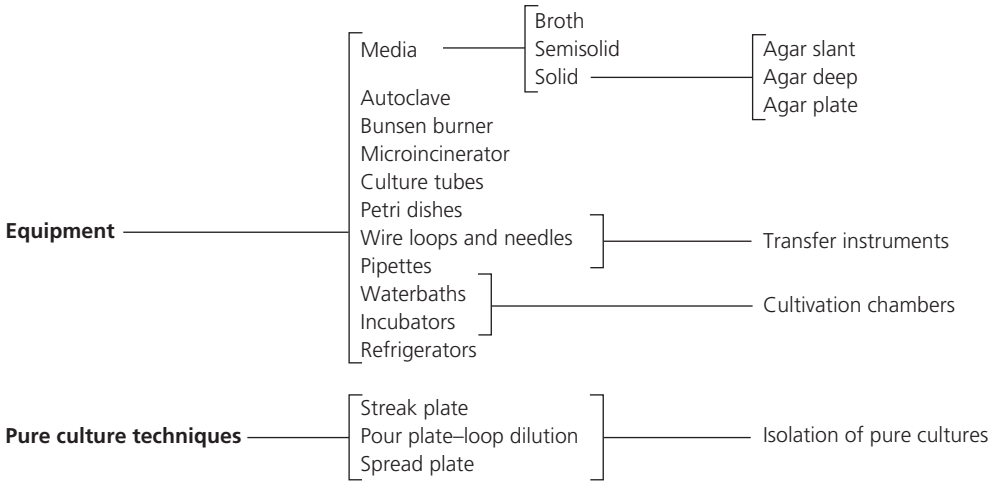
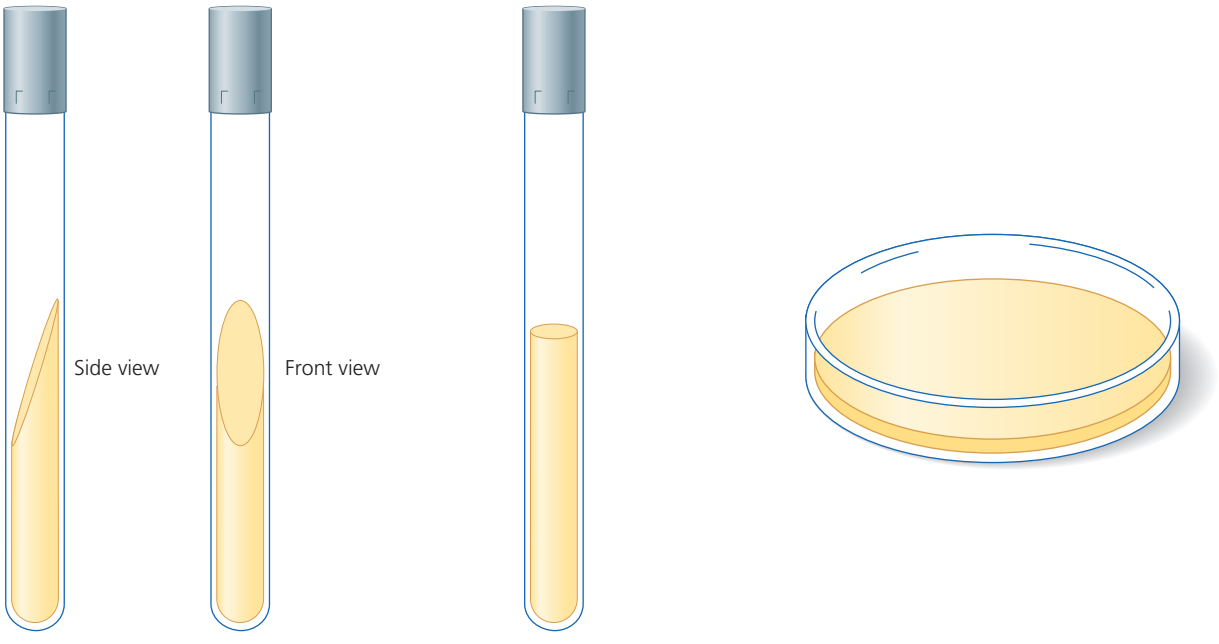


Figure P1.1 Laboratory apparatus and culture techniques

for testing the species' motility. A solid medium has the advantage that it presents a hardened surface on which microorganisms can be grown using specialized techniques for the isolation of discrete colonies. Each **colony** is a cluster of cells that originates from the multiplication of a single cell and represents the growth of a single species of microorganism. Such a defined and well-isolated colony is a pure culture. Also, while in the liquefied state, solid media can be placed in test tubes, which are then allowed to cool and harden in a slanted position, producing **agar slants**. These are useful for maintaining pure cultures. The slanted surface of the agar maximizes the available surface area for microorganism growth while minimizing the amount of medium required. Similar tubes that, following preparation, are allowed to harden in the upright position are designated as **agar deep tubes**. Agar deep tubes are used primarily for the study of the gaseous requirements of microorganisms since gas exchange between the agar at the butt of the test tube and the external environment is impeded by the height of the agar. Liquid agar medium can also be poured into Petri dishes, producing **agar plates**, which provide large surface areas for the isolation and study of microorganisms. The various forms of solid media are illustrated in Figure P1.2.



(a) Agar slants **(b)** Agar deep tube **(c)** Agar plate

Figure P1.2 Forms of solid (agar) media

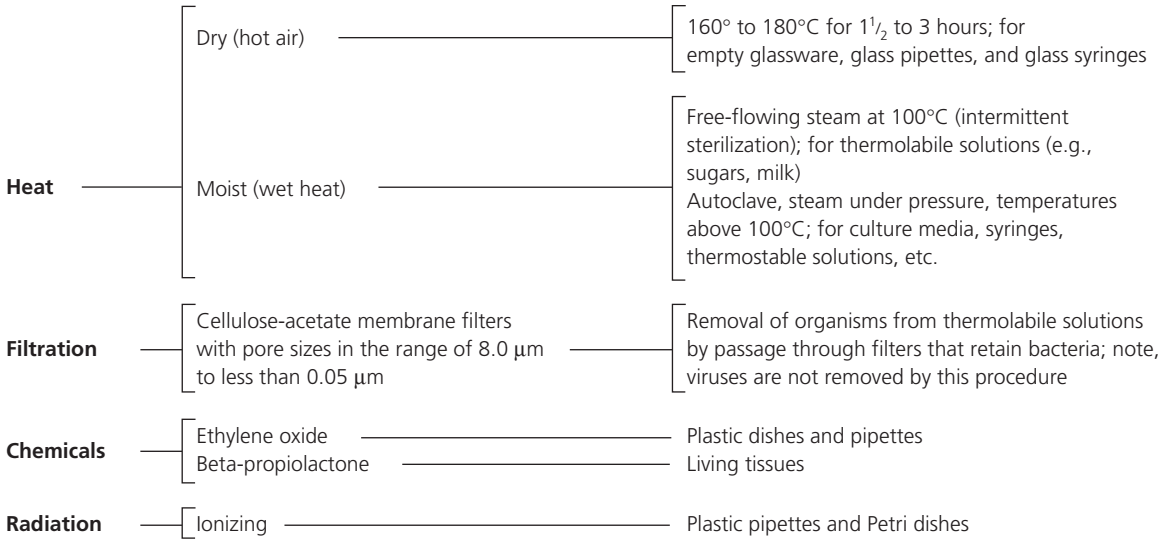


Figure P1.3 Sterilization techniques

In addition to nutritional needs, the environmental factors must also be regulated, including proper pH, temperature, gaseous requirements, and osmotic pressure. A more detailed explanation is presented in Part 4, which deals with cultivation of microorganisms; for now, you should simply bear in mind that numerous types of media are available.

Aseptic Technique

Sterility is the hallmark of successful work in the microbiology laboratory, and **sterilization** is the process of rendering a medium or material free of all forms of life. To achieve sterility, it is mandatory that you use sterile equipment and employ **aseptic techniques** when handling bacterial cultures. Using correct aseptic techniques minimizes the likelihood that bacterial cultures will be contaminated, and reduces the opportunity for students to be exposed to potential pathogens. Although a more detailed discussion is presented in Part 9, which describes the control of microorganisms, Figure P1.3 is a brief outline of the routine techniques used in the microbiology laboratory.

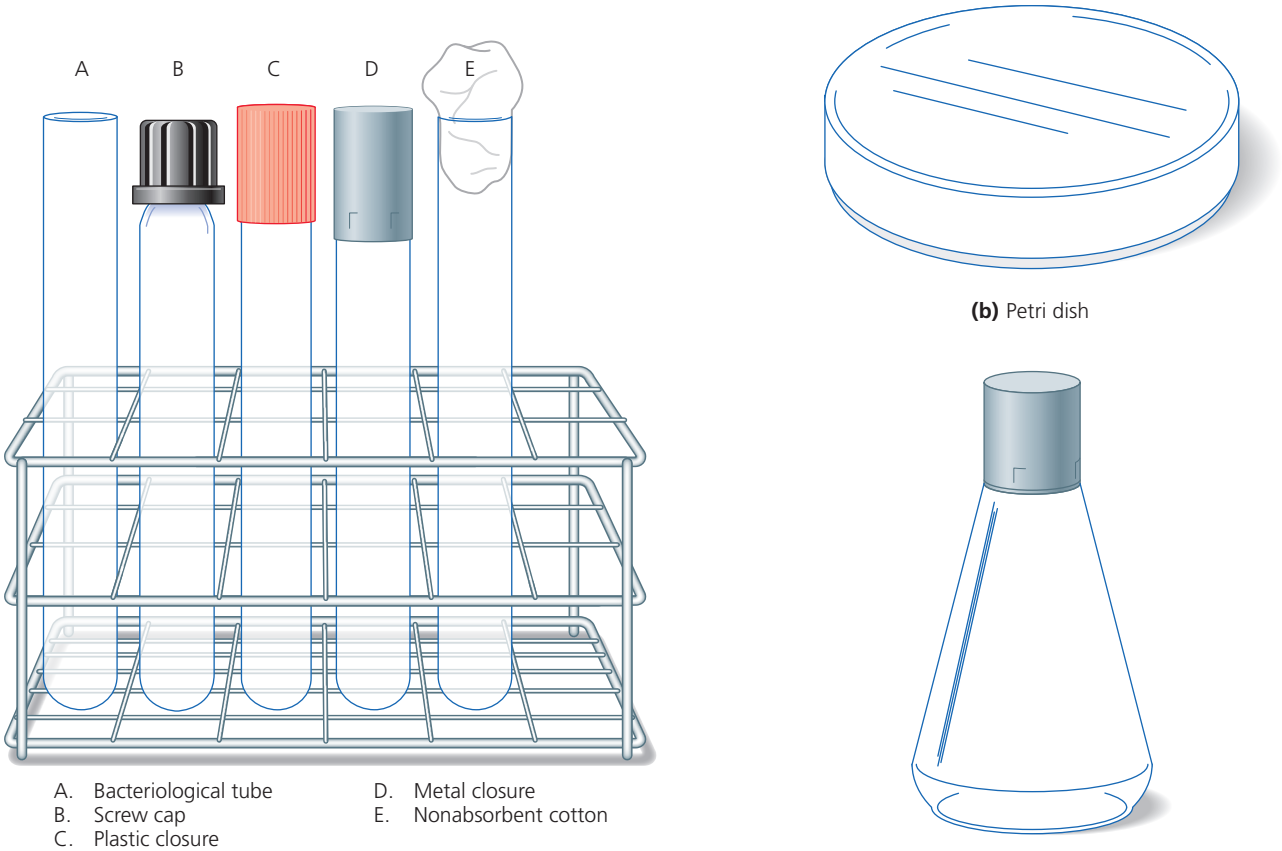
Culture Tubes and Petri Dishes

Glass **test tubes** and glass or plastic **Petri dishes** are used to cultivate microorganisms. A suitable nutrient medium in the form of broth or agar may be added to the tubes, while only a solid medium is used in Petri dishes. A sterile environment is maintained in culture tubes by various types of closures. Historically, the first type, a cotton plug, was developed by Schröeder and von Dusch in the nineteenth century. Today most laboratories use sleeve-like caps (Morton closures) made of metal, such as stainless steel, or heat-resistant plastics. The advantage of these closures over the cotton plug is that they are labor-saving and, most of all, slip on and off the test tubes easily.

Petri dishes provide a larger surface area for growth and cultivation. They consist of a bottom dish portion that contains the medium and a larger top portion that serves as a loose cover. Petri dishes are manufactured in various sizes to meet different experimental requirements. For routine purposes, dishes approximately 15 cm in diameter are used. The sterile agar medium is dispensed to previously sterilized dishes from molten agar deep tubes containing 15 ml to 20 ml of medium, or from a molten sterile medium prepared in bulk and contained in 250-, 500-, and 1000-ml flasks, depending on the volume of medium required. When cooled to 40°C, the medium will solidify. Remember that *after inoculation, Petri dishes are incubated in an inverted position* (top down) to prevent condensation formed on the cover during solidification from dropping down onto the surface of the hardened agar. For this reason, Petri dishes should be labeled on the bottom of the dish. This makes it easier to read the label and minimizes confusion if two Petri dish covers are interchanged. Figure P1.4 illustrates some of the culture vessels used in the laboratory. Built-in ridges on tube closures and Petri dishes provide small gaps necessary for the exchange of air.

Transfer Instruments

Microorganisms must be transferred from one vessel to another or from stock cultures to various media for maintenance and study. This transfer



(a) Test tube rack with tubes showing various closures

(b) Petri dish

(c) DeLong shaker flask with closure

Figure P1.4 Culture vessels

is called **subculturing** and must be carried out under aseptic conditions to prevent possible contamination.

Wire loops and needles are made from inert metals such as Nichrome or platinum and are inserted into metal shafts that serve as handles. They are extremely durable instruments and are easily sterilized by incineration in the blue (hottest) portion of the Bunsen burner flame. A wire loop is useful for transferring a small volume of bacteria onto the surface of an agar plate or slant. A needle is used primarily to inoculate a culture into a broth medium or into an agar deep tube.

A **pipette** is another instrument used for aseptic transfers. Pipettes are similar in function to straws; that is, they draw up liquids. They are glass or plastic and drawn out to a tip at one end, with a mouthpiece forming the other end. They are calibrated to deliver different volumes depending on requirements. Pipettes may be sterilized in bulk inside canisters, or they may be wrapped individually in brown paper and sterilized in an autoclave or dry-heat oven. A micropipette (commonly called a "pipetter") with a disposable, single-use plastic tip is useful for transferring small volumes of liquid (less than ≤1 ml).

Figure P1.5 illustrates these transfer instruments. Your instructor will demonstrate the proper procedure for using pipettes.

> ⚠ **Pipetting by mouth is not permissible! Pipetting must be performed with mechanical pipette aspirators.**

Cultivation Chambers

The specific temperature requirements for growth are discussed in detail in Part 4. However, a prime requirement for the cultivation of microorganisms is that they be grown at their optimum temperature. An **incubator** is used to maintain optimum temperature during the necessary growth period. It resembles an oven and is thermostatically

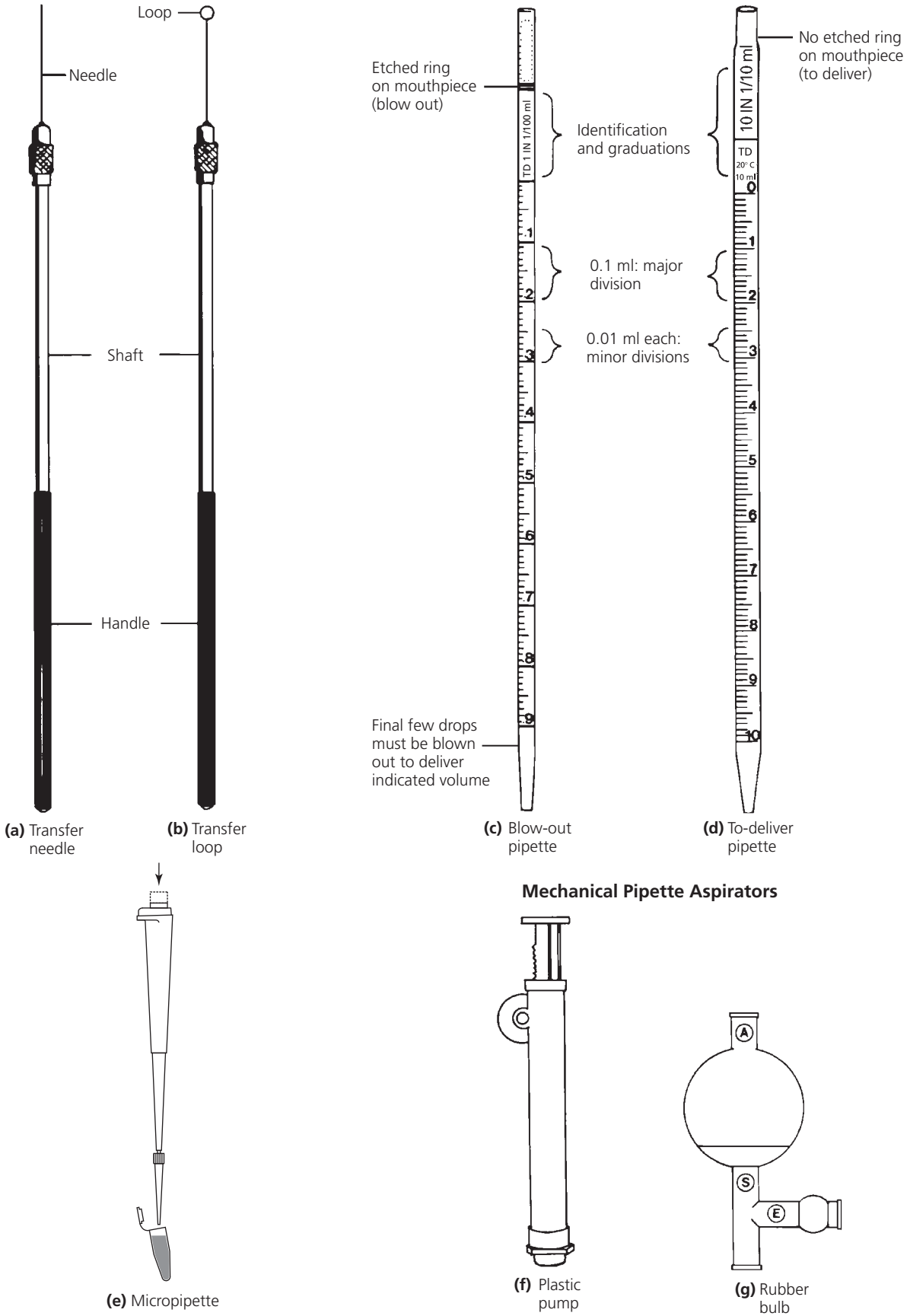


Figure P1.5 **Transfer instruments**

controlled so that temperature can be varied depending on the requirements of specific microorganisms. Most incubators use dry heat. Moisture is supplied by placing a beaker of water in the incubator during the growth period. A moist environment retards dehydration of the medium and thereby avoids misleading experimental results.

A thermostatically controlled **shaking waterbath** is another piece of apparatus used to cultivate microorganisms. Its advantage is that it provides a rapid and uniform transfer of heat to the culture vessel, and its agitation provides increased aeration, resulting in acceleration of growth. The primary disadvantage of this instrument is that it can be used only for cultivation of organisms in a broth medium.

Many laboratories also use shaking incubators that utilize dry air incubation to promote aeration of the broth medium. This method has a distinct advantage over a shaking waterbath since there is no chance of cross contamination from microorganisms that might grow in the waterbath.

Refrigerator

A refrigerator is used for a wide variety of purposes such as maintenance and storage of stock cultures between subculturing periods, and storage of sterile media to prevent dehydration. It is also used as a repository for thermolabile solutions, antibiotics, serums, and biochemical reagents.

EXPERIMENT 1

Culture Transfer Techniques

LEARNING OBJECTIVES

Once you have completed this experiment, you should be able to

1. Carry out the technique for aseptic removal and transfer of microorganisms for subculturing.
2. Correctly sterilize inoculating instruments in a microincinerator or the flame of a Bunsen burner.
3. Correctly remove and replace the test tube closure.

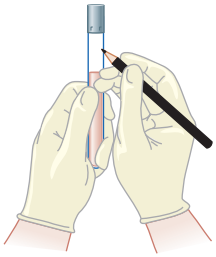
Principle

Microorganisms are transferred from one medium to another by **subculturing**. This technique is of basic importance and is used routinely in preparing and maintaining stock cultures, as well as in microbiological test procedures.

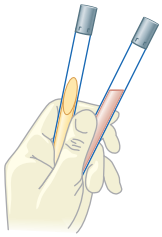
Microorganisms are always present in the air and on laboratory surfaces, benches, and equipment. These ambient microorganisms can serve as a source of external contamination and interfere with experimental results unless proper aseptic techniques are used during subculturing. Described below are essential steps that you must follow for aseptic transfer of microorganisms. The complete procedure is illustrated in Figure 1.1.

1. Label the tube to be inoculated with the name of the organism and your initials.
2. Hold the stock culture tube and the tube to be inoculated in the palm of your hand, secure with your thumb, and separate the two tubes to form a V in your hand.
3. Sterilize an inoculating needle or loop by holding it in the microincinerator or the hottest portion of the Bunsen burner flame, until the wire becomes red hot. Once sterilized, the loop is held in the hand and allowed to cool for 10 to 20 seconds; it is never put down.
4. Uncap each tube by grasping the first cap with your little finger and the second cap with your next finger and lifting the closure upward. *Note: Once removed, these caps must be kept in the hand that holds the sterile inoculating loop or needle; the inner aspects of the caps point away from the palm of the hand.* The caps must never be placed on the laboratory bench because that would compromise the aseptic procedure.
5. After removing the caps, flame the necks and mouths of the tubes by briefly passing them through the opening of the microincinerator or through the Bunsen burner flame two to three times rapidly. The sterile transfer instrument is further cooled by touching it to the sterile inside wall of the culture tube before removing a small sample of the inoculum.
6. Depending on the culture medium, a loop or needle is used for removal of the inoculum. Loops are commonly used to obtain a sample from a broth culture. Either instrument can be used to obtain the inoculum from an agar slant culture by carefully touching the surface of the solid medium in an area exhibiting growth so as not to gouge the agar. A straight needle is always used when transferring microorganisms to an agar deep tube from both solid and liquid cultures.
   a. For a slant-to-broth transfer, obtain inoculum from the slant and lightly shake the loop or needle in the broth culture to dislodge the microorganisms.
   b. For a broth-to-slant transfer, obtain a loopfull of broth and place at the base of an agar slant medium. Lightly draw the loop over the hardened surface in a straight or zig-zag line, from the base of the agar slant to the top.
   c. For a slant-to-agar deep tube transfer, obtain the inoculum from the agar slant. Insert a straight needle to the bottom of the tube in a straight line and rapidly withdraw along the line of insertion. This is called a stab inoculation.
7. Following inoculation, remove the instrument and reheat or reflame the necks of the tubes.

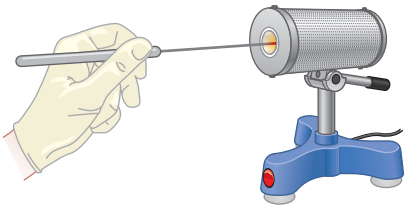
PROCEDURE



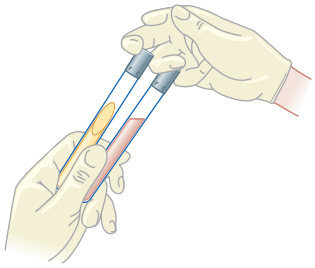
1 Label the tube to be inoculated with the name of the organism and your initials.



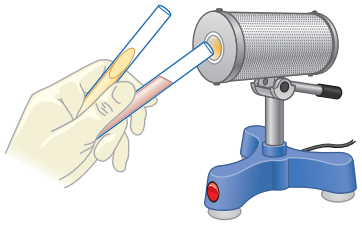
2 Place the tubes in the palm of your hand, secure with your thumb, and separate to form a V.



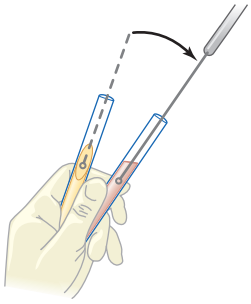
3 Flame the needle or loop until the wire is red.



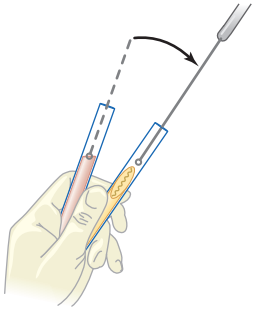
4 With the sterile loop or needle in hand, uncap the tubes.



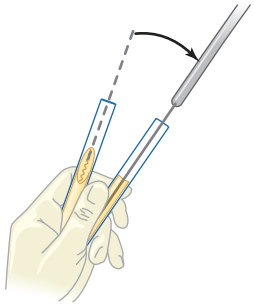
5 Flame the necks of the tubes by rapidly passing them through the flame once.



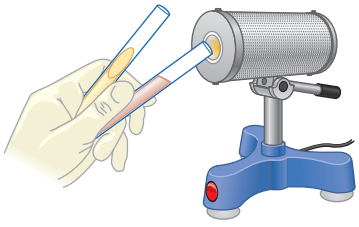
6 **Slant-to-broth transfer:** Obtain inoculum from slant and dislodge inoculum in the broth with a slight agitation.



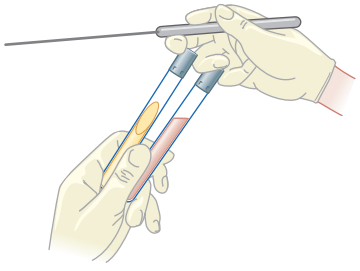
Broth-to-slant transfer: Obtain a loopful of broth and place at base of slant. Withdraw the loop in a zigzag motion.



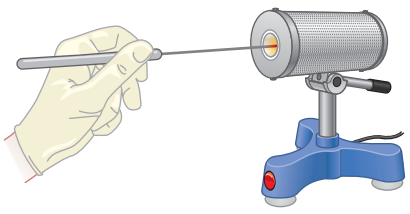
Slant-to-agar deep transfer: Obtain inoculum from slant. Insert the needle to the bottom of the tube and withdraw along the line of insertion.



7 Flame the necks of the tubes by rapidly passing them through the flame once.



8 Recap the tubes.



9 Reflame the loop or needle.

Figure 1.1 **Subculturing procedure**

8. Replace the caps on the same tubes from which they were removed.
9. Resterilize the loop or needle to destroy any remaining organisms.

In this experiment, you will master the manipulations required for aseptic transfer of microorganisms in broth-to-slant, slant-to-broth, and slant-to-agar deep tubes. You will be using a positive and a negative control to test your ability to maintain aseptic techniques while transferring cultures. The technique for transfer to and from agar plates is discussed in Experiment 2.

CLINICAL APPLICATION

Aseptic Inoculation and Transfer

It is mandatory that microbiology laboratory workers learn and perfect the skill of inoculating bacterial specimens on agar plates, in liquid broth, or in semisolid medium, and be able to subculture the organism from one medium to another. A sterile inoculating needle or loop is the basic instrument of transfer. Keep in mind that, transferring bacterial cultures requires aseptic or sterile techniques at all times, especially if you are working with pathogens. Do not contaminate what you are working with and do not contaminate yourself.

AT THE BENCH

Materials

Cultures

Twenty-four–hour nutrient broth and nutrient agar slant cultures of *Serratia marcescens* and a sterile tube of nutrient broth. The nutrient broth tubes will be labeled "A" and "B," and the contents will be known only by the instructor.

Media

Per student: three nutrient broth tubes, three nutrient agar slants, and three nutrient agar deep tubes.

Equipment

Microincinerator or Bunsen burner, inoculating loop and needle, and glassware marking pencil.

Procedure Lab One

1. Label all tubes of sterile media as described in the Laboratory Protocol section on page 15.
2. Following the procedure outlined and illustrated previously (Figure 1.1), perform the following transfers:
   a. *Broth culture "A" to a nutrient agar slant, nutrient agar deep tube, and nutrient broth.*
   b. *Broth culture "B" to a nutrient agar slant, nutrient agar deep tube, and nutrient broth.*
   c. *S. marcescens agar slant culture to a nutrient agar slant, nutrient agar deep tube and nutrient broth.*
3. Incubate all cultures at 25°C for 24 to 48 hours.

Procedure Lab Two

1. Examine all cultures for the appearance of growth, which is indicated by turbidity in the broth culture and the appearance of an orange-red growth on the surface of the slant and along the line of inoculation in the agar deep tube.
2. Record your observations in the chart provided in the Lab Report.
3. Confirm your results with the instructor to determine the negative control tube.

TIPS FOR SUCCESS

1. **It is imperative that you maintain sterility and utilize aseptic techniques at all times.** If you allow a contaminating organism into your bacterial culture, you will see a positive growth in media that was inoculated with the negative control.

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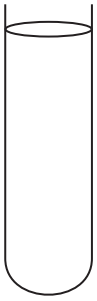
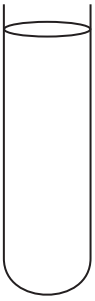
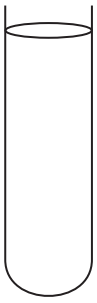
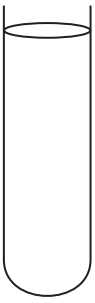
Name: _____

Date: _____ Section: _____

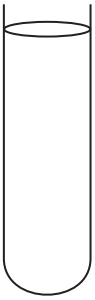
Observations and Results Culture "A"

	Nutrient Broth	Nutrient Agar Slant	Nutrient Agar Deep
Growth (+) or (–)	_____	_____	_____
Orange-red pigmentation (+) or (–)	_____	_____	_____
Draw the distribution of growth.			

Observations and Results Culture "B"

	Nutrient Broth	Nutrient Agar Slant	Nutrient Agar Deep
Growth (+) or (–)	_____	_____	_____
Orange-red pigmentation (+) or (–)	_____	_____	_____
Draw the distribution of growth.			

Observations and Results *S. marcescens*

	Nutrient Broth	Nutrient Agar Slant	Nutrient Agar Deep
Growth (+) or (–)	_____	_____	_____
Orange-red pigmentation (+) or (–)	_____	_____	_____
Draw the distribution of growth.			

1. Explain why the following steps are essential during subculturing:

a. Flaming the inoculating instrument *prior to and after* each inoculation.

b. Holding the test tube caps in the hand as illustrated in Figure 1.1 on page 24.

c. Cooling the inoculating instrument prior to obtaining the inoculum.

d. Flaming the neck of the tubes immediately after uncapping and before recapping.

2. What are ambient microorganisms? Why should they not be present during subculturing?

3. Explain why a straight inoculating needle is used to inoculate an agar deep tube.

4.  There is a lack of orange-red pigmentation in some of the growth on your agar slant labeled *S. marcescens*. Does this necessarily indicate the presence of a contaminant? Explain.

5.  Upon observation of the nutrient agar slant culture, you strongly suspect that the culture is contaminated. Outline the method you would follow to ascertain whether your suspicion is justified.

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Techniques for Isolation of Pure Cultures

EXPERIMENT 2

In nature, microbial populations do not segregate themselves by species, but exist with a mixture of many other cell types. In the laboratory, these populations can be separated into **pure cultures**. These cultures contain only one type of organism and are suitable for the study of their cultural, morphological, and biochemical properties.

In this experiment, you will first use one of the techniques designed to produce discrete colonies. Colonies are individual, macroscopically visible masses of microbial growth on a solid medium surface, each representing the multiplication of a single organism. Once you have obtained these discrete colonies, you will make an aseptic transfer onto nutrient agar slants for the isolation of pure cultures.

PART A Isolation of Discrete Colonies from a Mixed Culture

LEARNING OBJECTIVE

Once you have completed this experiment, you should be able to

1. Perform the streak-plate and/or the spread-plate inoculation procedure to separate the cells of a mixed culture so that discrete colonies can be isolated.

Principle

The techniques commonly used for isolation of discrete colonies initially require that the number of organisms in the inoculum be reduced. The resulting diminution of the population size ensures that, following inoculation, individual cells will be sufficiently far apart on the surface of the agar medium to separate the different species. The following are techniques that can be used to accomplish this necessary dilution:

1. The **streak-plate** method is a rapid qualitative isolation method. It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate. Although many types of procedures are performed, the four-way, or quadrant, streak is described. Refer to Figure 2.1, which schematically illustrates this technique.
   - **a.** Place a loopful of culture on the agar surface in Area 1. Flame the loop, cool it by touching an unused part of the agar surface close to the periphery of the plate, and then drag it rapidly several times across the surface of Area 1.
   - **b.** Reflame and cool the loop, and turn the Petri dish 90°. Then touch the loop to a corner of the culture in Area 1 and drag it several times across the agar in Area 2. The loop should never enter Area 1 again.
   - **c.** Reflame and cool the loop and again; turn the dish 90°. Streak Area 3 in the same manner as Area 2.

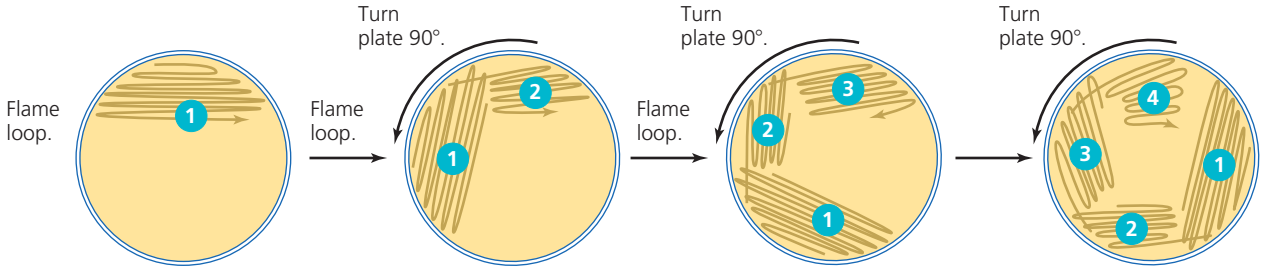


Figure 2.1 Four-way streak-plate technique

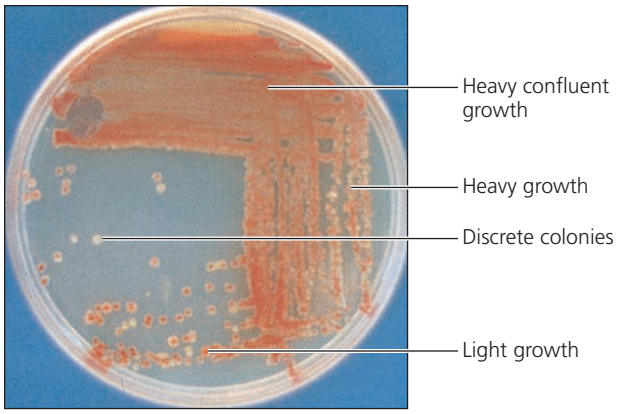


Figure 2.2 Four-way streak-plate inoculation with *Serratia marcescens*

d. Without reflaming the loop, again turn the dish 90° and then drag the culture from a corner of Area 3 across Area 4, using a wider streak. Don't let the loop touch any of the previously streaked areas. The flaming of the loop at the points indicated is to dilute the culture so that fewer organisms are streaked in each area, resulting in the final desired separation. A photograph of a streak-plate inoculation is shown in Figure 2.2.

2. An alternative streak-plate method is for students new to the laboratory who have yet to master the necessary lab skills that would allow them to use the rapid method listed above. This alternative method involves spreading a loopful of culture over the surface of an agar plate that has the quadrants laid out visibly for quick reference. Refer to Figure 2.3, which illustrates this technique.

a. Using a marker, draw two bisecting lines on the bottom of the Petri dish to divide the plate into 4 equal parts. Label each quadrant 1 through 4, starting with the top right quadrant and labeling counter-clockwise. Sterilizing the loop at the points indicated is to dilute the culture due to fewer organisms available to be streaked into each area, resulting in the final desired separation.

b. Turn the Petri dish over and place a loopful of culture on the agar surface in quadrant 1. Using the edge of the loop and holding the loop at a shallow angle so as not to gouge the agar, quickly spread the bacteria throughout the quadrant.

c. Reflame and cool the loop, and turn the Petri dish 90°. Then touch the loop into an area that has been streaked in quadrant 1 and drag it across the agar into quadrant 2, repeat this twice without flaming the loop.

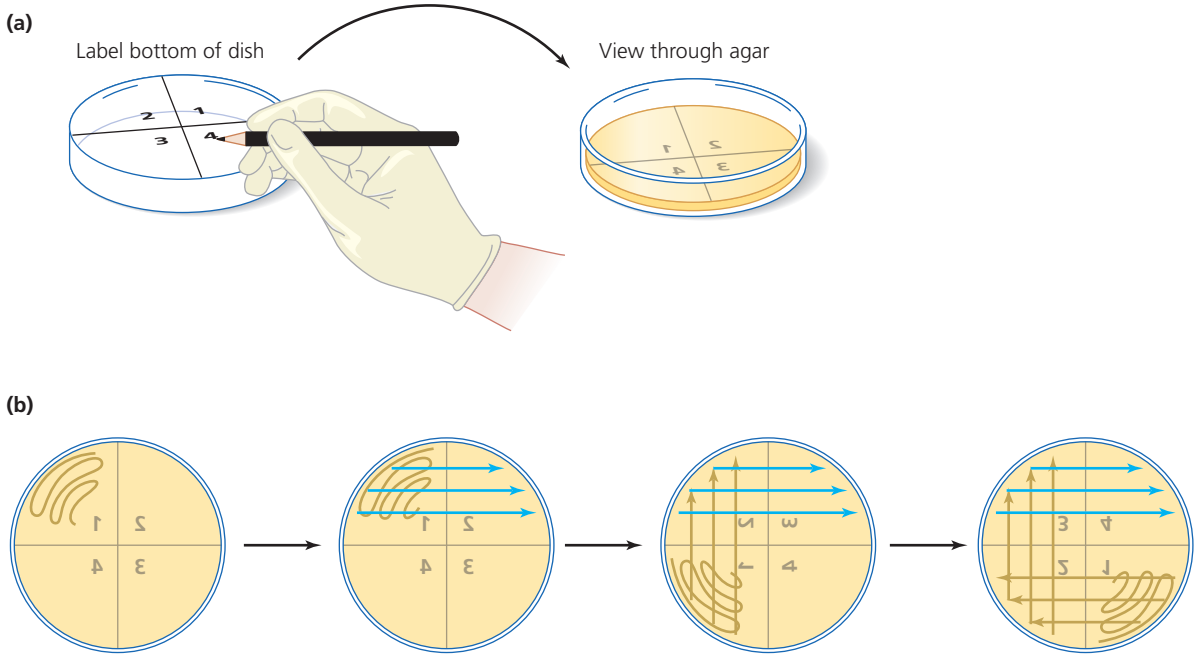


Figure 2.3 Alternate streak-plate method

d. Reflame and cool the loop and again turn the dish 90°. Streak the bacteria into quadrant 3 in the same manner used for quadrant 2.

e. Reflame and cool the loop and again turn the dish 90°. Streak the bacteria into quadrant 4 in the same manner used for quadrant 3.

3. The **spread-plate** technique requires that a previously diluted mixture of microorganisms be used. During inoculation, the cells are spread over the surface of a solid agar medium with a sterile, L-shaped bent glass rod while the Petri dish is spun on a "lazy Susan" turntable. The step-by-step procedure for this technique is as follows:

a. Place the bent glass rod into a beaker and add a sufficient amount of 95% ethyl alcohol to cover the lower, bent portion.

b. Place an appropriately labeled nutrient agar plate on the turntable. With a sterile pipette, place one drop of sterile water on the center of the plate, followed by a sterile loopful of *Micrococcus luteus*. Mix gently with the loop and replace the cover.

c. Remove the glass rod from the beaker, and pass it through the Bunsen burner flame with the bent portion of the rod pointing downward to prevent the burning alcohol from running down your arm. Allow the alcohol to burn off the rod completely. Cool the rod for 10 to 15 seconds.

d. Remove the Petri dish cover and spin the turntable.

e. While the turntable is spinning, lightly touch the sterile bent rod to the surface of the agar and move it back and forth. This will spread the culture over the agar surface.

f. When the turntable comes to a stop, replace the cover. Immerse the rod in alcohol and reflame.

g. In the absence of a turntable, turn the Petri dish manually and spread the culture with the sterile bent glass rod.

4. The **pour-plate** technique requires a serial dilution of the mixed culture by means of a loop or pipette. The diluted inoculum is then added to a molten agar medium in a Petri dish, mixed, and allowed to solidify. The serial dilution and pour-plate procedures are outlined in Experiment 18.

CLINICAL APPLICATION

Isolation of Cultures as a Diagnostic Technique

The isolation of pure cultures is the most important diagnostic tool used in a clinical or research laboratory to uncover the cause of an infection or disease. Before any biochemical or molecular techniques may be used to identify or characterize the causative organism, an individual bacterial colony must be isolated for testing. The isolation of *Staphylococcus aureus* from cultures taken from abscesses or *Streptococcus pyogenes* from a throat culture are two examples of clinical applications of this technique.

AT THE BENCH

Materials

Cultures

24- to 48-hour nutrient broth cultures of a mixture of one part *Serratia marcescens* and three parts *Micrococcus luteus* and a mixture of one part *Escherichia coli* and ten parts *Micrococcus luteus*.

Sources of mixed cultures from the environment could include cultures from a tabletop, bathroom sink, water fountain, or inside of an incubator. Each student should obtain a mixed culture from one of the environmental sources listed above.

Media

Three Trypticase™ soy agar plates per designated student group for each inoculation technique to be performed.

Equipment

Microincinerator or Bunsen burner, inoculating loop, turntable, glassware marking pencil, culture tubes containing 1 ml of sterile water, test tube rack, and sterile cotton swabs.

Procedure Lab One

1. Following the procedures previously described, prepare a spread-plate and/or streak-plate inoculation of each test culture on an appropriately labeled plate.
2. Prepare an environmental mixed culture.
   - **a.** Dampen a sterile cotton swab with sterile water. Wring out the excess water by pressing the wet swab against the walls of the tube.
   - **b.** With the moistened cotton swab, obtain your mixed-culture specimen from one of the selected environmental sources listed in the section on cultures.
   - **c.** Place the contaminated swab back into the tube of sterile water. Mix gently and let stand for 5 minutes.
   - **d.** Perform spread-plate and/or streak-plate inoculation on an appropriately labeled plate.
3. **Incubate all plates in an inverted position** for 48 to 72 hours at 25°C.

Procedure Lab Two

1. Examine all agar plate cultures to identify the distribution of colonies. In the charts provided in Part A of the Lab Report, complete the following:
   - **a.** Draw the distribution of colonies appearing on each of the agar plate cultures.
   - **b.** On each of the agar plate cultures, select two discrete colonies that differ in appearance. Using Figure 3.1 on page 42 as a reference, describe each colony as to its

     Form: Circular, irregular, or spreading.

     Elevation: Flat, slightly raised, or markedly raised.

     Pigmentation.

     Size: Pinpoint, small, medium, or large.
2. Retain the mixed-culture plates to perform Part B of this experiment.

TIPS FOR SUCCESS

1. **An isolation plate has isolated distinct, individual colonies.** If your technique results in isolated colonies in a quadrant that was not the last one to be streaked, that is okay. The point of using this method is to get those individual colonies somewhere on the plate.
2. **Pay attention to how well you sterilize your loop and maintain your aseptic technique.** If the loop is not properly sterilized between streaks, or your aseptic technique is not maintained, the resulting plate will not exhibit a decrease in bacteria leading to individual colonies. With that in mind, if a plate you have streaked or poured does not exhibit a decrease in bacterial colonies area-to-area, you may want to re-examine your technique for maintaining sterilization.

PART B Isolation of Pure Cultures from a Spread-Plate or Streak-Plate Preparation

LEARNING OBJECTIVE

Once you have completed this experiment, you should be able to

1. Prepare a stock culture of an organism using isolates from mixed cultures prepared on an agar streak plate and/or spread plate.

Principle

Once discrete, well-separated colonies develop on the surface of a nutrient agar plate culture, each may be picked up with a sterile needle and transferred to separate nutrient agar slants. Each of these new slant cultures represents the growth of a single bacterial species and is designated as a **pure** or **stock culture**.

CLINICAL APPLICATION

Transferring a Colony of Bacteria Daughter Cells

For identification of a bacterial pathogen, a discrete bacterial colony must be transferred from a streak or spread plate to the new testing media. This new culture will consist of daughter cells that are genetic and metabolic clones of the original bacterial cells that were transferred to the plate. This will allow for identification of the unknown bacterial species through its biochemical and molecular characteristics.

AT THE BENCH

Materials

Cultures

Mixed-culture, nutrient agar streak-plate and/or spread-plate preparations of *S. marcescens* and *M. luteus*, *M. luteus* and *E. coli*, and the environmental specimen plate from Part A.

Media

Four Trypticase™ soy agar slants per designated student group.

Equipment

Microincinerator or Bunsen burner, inoculating needle, and glassware marking pencil.

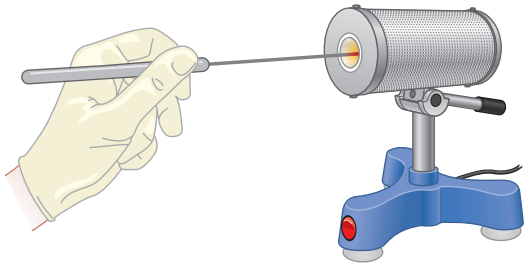
Procedure Lab One

1. Aseptically transfer, from visibly discrete colonies, the yellow *M. luteus*, the white *E. coli*, the red *S. marcescens*, and a discrete colony from the environmental agar plate specimen to the appropriately labeled agar slants as shown in **Figure 2.4**.
2. Incubate all slants at 37°C for 18 to 24 hours.

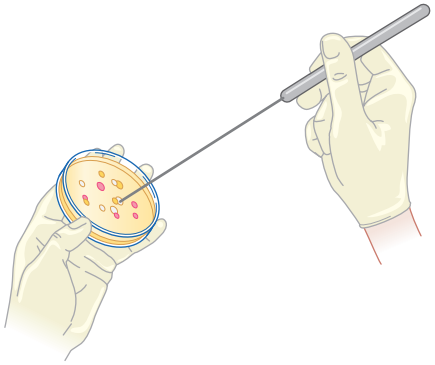
Procedure Lab Two

1. In the chart provided in Part B of the Lab Report, complete the following:
   - **a.** Draw and indicate the type of growth of each pure-culture isolate, using Figure 3.1 on page 42 as a reference.
   - **b.** Observe the color of the growth and record its pigmentation.
   - **c.** Indicate the name of the isolated organisms.

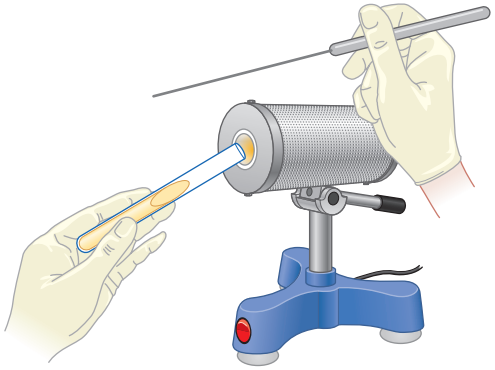
PROCEDURE



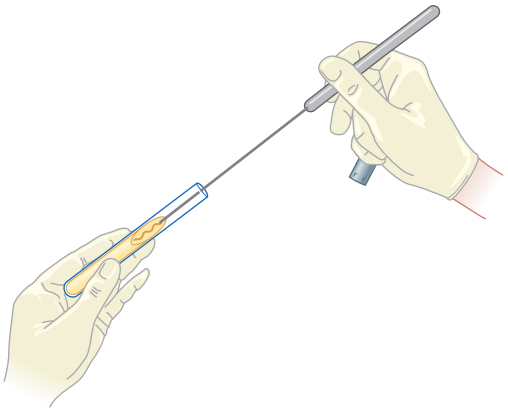
1 Flame the straight needle until the entire wire is red.



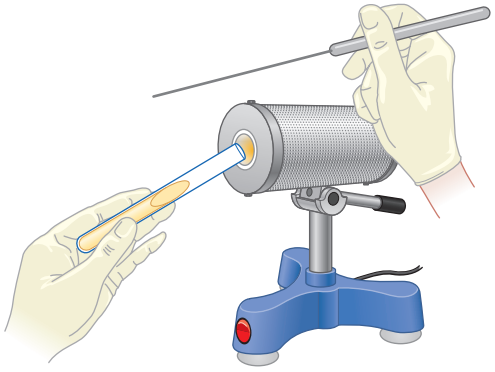
2 After isolating a discrete colony on the agar streak plate, touch the straight needle to the surface of the selected colony.



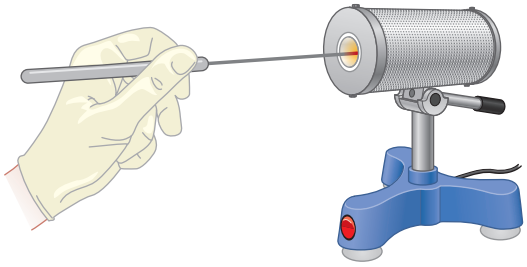
3 Uncap the agar slant and pass the neck of the tube rapidly over the Bunsen burner flame.



4 Inoculate the slant by drawing the needle upward in a zigzag motion along the surface of the agar. Do not dig into the agar.



5 Flame the neck of the tube and recap.



6 Flame the inoculating needle.

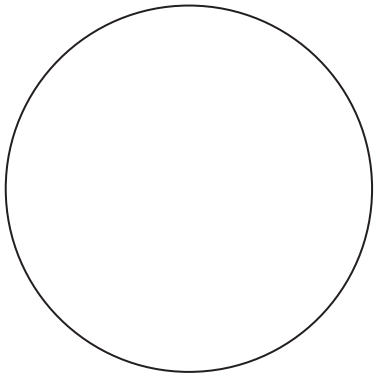
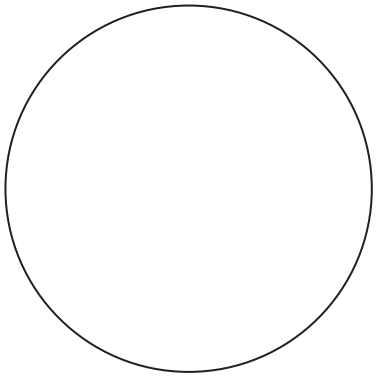
Figure 2.4 **Procedure for the preparation of a pure culture**

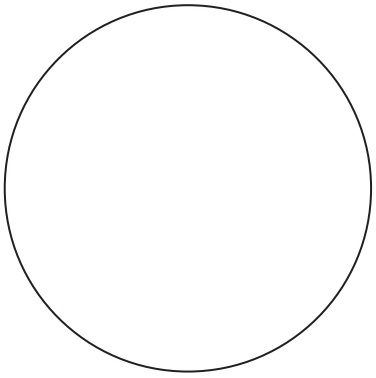
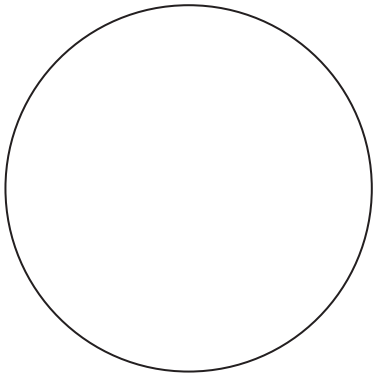
Name: _____

Date: _____ Section: _____

Observations and Results

PART A: Isolation of Discrete Colonies from a Mixed Culture

	STREAK-PLATE TECHNIQUE			
	S. marcescens* and *M. luteus		***M. luteus* and *E. coli***	
Draw the colonies that appear on each agar plate.				
Colony description:	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Form	_____	_____	_____	_____
Elevation	_____	_____	_____	_____
Pigmentation	_____	_____	_____	_____
Size	_____	_____	_____	_____

	ENVIRONMENTAL SPECIMEN	
	Spread-Plate Technique	**Streak-Plate Technique**
Draw the colonies that appear on each agar plate.		
Colony description: Form Elevation Pigmentation Size	_____ _____ _____ _____	_____ _____ _____ _____

PART B: Isolation of Pure Cultures from a Spread-Plate or Streak-Plate Preparation

Draw the distribution of growth on the slant surface.				
Type of growth Pigmentation Name of organism	_____ _____ _____	_____ _____ _____	_____ _____ _____	_____ _____ _____

Review Questions

1. Why is it important to use a sterilized loop between streaks when preparing a streak-plate?

2. Observation of a streak-plate culture shows more growth in Quadrant 4 than in Quadrant 3. Account for this observation.

3. Describe the way in which you can isolate an individual colony from a spread-plate or a streak-plate that holds multiple colonies.

4. Outline the differences between a streak plate and a spread plate.

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Cultural Characteristics of Microorganisms

EXPERIMENT 3

LEARNING OBJECTIVE

Once you have completed this experiment, you should be able to

1. Determine the cultural characteristics of microorganisms as an aid in identifying and classifying organisms into taxonomic groups.

Principle

When grown on a variety of media, microorganisms will exhibit differences in the macroscopic appearance of their growth. These differences, called **cultural characteristics**, are used as a basis for separating microorganisms into taxonomic groups. The cultural characteristics for all known microorganisms are contained in *Bergey's Manual of Systematic Bacteriology.* They are determined by culturing the organisms on nutrient agar slants and plates, in nutrient broth, and in nutrient gelatin. The patterns of growth to be considered in each of these media are described below, and some are illustrated in Figure 3.1.

Nutrient Agar Slants

These have a single straight line of inoculation on the surface and are evaluated in the following manner:

1. **Abundance of growth:** The amount of growth is designated as none, slight, moderate, or large.
2. **Pigmentation:** Chromogenic microorganisms may produce intracellular pigments that are responsible for the coloration of the organisms as seen in surface colonies. Other organisms produce extracellular soluble pigments that are excreted into the medium and also produce a color. Most organisms, however, are nonchromogenic and will appear white to gray.
3. **Optical characteristics:** Optical characteristics may be evaluated on the basis of the amount of light transmitted through the growth. These characteristics are described as **opaque** (no light transmission), **translucent** (partial transmission), or **transparent** (full transmission).
4. **Form:** The appearance of the single-line streak of growth on the agar surface is designated as:
   a. **Filiform:** Continuous, threadlike growth with smooth edges.
   b. **Echinulate:** Continuous, threadlike growth with irregular edges.
   c. **Beaded:** Nonconfluent to semiconfluent colonies.
   d. **Effuse:** Thin, spreading growth.
   e. **Arborescent:** Treelike growth.
   f. **Rhizoid:** Rootlike growth.
5. **Consistency:**
   a. **Dry:** Free from moisture.
   b. **Buttery:** Moist and shiny.
   c. **Mucoid:** Slimy and glistening.

Nutrient Agar Plates

These demonstrate well-isolated colonies and are evaluated in the following manner:

1. **Size:** Pinpoint, small, moderate, or large.
2. **Pigmentation:** Color of colony.
3. **Form:** The shape of the colony is described as follows:
   a. **Circular:** Unbroken, peripheral edge.
   b. **Irregular:** Indented, peripheral edge.
   c. **Rhizoid:** Rootlike, spreading growth.
4. **Margin:** The appearance of the outer edge of the colony is described as follows:
   a. **Entire:** Sharply defined, even.
   b. **Lobate:** Marked indentations.
   c. **Undulate:** Wavy indentations.

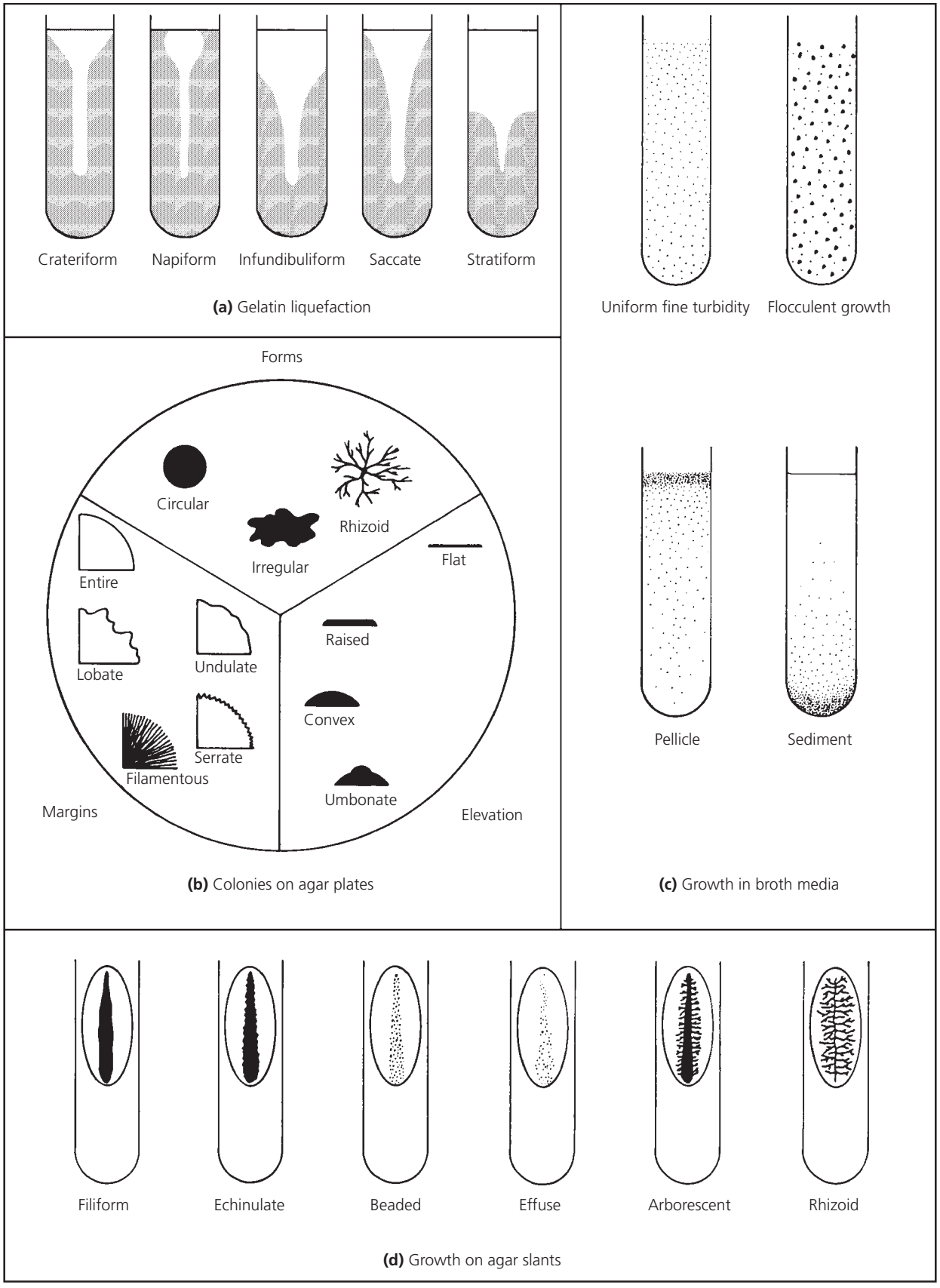


Figure 3.1 **Cultural characteristics of bacteria**

d. **Serrate:** Toothlike appearance.

e. **Filamentous:** Threadlike, spreading edge.

5. **Elevation:** The degree to which colony growth is raised on the agar surface is described as follows:

   a. **Flat:** Elevation not discernible.

   b. **Raised:** Slightly elevated.

   c. **Convex:** Dome-shaped elevation.

   d. **Umbonate:** Raised, with elevated convex central region.

Nutrient Broth Cultures

These are evaluated as to the distribution and appearance of the growth as follows:

1. **Uniform fine turbidity:** Finely dispersed growth throughout.
2. **Flocculent:** Flaky aggregates dispersed throughout.
3. **Pellicle:** Thick, padlike growth on surface.
4. **Sediment:** Concentration of growth at the bottom of broth culture may be granular, flaky, or flocculent.

Nutrient Gelatin

This solid medium may be liquefied by the enzymatic action of gelatinase. Liquefaction occurs in a variety of patterns:

1. **Crateriform:** Liquefied surface area is saucer-shaped.
2. **Napiform:** Bulbous-shaped liquefaction at surface.
3. **Infundibuliform:** Funnel-shaped.
4. **Saccate:** Elongated, tubular.
5. **Stratiform:** Complete liquefaction of the upper half of the medium.

CLINICAL APPLICATION

Examining Colony Growth Characteristics to Aid Identification

Bacterial species each have a characteristic pattern of colony growth in a liquid culture or on a solid medium. While not truly a diagnostic tool, recognition of these patterns of characteristics will aid in a clinical lab setting by helping to minimize the list of potential bacterial species to test for.

AT THE BENCH

Materials

Cultures

Twenty-four–hour nutrient broth cultures of *Pseudomonas aeruginosa* BSL-2, *Bacillus cereus*, *Micrococcus luteus*, and *Escherichia coli*. Seventy-two– to 96-hour Trypticase™ soy broth culture of *Mycobacterium smegmatis*.

Media

Per designated student group: five each of nutrient agar slants, nutrient agar plates, nutrient broth tubes, and nutrient gelatin tubes.

Equipment

Microincinerator or Bunsen burner, inoculating loop and needle, and glassware marking pencil.

Procedure Lab One

1. Using aseptic technique, inoculate each of the appropriately labeled media listed below in the following manner:

   a. Nutrient agar slants: With a sterile needle, make a single-line streak of each of the cultures provided, starting at the butt and drawing the needle up the center of the slanted agar surface.

   b. Nutrient agar plates: With a sterile loop, prepare a streak-plate inoculation of each of the cultures for the isolation of discrete colonies.

   c. Nutrient broth cultures: Using a sterile loop, inoculate each organism into a tube of nutrient broth. Shake the loop a few times to dislodge the inoculum.

   d. Nutrient gelatin: Using a sterile needle, prepare a stab inoculation of each of the cultures provided.

2. Incubate all cultures at 37°C for 24 to 48 hours.

Procedure Lab Two

1. Before beginning observation of all the cultures, place the gelatin cultures in a refrigerator for 30 minutes or in a beaker of crushed ice for a few minutes. The gelatin culture will be the last to be observed.

2. Refer to Figure 3.1 on page 42 and the descriptions presented in the introductory section of Experiment 3 while making the following observations:
   a. Nutrient agar slants: Observe each of the nutrient agar slant cultures for the amount, pigmentation, form, and consistency of the growth. Record your observations in the chart provided in the Lab Report.
   b. Nutrient agar plates: Observe a single, well-isolated colony on each of the nutrient agar plate cultures and identify its size, elevation, margin, form, and pigmentation. Record your observations in the chart provided in the Lab Report.
   c. Nutrient broth cultures: Observe each of the nutrient broth cultures for the appearance of growth (flocculation, turbidity, sediment, or pellicle). Record your observations in the chart provided in the Lab Report.
   d. Nutrient gelatin: Remove gelatin cultures from the refrigerator or beaker of crushed ice, and observe whether liquefaction of the medium has developed and whether the organism has produced gelatinase. Record your observations in the chart provided in the Lab Report.

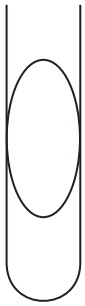
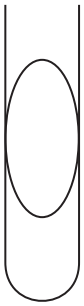
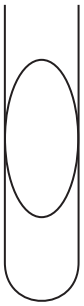
EXPERIMENT
3
Lab Report

Name: _____

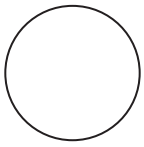
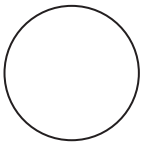
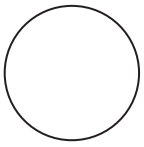
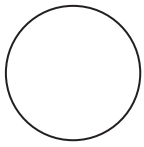
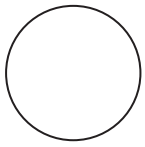
Date: _____ Section: _____

Observations and Results

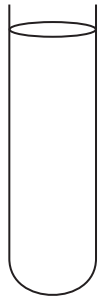
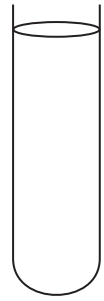
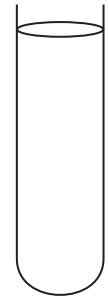
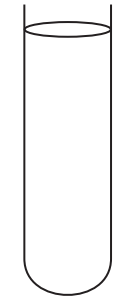
Nutrient Agar Slants

	NUTRIENT AGAR SLANT CULTURES				
	M. luteus	***P. aeruginosa***	***M. smegmatis***	***E. coli***	***B. cereus***
Draw the distribution of growth on the slant surface.					
Amount of growth	_____	_____	_____	_____	_____
Pigmentation	_____	_____	_____	_____	_____
Form	_____	_____	_____	_____	_____
Consistency	_____	_____	_____	_____	_____

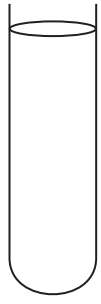
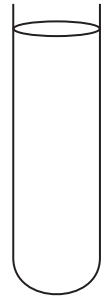
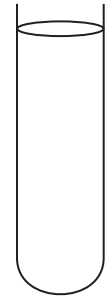
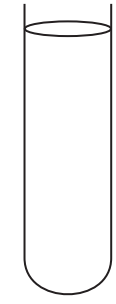
Nutrient Agar Plates

	NUTRIENT AGAR PLATES				
	M. luteus	***P. aeruginosa***	***M. smegmatis***	***E. coli***	***B. cereus***
Draw distribution of colonies.					
Size	_____	_____	_____	_____	_____
Elevation	_____	_____	_____	_____	_____
Margin	_____	_____	_____	_____	_____
Form	_____	_____	_____	_____	_____
Pigmentation	_____	_____	_____	_____	_____

Nutrient Broth Cultures

	NUTRIENT BROTH CULTURES				
	M. luteus	***P. aeruginosa***	***M. smegmatis***	***E. coli***	***B. cereus***
Draw the distribution of growth.					
Appearance of growth	_____	_____	_____	_____	_____

Nutrient Gelatin

	NUTRIENT GELATIN CULTURES				
	M. luteus	***P. aeruginosa***	***M. smegmatis***	***E. coli***	***B. cereus***
Draw liquefaction patterns.					
Liquefaction (+) or (–)	_____	_____	_____	_____	_____
Type of liquefaction	_____	_____	_____	_____	_____

PART 2

Microscopy

LEARNING OBJECTIVES

Once you have completed the experiments in this section, you should be

1. Familiar with the history and diversity of microscopic instruments.
2. Able to understand the components, use, and care of the brightfield microscope.
3. Able to correctly use the microscope for observation and measurement of microorganisms.

Introduction

Microbiology, the branch of science that has so vastly extended and expanded our knowledge of the living world, owes its existence to Antoni van Leeuwenhoek. In 1673, with the aid of a crude microscope consisting of a biconcave lens enclosed in two metal plates, Leeuwenhoek introduced the world to the existence of microbial forms of life. Over the years, microscopes have evolved from the simple, single-lens instrument of Leeuwenhoek, with a magnification of 300×, to the present-day electron microscopes capable of magnifications greater than 250,000×.

Microscopes are designated as either light microscopes or electron microscopes. The former use visible light or ultraviolet rays to illuminate specimens. They include brightfield, darkfield, phase-contrast, and fluorescent instruments. Fluorescent microscopes use ultraviolet radiations whose wavelengths are shorter than those of visible light and are not directly perceptible to the human eye. Electron microscopes use electron beams (instead of light rays) and magnets (instead of lenses) to observe submicroscopic particles.

Essential Features of Various Microscopes

Brightfield Microscope This instrument contains two-lens systems for magnifying specimens: the ocular lens in the eyepiece and the objective lens located in the nosepiece. The specimen is illuminated by a beam of tungsten light focused on it by a substage lens called a condenser; the result is a specimen that appears dark against a bright background. A major limitation of this system is the absence of contrast between the specimen and the surrounding medium, which makes it difficult to observe living cells. Therefore, most brightfield observations are performed on nonviable, stained preparations.

Darkfield Microscope This is similar to the ordinary light microscope; however, the condenser system is modified so that the specimen is not illuminated directly. The condenser directs the light obliquely so that the light is deflected or scattered from the specimen, which then appears bright against a dark background. Living specimens may be observed more readily with darkfield than with brightfield microscopy.

Phase-Contrast Microscope Observation of microorganisms in an unstained state is possible with this microscope. The optics include special objectives and a condenser that make visible cellular components that differ only slightly in their refractive indexes. As light is transmitted through a specimen with a refractive index different from that of the surrounding medium, a portion of the light is refracted (bent) due to slight variations in density and thickness of the cellular components. The special optics convert the difference between transmitted light and refracted rays, resulting in a significant variation in the intensity of light and thereby producing a discernible image of the structure under study. The image appears dark against a light background.

Fluorescent Microscope This microscope is used most frequently to visualize specimens that are chemically tagged with a fluorescent dye. The source of illumination is an ultraviolet (UV) light obtained from a high-pressure mercury lamp or hydrogen quartz lamp. The ocular lens is fitted with a filter that permits the longer ultraviolet wavelengths to pass, while the shorter wavelengths are blocked or eliminated. Ultraviolet radiations are absorbed by the fluorescent label, and the energy is re-emitted in the form of a different wavelength in the visible light range. The fluorescent dyes absorb at wavelengths between 230 and 350 nanometers (nm) and emit orange, yellow, or greenish light. This microscope is used primarily for the detection of antigen-antibody reactions. Antibodies are conjugated with a fluorescent dye that becomes excited in the presence of ultraviolet light, and the fluorescent portion of the dye becomes visible against a black background.

Electron Microscope This instrument provides a revolutionary method of microscopy, with magnifications up to 1 million ×. This permits visualization of submicroscopic cellular particles as well as viral agents. In the electron microscope, the specimen is illuminated by a beam of electrons rather than light, and the focusing is carried out by electromagnets instead of a set of optics. These components are sealed in a tube in which a complete vacuum is established. Transmission electron microscopes require specimens that are prepared as thin filaments, fixed and dehydrated for the electron beam to pass freely through them. As the electrons pass through the specimen, images are formed by directing the electrons onto photographic film, thus making internal cellular structures visible. Scanning electron microscopes are used for visualizing surface characteristics rather than intracellular structures. A narrow beam of electrons scans back and forth, producing a three-dimensional image as the electrons are reflected off the specimen's surface.

While scientists have a variety of optical instruments with which to perform routine laboratory procedures and sophisticated research, the compound brightfield microscope is the "workhorse" and is commonly found in all biological laboratories. Although you should be familiar with the basic principles of microscopy, you probably have not been exposed to this diverse array of complex and expensive equipment. Therefore, only the compound brightfield microscope will be discussed in depth and used to examine specimens.

Microscopic Examination of Stained Cell Preparations

EXPERIMENT 4

LEARNING OBJECTIVES

Once you have completed this experiment, you should be familiar with them

1. Theoretical principles of brightfield microscopy.
2. Component parts of the compound microscope.
3. Use and care of the compound microscope.
4. Practical use of the compound microscope for visualization of cellular morphology from stained slide preparations.

Principle

Microbiology is a science that studies living organisms that are too small to be seen with the naked eye. Needless to say, such a study must involve the use of a good compound microscope. Although there are many types and variations, they all fundamentally consist of a two-lens system, a variable but controllable light source, and mechanical adjustable parts for determining focal length between the lenses and specimen (Figure 4.1).

Components of the Microscope

Stage A fixed platform with an opening in the center allows the passage of light from an illuminating source below to the lens system above the stage. This platform provides a surface for the placement of a slide with its specimen over the central opening. In addition to the fixed stage, most microscopes have a **mechanical stage** that can be moved vertically or horizontally by means of adjustment controls. Less sophisticated microscopes have clips on the fixed stage, and the slide must be positioned manually over the central opening.

Illumination The light source is positioned in the base of the instrument. Some microscopes are equipped with a built-in light source to provide direct illumination. Others are provided with a reversible mirror that has one side flat and the other concave. An external light source, such as a lamp, is placed in front of the mirror to direct the light upward into the lens system. The flat side of the mirror is used for artificial light, and the concave side for sunlight.

Abbé Condenser This component is found directly under the stage and contains two sets of lenses that collect and concentrate light as it passes upward from the light source into the lens systems. The condenser is equipped with an **iris diaphragm**, a shutter controlled by a lever that is used to regulate the amount of light entering the lens system.

Body Tube Above the stage and attached to the arm of the microscope is the body tube. This structure houses the lens system that magnifies the specimen. The upper end of the tube contains the **ocular** or **eyepiece** lens. The lower portion consists of a movable **nosepiece** containing the **objective lenses**. Rotation of the nosepiece positions objectives above the stage opening. The body tube may be raised or lowered with the aid of **coarse-adjustment** and **fine-adjustment knobs** that are located above or below the stage, depending on the type and make of the instrument.

Theoretical Principles of Microscopy

To use the microscope efficiently and with minimal frustration, you should understand the basic principles of microscopy: magnification, resolution, numerical aperture, illumination, and focusing.

Magnification Enlargement, or magnification, of a specimen is the function of a two-lens system; the **ocular lens** is found in the eyepiece, and the **objective lens** is situated in a revolving nosepiece. These lenses are separated by the **body tube**. The objective lens is nearer the specimen and magnifies it, producing the **real image** that is projected up into the focal plane and then magnified by the ocular lens to produce the final image.

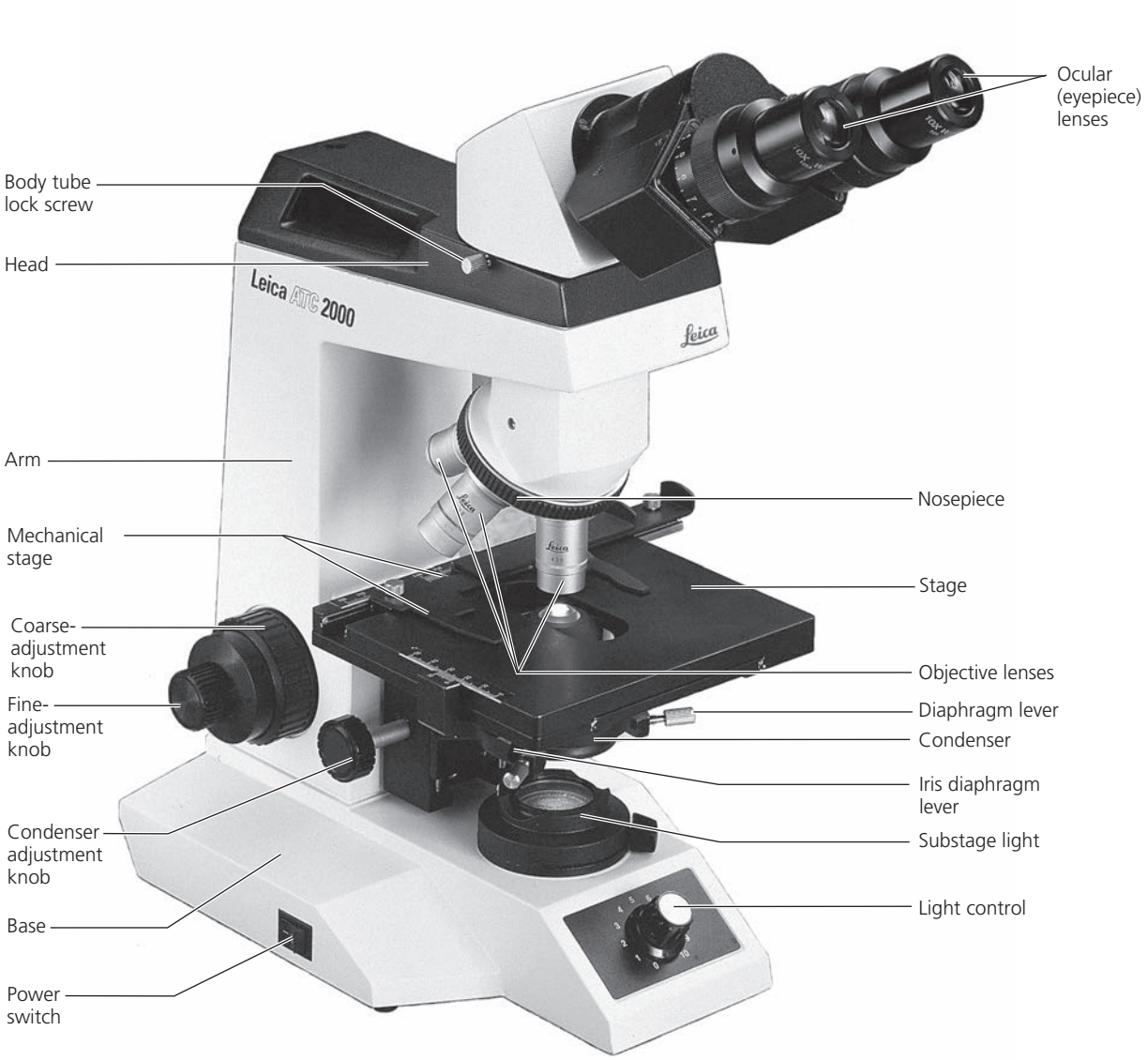


Figure 4.1 **A compound microscope**

The most commonly used microscopes are equipped with a revolving nosepiece containing four objective lenses, each possessing a different degree of magnification. When these are combined with the magnification of the ocular lens, the total or overall linear magnification of the specimen is obtained. This is shown in **Table 4.1**.

Resolving Power or Resolution Although magnification is important, you must be aware that unlimited enlargement is not possible by merely increasing the magnifying power of the lenses or by using additional lenses, because lenses are limited by a property called **resolving power**. By definition, resolving power is how far apart two adjacent objects must be before a given lens shows them as discrete entities. When a lens cannot discriminate, that is, when the two objects appear as one, it has lost resolution. Increased magnification will not rectify the loss and will, in fact, blur the object. The resolving power of a lens is dependent on the wavelength of light used and the **numerical aperture**, which is a characteristic of each lens and imprinted on each objective. The numerical aperture is defined as a function of the diameter of the objective lens in relation to its focal length. It is doubled by use of the substage condenser, which illuminates the object with rays of light that pass through the specimen obliquely as well as directly. Thus, resolving power is expressed mathematically as follows:

$$\text{resolving power} = \frac{\text{wavelength of light}}{2 \times \text{numerical aperture}}$$

TABLE 4.1 Overall Linear Magnification

MAGNIFICATION		TOTAL MAGNIFICATION
OBJECTIVE LENSES	OCULAR LENS	OBJECTIVE MULTIPLIED BY OCULAR
Scanning 4×	10×	40×
Low-power 10×	10×	100×
High-power 40×	10×	400×
Oil-immersion 100×	10×	1000×

Based on this formula, the shorter the wavelength, the greater the resolving power of the lens. Thus, for the same numerical aperture, short wavelengths of the electromagnetic spectrum are better suited for higher resolution than are longer wavelengths.

However, as with magnification, resolving power also has limits. Decreasing the wavelength will not automatically increase the resolving power of a lens, because the visible portion of the electromagnetic spectrum is very narrow and borders on the very short wavelengths found in the ultraviolet portion of the spectrum.

The relationship between wavelength and numerical aperture is valid only for increased resolving power when light rays are parallel. Therefore, the resolving power is also dependent on another factor, the **refractive index**. This is the bending power of light passing through air from the glass slide to the objective lens. The refractive index of air is lower than that of glass; as light rays pass from the glass slide into the air, they are bent or refracted so that they do not pass into the objective lens. This would cause a loss of light, which would reduce the numerical aperture and diminish the resolving power of the objective lens. Loss of refracted light can be compensated for by interposing mineral oil, which has the same refractive index as glass, between the slide and the objective lens. In this way, decreased light refraction occurs and more light rays enter directly into the objective lens, producing a vivid image with high resolution (Figure 4.2).

Illumination Effective illumination is required for efficient magnification and resolving power. Since the intensity of daylight is an uncontrolled variable, artificial light from a tungsten lamp is the most commonly used light source in microscopy. The light is passed through the condenser located beneath the stage. The condenser contains two lenses that are necessary to produce a maximum numerical aperture. The height of the condenser can be adjusted with the **condenser knob**. Always keep the condenser close to the stage, especially when using the oil-immersion objective.

Between the light source and the condenser is the iris diaphragm, which can be opened and closed by means of a lever, thereby regulating the amount of light entering the condenser. Excessive illumination may actually obscure the specimen because of lack of contrast. The amount of light entering the microscope differs with each objective lens used. A rule of thumb is that *as the magnification of the lens increases, the distance between the objective lens and slide, called working distance, decreases, whereas the numerical aperture of the objective lens increases* (Figure 4.3).

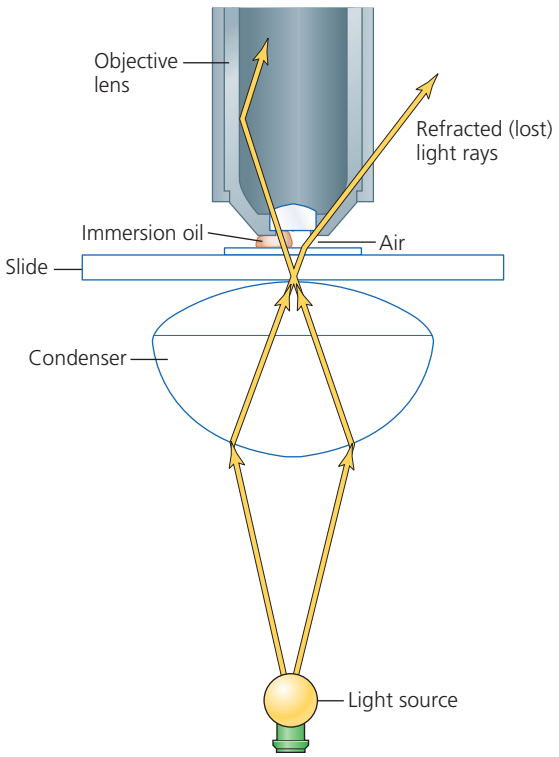


Figure 4.2 Refractive index in air and in mineral oil

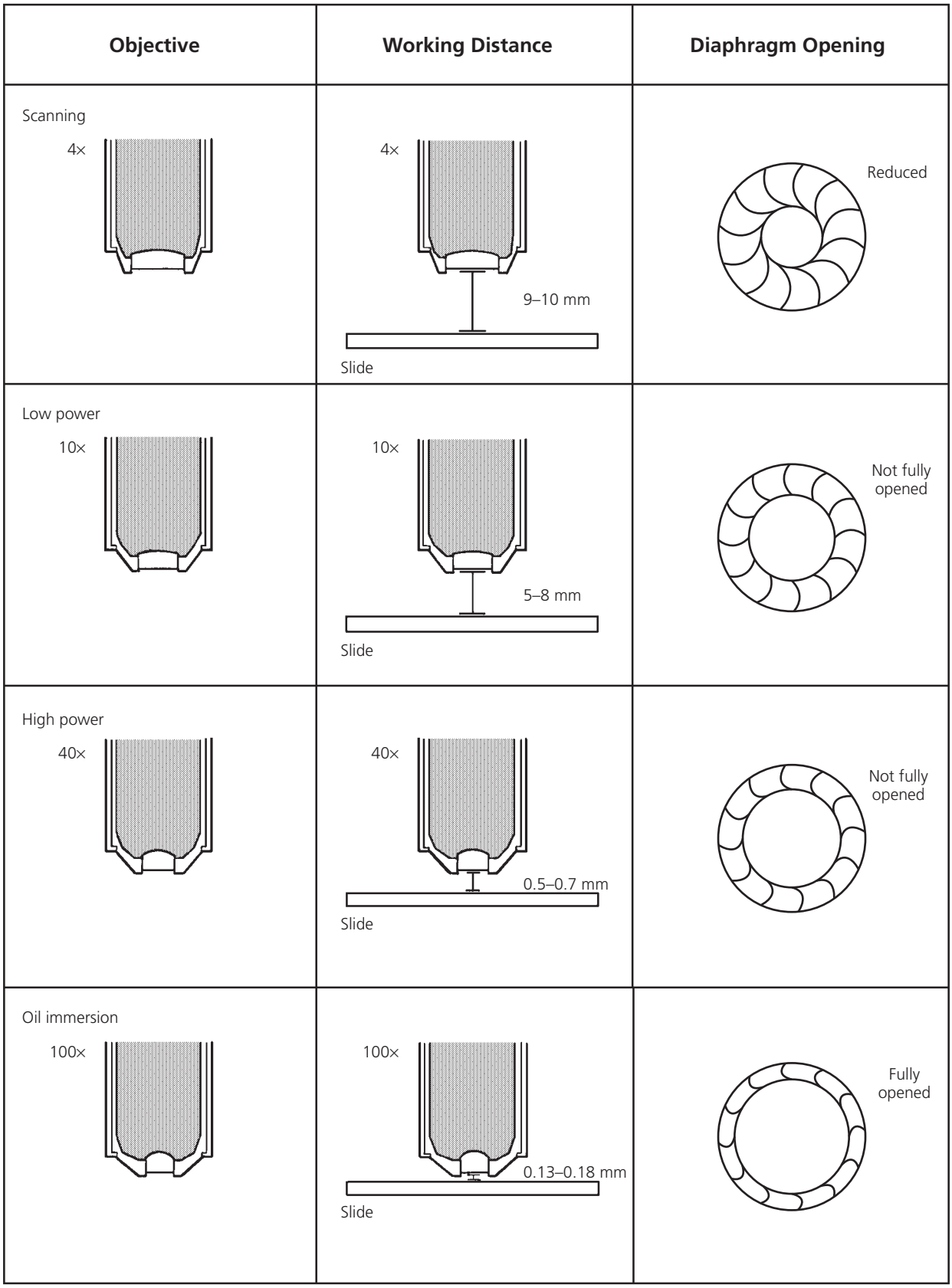


Figure 4.3 **Relationship between working distance, objective, and diaphragm opening**

Use and Care of the Microscope

You will be responsible for the proper care and use of microscopes. Since microscopes are expensive, you must observe the following regulations and procedures.

The instruments are housed in special cabinets and must be moved by users to their laboratory benches. The correct and only acceptable way to do this is to grip the microscope arm firmly with the right hand and the base with the left hand, and lift the instrument from the cabinet shelf. Carry it close to the body and gently place it on the laboratory bench. This will prevent collision with furniture or coworkers and will protect the instrument against damage.

Once the microscope is placed on the laboratory bench, observe the following rules:

1. Remove all unnecessary materials (including books, papers, purses, and hats) from the laboratory bench.
2. Uncoil the microscope's electric cord and plug it into an electrical outlet.
3. Clean all lens systems; the smallest bit of dust, oil, lint, or eyelash will decrease the efficiency of the microscope. The ocular, scanning, low-power, and high-power lenses may be cleaned by wiping several times with acceptable lens tissue. Never use paper toweling or cloth on a lens surface. If the oil-immersion lens is gummy or tacky, a piece of lens paper moistened with xylol is used to wipe it clean. The xylol is immediately removed with a tissue moistened with 95% alcohol, and the lens is wiped dry with lens paper. *Note: This xylol cleansing procedure should be performed only by the instructor and only if necessary; consistent use of xylol may loosen the lens.*

The following routine procedures must be followed to ensure correct and efficient use of the microscope.

1. Place the microscope slide with the specimen within the stage clips on the fixed stage. Move the slide to center the specimen over the opening in the stage directly over the light source.
2. Raise the microscope stage up as far as it will go. Rotate the scanning lens or low-power lens into position. Lower the body tube with the coarse-adjustment knob to its lowest position. *Note: Never lower the body tube while looking through the ocular lens.*
3. While looking through the ocular lens, use the fine-adjustment knob, rotating it back and forth slightly, to bring the specimen into sharp focus.
4. Adjust the substage condenser to achieve optimal focus.
5. Routinely adjust the light source by means of the light-source transformer setting, and/or the iris diaphragm, for optimum illumination for each new slide and for each change in magnification.
6. Most microscopes are **parfocal**, which means that when one lens is in focus, other lenses will also have the same focal length and can be rotated into position without further major adjustment. In practice, however, usually a half-turn of the fine-adjustment knob in either direction is necessary for sharp focus.
7. Once you have brought the specimen into sharp focus with a low-powered lens, preparation may be made for visualizing the specimen under oil immersion. Place a drop of oil on the slide directly over the area to be viewed. Rotate the nosepiece until the oil-immersion objective locks into position. *Note: Care should be taken not to allow the high-power objective to touch the drop of oil.* The slide is observed from the side as the objective is rotated slowly into position. This will ensure that the objective will be properly immersed in the oil. The fine-adjustment knob is readjusted to bring the image into sharp focus.
8. During microscopic examination of microbial organisms, it is always necessary to observe several areas of the preparation. This is accomplished by scanning the slide without the application of additional immersion oil. *Note: This will require continuous, very fine adjustments by the slow, back-and-forth rotation of the fine-adjustment knob only.*

On completion of the laboratory exercise, return the microscope to its cabinet in its original condition. The following steps are recommended:

1. Clean all lenses with dry, clean lens paper. *Note: Use xylol to remove oil from the stage only.*
2. Place the low-power objective in position and lower the body tube completely.
3. Center the mechanical stage.
4. Coil the electric cord around the body tube and the stage.
5. Carry the microscope to its position in its cabinet in the manner previously described.

CLINICAL APPLICATION

Using Microscopic Examination in the Diagnosis of Tuberculosis

The visualization of stained bacterial cells using a compound light microscope can be the first step in diagnosing microbial infections. For example, a rapid diagnosis for tuberculosis can be made by identifying the unique characteristics of *Mycobacterium tuberculosis* in a stained sample of patient sputum.

AT THE BENCH

Materials

Slides

Commercially prepared slides of *Staphylococcus aureus*, *Bacillus subtilis*, *Aquaspirillum itersonii*, and other alternate slides.

Equipment

Compound microscope, lens paper, and immersion oil.

Procedure

1. Review the parts of the microscope, making sure you know the names and understand the function of each of these components.
2. Review instructions for the use of the microscope, giving special attention to the use of the oil-immersion objective.
3. Examine the prepared slides, noting the shapes and the relative sizes of the cells under the high-power (also called high-dry, because it is the highest power that does not use oil) and oil-immersion objectives.
4. Record your observations in the Lab Report.

EXPERIMENT
4

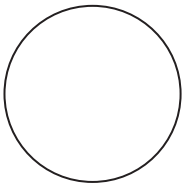
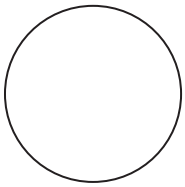
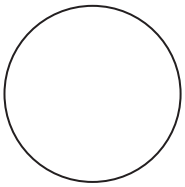
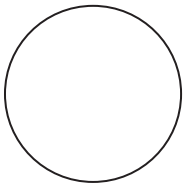
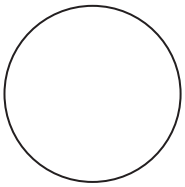
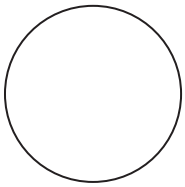
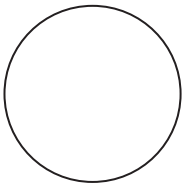
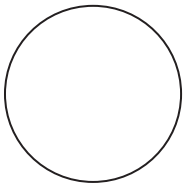
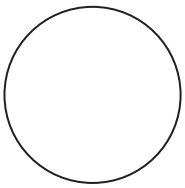
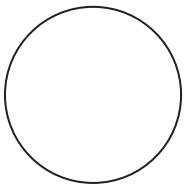
Lab Report

Name: _____

Date: _____ Section: _____

Observations and Results

Draw several cells from a typical microscopic field as viewed under each magnification, and give the total magnification for each objective.

	High Power	Oil Immersion
S. aureus Magnification	 _____	 _____
B. subtilis Magnification	 _____	 _____
S. cerevisiae Magnification	 _____	 _____
_____ Magnification	 _____	 _____
_____ Magnification	 _____	 _____

Review Questions

1. How is xylol used to clean the lens of a microscope? What may happen if xylol is constantly used for this purpose?

2. For what purpose would you adjust each of the following microscope components during a microscopy exercise?

a. Iris diaphragm:

b. Coarse-adjustment knob:

c. Fine-adjustment knob:

d. Condenser:

e. Mechanical stage control:

3.  As a beginning student in the microbiology laboratory, you experience some difficulties in using the oil-immersion lens. Describe the steps you would take to correct the following problems:

a. Inability to bring the specimen into sharp focus.

b. Insufficient light while viewing the specimen.

c. Artifacts in the microscopic field.