Microbiological Procedures

Notes for Lecture and Laboratory

Prepared for Micro 3053
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1 WHAT IS “SCIENCE?”

The Meaning of “Science”

The term *science* has come to mean several things in popular usage. For example, many students are “good at science,” usually meaning that they can memorize and understand the information presented in science classes. Another common usage is where *science* is used to describe the general activities performed at so-called scientific institutions. Some may claim that *science* is the search for truth. Although these views touch on things scientific, the proper definition of *science* is that *science is an investigative method for understanding the world around us by evaluating evidence through reason*. Evidence is collected by observing phenomena that may occur naturally or through experimental procedures or surveys. The evidence is analyzed within a systematic and logical framework, generally termed the *scientific method*.

In the scientific method, initial observations are used as the basis for making testable explanations or *hypotheses*. The validity of a hypothesis is tested with additional observations. If the new observations do not agree with the hypothesis, it is rejected and a new one developed. If the new observations support the hypothesis, it is accepted, but it is always susceptible to additional testing. These steps are summarized in Figure 1.1.

![Figure 1.1. Steps in the scientific method.](image)

Microbiological Procedures 1
Recognize that although hypotheses can never be proven, they can be disproved. It may sound contradictory, but scientific progress is made by disproving false hypotheses.

Hypotheses that stand the test of time are usually considered theories. In addition to theories, which provide explanations of phenomena, there are also laws, which summarize a large number of individual facts.

**The Structure of Science**

The logical approach to understanding nature, the *scientific method*, has its roots in ancient Greece. Aristotle invented the formal concept of deductive logic where consequences can be predicted from generalizations; the foundation of deductive logic is to draw conclusions based on premises. The classic example of this:

- All men are mortal. \textit{(premise 1)}
- Socrates is a man. \textit{(premise 2)}
- Socrates is mortal. \textit{(conclusion)}

Notice that the truth of the premises is not considered. They are assumed to be true, with the conclusion following these assumptions. This was the earliest form of logic and arose in opposition to myth. Myths are useful fictions used to provide explanations while maintaining established social orders and recognizing the mystery of the universe. Logic allows truth to be demonstrated and debated.

Aristotle’s approach was to draw specific conclusions from general observations. This was challenged by Francis Bacon in the seventeenth century. Bacon introduced *inductive logic*, where general rules are inferred from specific observations. That is, assumptions about a whole group is based on observing a few of its members. For example, we infer taxonomic traits for all white pines based on observations of a few individuals. Induction is the basis of the premises listed above to illustrate deductive logic. Are all men mortal? More people are alive today than existed in the past. That means that the majority of people who were born are still alive! So, why do we believe that all men are mortal? The problem with inductive logic is that there is really no justification for believing the conclusions. However, induction is something we have learned to rely on as a part of everyday life and in science it continues to be a useful tool.

At the same time Bacon was thinking about logic, Galileo developed the idea of a *controlled experiment*. In an experiment designed to study a specific phenomenon all of the variables except the one being investigated are controlled. For example, to investigate the effect of a fertilizer on your rose garden, a simple experiment would be to mix fertilizer into the soil and wait to see if more roses are produced than in the past. But, how would one know if a difference was due to the fertilizer and not to something else, like the weather? A better experiment, where the effect of weather is controlled, would be to fertilize one portion of the rose garden and to leave another portion unamended. Since both portions are exposed to the same weather, it was removed as a variable. Any difference in the yield of roses between the two plots could not be due to the weather and therefore, might support fertilizer as the cause. Of course, other variables, such as watering, would have to be controlled as well.
Karl Popper introduced the concept of **falsification**. Popper believed that nature is objective and that accurate details could be determined through science. Falsification relies on constructing a hypothesis and then looking for evidence to falsify it. The hypothesis that withstands falsification attempts best is accepted. Related to this is **Occam’s Razor**, which suggests that “it is wasteful to do with more that which can be done with less.” In other words, the simplest explanation is the one to choose.

Isaac Newton introduced the **mathematical model**, where a physical phenomenon is described in terms of mathematical relationships. A good model helps summarize and describe phenomena and is an aid to making predictions. Models can have tremendous value, but their construction requires large amounts of data, determination of the key variables, and elucidation of the relationships between those variables. The equation that describes bacterial growth in exponential phase, \( N = N_0 2^n \), is a straightforward model of what to expect during a particular growth mode. On the other hand, a model describing weather patterns contains many more variables, the relationships of which are not always clear, sometimes resulting in inaccurate predictions. A good place to observe modeling in action is a racetrack; finding people trying to predict future performance using variables from prior races is easy.

In addition to using the scientific method, the scientific community also places social requirements on scientists. A peer review system evaluates the merit and validity of scientific investigations according to the following criteria:

1. Scientific results should be original. Although it does not hurt to corroborate hypotheses, only novel results are worth reporting. (Most lab classes perform **exercises** rather than experiments, so that specific results can be anticipated. Exercises are most useful for learning new techniques.)

2. The scientist should be free of bias regarding the outcome of the problems investigated. The validity of the data should be the main concern, not whether or not they support a pet hypothesis. In other words, no motivation should be operating other than the desire to understand a phenomenon.

3. Results and conclusions should stand on their own merit, without relying on social, ethnic, religious, or personal factors to gain acceptance.

4. Scientific statements are not to be accepted on faith, but are to be subjected to close scrutiny and falsification attempts. This is why the scientist must play her own Devil’s advocate, trying to disprove hypotheses before going public.

5. Scientific knowledge, including methodology, should be freely available to the public. It is critical that observations can be replicated by anyone with the necessary technical expertise.

6. The language used to describe all aspects of research must be precise and unambiguous. In science, a report is not subject to interpretation, but must be accepted or rejected solely on the information it presents. (“Say what you mean and mean what you say.”)
Interestingly, even when all of the conditions outlined above are met, introducing information that contradicts existing paradigms is difficult. For example, in the late 1970's when Carl Woese determined that rRNA sequences suggested three evolutionary lines (two prokaryotic, the Bacteria and Archaea, and one eukaryotic, the Eukarya), there was a tremendous amount of resistance from established scientists who had not evaluated the data but had learned and accepted Whitaker’s five Kingdom scheme. There followed a period of rigorous testing in many labs, all lending support to Woese’s conclusions. Today, the three Domain scheme, based on evolutionary relatedness of organisms, is considered correct. (Of course, technological refinements and increasing phylogenetic information continue to “fine tune” the original scheme.) The reaction that Woese faced is common. It is not unusual for a controversial hypothesis to be ignored by the established scientific community. Very often acceptance is not based on the strength of the data, but also requires a shift in paradigms arising from a change in the scientific order, frequently through the demise of older scientists.

**How is Science Done?**

Science is done by testing hypotheses. The concept of a hypothesis is often unclear, so before proceeding, let’s define a hypothesis as a testable explanation for an observed phenomenon. Many times people will define a hypothesis as a prediction or an educated guess, which are not really incorrect, only less precise, in regard to science, than our definition. Predictions play an important part in hypothesis testing, but do not explain a phenomenon. Science requires very precise, unambiguous language, as will be discussed later.

The scientific method requires postulating and testing hypotheses. Hypotheses are tested using deductive logic (if . . ., then . . .) applied to predictions. This approach assumes that there is order in the universe. That is, identical conditions will produce identical results. An important point to recognize is that true predictions may arise from true or false hypotheses. For example, consider the joke where a frog is told to jump. After jumping, one limb is removed and the frog jumps only half the distance. Removal of another limb halves the distance again. Removal of the third limb halves the previous distance. Finally, with all limbs removed, the frog doesn’t jump at all, no matter how loudly the scientist yells. **Hypothesis:** Without legs, a frog becomes deaf. **Prediction:** No amount of noise will make a deaf (limbless) frog jump. Therefore, it is essential (not just important) to try and falsify a hypothesis and determine if alternative hypotheses exist that explain the data better.

Scientific advancement depends on rejecting (not accepting) hypotheses. Acceptance of a hypothesis is always conditional, subject to being refuted at some later time. So, **nothing can ever be proven.** Good scientific research may solve a particularly important problem or produce results from which generalizations can be made.

In any investigation (testing of hypotheses), it is important to control the influence of the factors not being examined. That is, it is desirable that any effects observed be due to the treatment being tested.

*If a hypothesis cannot be tested, the scientific method cannot be employed and the hypothesis lies outside the realm of science.* Such a hypothesis is not necessarily false, only nonscientific.
Making Observations

Observation is one of the foundations of science. The first step in any scientific investigation is to make an observation or series of observations that can be examined for an explanation (Figure 1.1). There are three ways that observations are made:

1) ordered observations,
2) surveys,
3) experimentation.

Selection of the appropriate strategy or combination of strategies depends on the information sought and the system being studied.

Ordered Observations
Ordered observations are the most common type of observation used by field scientists. Consider a rainforest. An alert observer has no problem finding many examples of species diversity or symbiotic relationships. In fact, the amount of information available is so overwhelming, the observer is forced to ask precise questions and narrow observations to those that bear upon the specific questions. That is the fundamental nature of ordered observations. Not only does the scientist try to answer questions in a specific order, but he also tries to avoid interfering with what is being observed. In other words, it is impossible to study natural behaviors or relationships by placing the subjects into unnatural situations.

Surveys
Unlike ordered observations, surveys require interactions between the observer (surveyor) and the subjects being studied. Surveys are common in, although not limited to, social sciences. The results of surveys can be seen on television news programs everyday. However, surveys are not limited to polls.

There are three basic forms of survey:

1) retrospective
2) current
3) prospective.

A retrospective survey gathers information from the past, for example, historical data or fossil records. The biggest problem with retrospective surveys is that data must be accepted as is. That means that information can be confounded by uncontrolled factors and that older information can be sketchy and less reliable. Consider constructing a family tree. The information used is limited to what’s available (documents, personal recollections), which could be biased and contain gaps.

A current survey investigates something as it is now, for example, student performance within a
class. Although the information is still subject to uncontrolled factors, it is usually easier to get complete information (no gaps).

A prospective survey follows the progress of a selected subject into the future. These are common in nutritional studies, for example the effect of fiber on colon cancer or the effect of wine on heart disease.

**Experiments**
In an experiment, the observer sets up a situation so that a phenomenon can be observed without being obscured by extraneous factors. This is the most common type of observation used in the laboratory. Well-constructed experiments allow for the unambiguous observation of specific phenomena, allowing the observer to examine cause and effect. Construction of a good experiment is not a trivial skill and will be explored in detail later this semester.
The Laboratory Notebook

Why Keep a Notebook?
One of the most important tasks a scientist has is to keep an accurate record of experiments. However, maintaining a clear, complete record is easily and commonly neglected. Lab work (except for cleaning glassware) is a lot more fun to do than it is to write about. Unfortunately, experiments that aren’t recorded properly are almost useless.

Good records are important to two people: you and anyone else who reads your lab notebook. For you, good records mean you won’t have to rely on your memory to recall details and could allow you to see patterns in the data. For someone else, good records document your work, allowing it to be repeated and providing support for patents and other credit claims.

A well-kept laboratory notebook saves the researcher time and usually provides the foundation for additional investigations. Faulty memories can lead to repeating the same experiment needlessly, expensive in terms of time and money. Furthermore, data can be misremembered, allowing the investigator to form unsupported hypotheses. Good records allow the scientist (or anyone else) to understand exactly what was done and what was observed, regardless of how long ago the experiment was conducted. An added benefit is that by following a series of experiments, an investigator can often extend the project, asking better, more precise questions. That is, future experiments can be conceived by building on previous knowledge contained in the lab notebook.

The Proper Tools
The value of the lab notebook cannot be overemphasized. As such, it is essential that the notebook itself be sturdy. Pages can be lost too easily from a looseleaf or spiral notebook, or from a notebook with a weak binding. Use a string-bound notebook with pages that are strongly and permanently bound together. The cover should be strong to protect the pages and, when necessary, to provide a stiff writing surface. Pages should be numbered. Although some people prefer unlined pages, horizontal or quad rules are more common. A quality notebook will use acid-free paper to prevent yellowing and flaking. The most expensive notebooks also have coated pages to protect against accidental spills. The coating doesn’t work well with pencil, but that shouldn’t be a concern since records should be kept with indelible ink.

Notebooks are permanent records and must be written with ink. Ballpoint pens are the preferred writing instrument. Their inks are composed of concentrated dyes in organic solvents, usually alcohols. The solvents evaporate almost immediately, leaving sharp lines of fairly permanent pigments. Inks from ballpoint pens are resistant to water and non-polar organic solvents. However, alcohols, such as methanol, can cause the ink to bleed.

Rollerballs, felt-tip pens, and fountain pens use water-based inks. As you might expect, they spread easily if they get wet. Another disadvantage is that the slower evaporation of the solvent (water) allows the ink to diffuse through the paper, so the writing tends to bleed and smudge. Some inks are more light-sensitive than others. Overall, black inks are most permanent; red inks are least permanent.
Sometimes it is necessary to include attachments, for example, photographs, stripcharts, photocopies, or notes recorded away from the notebook. Archival tape that is acid-free and permanent is the best solution, but it is expensive and can be difficult to find. Staples work for short periods but can work loose or tear pages. There are a number of glues or pastes available. They must be permanent and free from chemicals that could degrade paper. Photocopies and laser printer outputs are unstable and can leach onto surrounding papers. They should not come into contact with plastics, including adhesive tape.

Setting Up the Notebook
Professional notebooks start with a signout page that serves as a record of its issue and possession. The next few pages include printed instructions covering legal issues and instructions for making entries. A blank table of contents with spaces for date, title, and page numbers precedes the pages where experiments are recorded. All notebooks should have a table of contents that is filled in as experiments are recorded. Experiments are recorded consecutively in chronological order with no missing or blank pages. In the case where a new experiment is started before another one is completed:

a. Start recording the new experiment on the next blank page.

b. Data from the uncompleted experiment are recorded beginning on the first blank page following the record of the new experiment.

c. The broken records are linked by noting “continued on page . . .” or “continued from page . . .”

Although it is usually neglected, a preface that describes the author and purpose of the notebook is often useful. Typical information includes who is involved in the project (author, supervisors, assistants), the goal of the work, a summary of progress to date, location of the project, and funding. This information can help future researchers and provide support for legal ownership of ideas and inventions.

Ownership is also supported by having each page signed after completion by the author and an uninvolved individual who understands the material. This is a good idea in academic research and is absolutely required in industrial research.

Recording Experiments
The goal in recording an experiment is to be clear, concise, and complete. Clarity requires thinking about things before committing to a permanent record. Concise writing also requires forethought. The idea is to present a straightforward record that is easily understood. Complete records require that information be recorded while it is fresh. If a mistake is made, cross it out with a single line, initial it, and provide a brief explanation. The entry is left legible in case it turns out to be right. Speculation or annotations can be added at the end of the record.

The format for notebook records is essentially the same as that used for a laboratory report or manuscript:

**Title:** The topic of the experiment. Every experiment should be given a title that clearly
expresses the topic. This provides an easy way to find a specific experiment or its continuation.

**Date:** The date the experiment began should be recorded in a prominent location, usually at the top of the first page alongside the title. Without a date, it is difficult, if not impossible, to claim first observation.

**Purpose:** What the experiment is designed to investigate. A statement of purpose is included so that the reader, as well as you, can unambiguously understand what the experiment was designed to investigate. At the very least, the purpose will be described in a single sentence. However, a detailed introduction is clarifies your thinking and makes it easier to write a report. Typically, an introduction provides background information that can help jog your memory or provide the foundation for a reader to understand the experiment. It includes previous knowledge and states an existing problem. The statement of purpose or experimental objective is how you hope to solve that problem.

**Materials and Methods:** What supplies are needed for the experiment and how they are to be used. A description of materials and methods should provide enough information to allow you or any other capable scientist to repeat the experiment. The list of materials should include suppliers and lot numbers. Methods should be described in detail or properly referenced. This section is easiest to read if it’s divided into subsections (e.g., organisms, media, analytical procedures, calculations). **Tip:** Diagram the experiment in a flow chart. Seeing the individual steps will help you estimate the time required and the supplies you’ll consume.

**Results:** Observations made during the experiment. Raw data and observations are recorded in a results section. Data should be recorded as it’s observed. It’s best to set up data tables ahead of time and to fill them in as the experiment proceeds. Observations (e.g., colony morphology, odors, medium color) can be listed; unlike manuscripts, it is not necessary to explain the results in paragraph forms. The primary responsibility is to record the raw data, clearly and completely, but it is a good idea to summarize the data in tables or graphs, similar to how they would be presented in a finished report. This greatly aids manuscript preparation and saves time when going through old experiments.

**Discussion:** Interpretation of the results. Interpretations of the data can be recorded as a list of conclusions, but it is almost always more beneficial to write a detailed discussion that illustrates your thinking. In either case, the conclusions should address the purpose. It’s also appropriate to record conjectures or hypotheses. If possible, additional experiments can be described.

**References:** Literature cited in the previous sections. Reference citations should be complete, as they would be used in a manuscript

**Other Uses**
In addition to recording experiments, notebooks can also be used to record literature surveys or ideas. While it’s probably best to use note cards, a separate notebook, or a computer database for
lengthy literature reviews, your research notebook is appropriate for notes on a few articles directly related to your project. Notebooks that are witnessed and signed help substantiate ownership and time of origin of an idea. There are numerous accounts of notebooks being used to help determine credit and patent rights.

**Electronic Notebooks**

Custom software, spreadsheets, and word processors can all be used to maintain laboratory records. In actual practice, it’s often easier to use software that can perform calculations and plot data than to record data in a notebook, especially since computers are interfaced to lots of lab equipment. However, there are many drawbacks to using an electronic notebook. First and foremost, computer records alone are not accepted for patents or other legal positions; data can be too easily altered or falsified. Furthermore, electronic databases are not secure and several copies can coexist. Worst of all, many people have poor computer habits and can lose a complete record with a single mishap. The best solution is to use computer applications as necessary, but keep a written notebook and back up data frequently.

**Preparation of Scientific Manuscripts and Experimental Lab Reports**

The purpose of making a presentation is because you have something worthwhile to convey. Since the essence of communication is based on what the other person perceives and not what you say, it is critical that you express yourself in a clear, efficient manner designed to simplify the reader’s ability to understand your meaning. The three fundamental aspects for good communication (including science writing) are:

- plan your structure
- consider your audience
- choose your words

Many publications and journals have style guides for the preparation of manuscripts. Although some differences may occur between style guides, the format of a lab report or research manuscript is fairly specific and is made up of the following sections:

- Title
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
- Literature Cited
Subheadings are common within some of the sections, especially the Materials and Methods and the Results sections. Subheadings should help organize information into specific categories that make it easier for the reader to find. For example, Materials and Methods may have subheadings for source of organisms, media, stains, biochemical tests, and statistical analysis. The Results section may have different subheadings, for example cell and colony morphology, biochemical tests, nutritional requirements, growth rates, and DNA homology.

Type the section headings, such as **Introduction**, in boldface, centering them on a line separate from the text body. Subheadings should be in **boldface**, followed by a period, and placed at the beginning of the relevant section.

Science writing is lean and precise. Every sentence has a purpose and each word should be necessary. Metaphor, analogy, hyperbole, and other literary devices are rare. Good grammar and style are essential with such a spare, focused document.

Develop your professional writing skills by comparing your writing with published manuscripts. Pay particular attention to language, style, and organization. Professors, admissions boards, and potential employers will compare your work with others, most especially in regard to how well you demonstrate the clarity of your thinking.

**Title:** The title should be clear, concise, and unambiguous.

**Abstract:** The Abstract is a brief summary of what you found with some detail on how the investigation was performed. State the principle objective(s), provide general descriptions of the methods, summarize the results (use data; if “significant,” give p-values) and state the principle conclusions. In general, background information is kept to a minimum and tables and figures are not included. Try to summarize each relevant section in just a few sentences.

**Introduction:** The Introduction contains three areas of information. First, background information is provided to set the stage, furnishing readers with information that allows them to appreciate the investigation and to pique their interest. Think of this as sort of a sales pitch for your work, where you’re trying to get the reader interested and thinking about things along a specific line of reason. The writing should be clear, appropriate references should be cited, and meaningless phrases should be avoided (e.g., “Studying microorganisms is very important.”) Second, is a statement of the problem that is the focus of the paper, including a synopsis of previous studies and the rationale for your study. The problem statement usually contains the hypothesis of the study and should also indicate why the investigation was worth doing. Finally, the Introduction should include a statement about the purpose of the investigation, which would be to address some aspect of the problem statement.

Keep in mind that a good introduction will

- present the nature and scope of the problem
- acquaint the reader with pertinent literature
- describe the key methods
state the principle results and conclusions.

Common mistakes made when writing introductions are to try and include a complete review of the literature (leave that for the Discussion) or to go the opposite way and use a lot of empty filler, that is, sentences that do not add anything but words.

**Materials and Methods:** The information in this section should allow someone competent in microbiology to duplicate the study. Step by step procedures are not given unless they are original. For example, a competent worker does not need to have the procedure for a gram stain explained. If previously published methods are used, a complete citation should be provided along with any modifications used in the current study. Make sure to provide descriptions of how things were done and what equipment and supplies were used. Descriptions of test outcomes and their interpretations can be included. In most cases a flow chart can clarify the sequence of experimental procedures, although they tend to be used only in lab reports, posters, and oral presentations but not in journal manuscripts.

**Results:** Observations should be described in a narrative. It is almost as if a description of observations was being given over the telephone. The narrative should be supported (not replaced) with summary instruments, such as tables, graphs, and diagrams. Tables and graphs are particularly useful with quantitative data. The same data should not be expressed in a table and a graph. Do not present raw data. Avoid interpreting the results in this section.

**Discussion:** Interpret the results. What do they mean? Discuss the validity of the observations and techniques. Is there internal consistency between the results? Are the results consistent with external reports? Specific points should be supported by mention of the key results responsible for the conclusion but the goal is to discuss the results, not reiterate them. It is legitimate to make speculations and implications based on your results and the external literature. The first paragraph usually points out the contribution your study makes to your research area, summarizing current literature. It is typical for the Discussion section to end with a paragraph that summarizes the key points established by your research. These conclusions should be related to your introduction, purpose, and hypothesis.

**Literature cited:** Throughout the report, the sources for key items of information from external sources should be cited. A common way to do this is to follow a statement with the reference number in parentheses. Note that without specific citations, a reference list is a bibliography. In science writing, the references are cited within the text.

**Additional Information**

**Figures and Tables:** Figures and tables are used to summarize data described in the text. They should be clear and contribute to making your presentation more easily understood. Figures and tables should be accompanied by a caption that includes a number, title, and brief description of the information being presented. If the figure or table is from an external source, cite the reference and include the complete reference in the Literature Cited section. In the natural sciences it is common practice to place the caption below figures and above tables. The figures and tables must be referred to within the text, usually within parentheses following a statement (e.g., Generation time was 24 minutes (Fig. 3)).
Pay attention to the proper usage of terms:

1. The proper names of bacteria include the genus and the species. Genus is capitalized, species are lower case, and both are italicized or underlined. For example, *Escherichia coli*.

2. “Bacterium” is singular, “bacteria” are plural. (“datum” = singular, “data” = plural; “hypothesis” = singular, “hypotheses” = plural)

3. Use past tense to describe the work you did, present tense for information from an external source.

4. Use scientific notation for large numbers.

5. The scientific way to write dates is DD/MM/YYYY.

6. Only proper nouns get capitalized.

7. Expletives are words used to fill a vacancy (i.e., empty filler) and should be avoided. That is, remove words that are unnecessary. For example, sentences that begin with “It is,” “There is,” or “There are,” can be written without these empty words. Instead of “It is apparent that the results suggest . . .,” write “The results suggest that . . .”

8. Write dynamically. Although science writing tends to be in the passive voice (“It has been observed . . .”) rather than “It was observed . . .”), the active voice makes reading more interesting. Furthermore, do not use nominalization (changing a verb to a noun). Instead of “we reached the conclusion that . . .” where “reach” becomes the action, write “we concluded that . . .” so that “concluding” is the action.

9. Eschew obfuscation by using concise phrasing and precise wording. Avoid jargon and uncommon words, if possible.

**Quality Standards Checklist**

- **Typing.** All manuscripts and lab reports should be typed. Use a font size of 12 points in a standard typeface, such as Times Roman, for normal text. Margins should be one inch on all sides. Double space the text to make commenting easier; references may be single spaced.

- **Neatness.** The manuscript should not be crumpled, torn, or damaged in any other way. Pages should be fastened together with a staple or paper clip. Torn and folded corners do not look professional. Plastic report covers are not necessary.

- **Proofreading.** Do not submit manuscripts with spelling errors or poor grammar. Word processors contain spell checkers and grammar checkers. Use them. Proofread the paper before you leave the computer; handwritten corrections are not professional.

- **Graphics.** Tables, figures, and graphs should be of high quality. Computer-generated graphics are preferred but hand-drawn graphics are acceptable if they are done with a
high degree of professionalism (i.e., straight lines, even printing, regular proportions). Graphics do not need to be inserted inside the text but may be included on separate sheets attached to the end of the manuscript.

☐ **Plagiarism.** Do not submit someone else’s work as your own. Use citations with full references to give credit where it is due.

☐ **Honesty.** Include all relevant results. Do not “massage” data to create an erroneous interpretation. By the same token, avoid presenting irrelevant information.
### 3 MOCK WOUND

#### Background

The goal of this exercise is to isolate and identify the organism(s) considered to be the cause of a wound infection. For this purpose each student is provided with a mix simulating material from the infected wound. Gram positive cocci such as *Staphylococcus aureus* and *Streptococcus pyogenes* as well as the negative commensals of the intestinal tract are the most common aerobic organisms which cause wound infections. This exercise will be concerned with isolation and identification of the gram positive cocci.

*Staphylococcus* is a genus of gram positive cocci which appear singly, in pairs, or in irregular clusters. Two species, *S. aureus* and *S. epidermidis*, are frequently found on normal human skin. *S. aureus* is an opportunistic pathogen and can cause abscesses and pustules. *S. epidermidis* is considered nonpathogenic. They can be differentiated from each other very easily with the coagulase test or by observing whether mannitol is fermented. *S. aureus* is positive for both tests, *S. epidermidis* is negative for both.

*Streptococcus* that are responsible for wound abscesses usually form long chains. Differentiation between *Staphylococcus* and *Streptococcus* is straightforward by testing for catalase activity or growth on Mannitol Salts Agar (MSA). *Streptococcus* does not display catalase activity, *Staphylococcus* has catalase. The 7.5% NaCl in MSA inhibits the growth of *Streptococcus* but not *Staphylococcus*. *Enterococcus*, a genus that used to be grouped as a *Streptococcus*, is able to grow with high salt concentrations and will ferment weakly on MSA.

There are four groups of *Streptococcus*, A, B, C, and D. The most common streptococcal group found in human wounds are those in Group A. Members of this group can lyse red blood cells (hemolysis) in three different patterns on blood agar plates. Alpha hemolysis is a partial lysis, demonstrated by a greenish, indistinct zone around the colony. Beta hemolysis describes complete lysis of the red blood cells, producing a clear halo around the colony. Gamma hemolysis is actually the absence of hemolysis; no halo forms around the colony. Beta-hemolytic streptococci in Group A can be differentiated from other beta-hemolytic streptococci by their sensitivity to bacitracin. Although some alpha-hemolytic bacteria are sensitive to bacitracin, the only beta-hemolytic streptococci sensitive to bacitracin are those in Group A.

#### Materials

Bacterial mixtures (3 species/tube)
- *Staphylococcus aureus* (β-hemolytic)
- *Staphylococcus epidermidis* (γ)
- *Streptococcus pyogenes* (β)
- *Streptococcus sanguis or mitis* (α)
- *Enterococcus faecalis* (γ)

Blood Agar plates

Tryptic Soy Agar plates
Mannitol Salt Agar (MSA) plates
Bacitracin disks
Plasma for coagulase test

**Procedure**

**Pre-lab**

- Use your text to review information on *Staphylococcus* and *Streptococcus* (including *Enterococcus*).
- Review procedures for making quadrant streak plates and gram stains.
- Prepare your notebook by entering as much information as possible.
- Create a flow diagram illustrating the work to be done and the decision-making process.

**Day 1**

1. Each pair will receive a culture containing three different microorganisms.
2. Each person streaks one Blood Agar Plate for isolation. Each person does a gram stain of the mix.
3. Each table will inoculate Blood Agar Plates in a small cross pattern of known cultures for reference. Gram stain knowns. (What is the importance of this step?)
4. Incubate plates at 37°C for 24 hr.

**Day 2**

1. Record colony morphologies (See *Appendix 1*), hemolysis patterns, and gram reactions.
2. Streak one of each colony type on TSA plates. (Why?)

**Day 3**

1. Test each isolate for catalase activity.
   a. transfer a small amount of cells from a TSA plate to a clean microscope slide
   b. apply a drop of fresh 3% hydrogen peroxide (H₂O₂) to the cells
   c. note the production of bubbles (oxygen) that indicates the presence of catalase
2. Innoculate one MSA plate with catalase-positive isolates and catalase-positive reference cultures. (Why only the catalase-positive isolates?)
3. Perform a bound (slide) coagulase test on the catalase-positive isolates and the *Staphylococcus* reference cultures.
   a. add a small amount of cells from the TSA plate to a drop of water on a slide
   b. add a loopful of coagulase plasma to the cell suspension and mix for 20 seconds
   c. formation of clumps is a positive result for coagulase activity and *S. aureus* is usually positive for coagulase activity and *S. epidermidis* is usually negative
4. Perform a bacitracin sensitivity test on any isolate that is a gram positive, catalase negative, beta hemolytic coccus. (What organism should this be?)
   1. make a normal quadrant streak of the isolate on Blood Agar
   2. aseptically press a bacitracin antibiotic disk onto the first two quadrants
   3. incubate at 37°C for 24 hr

**Day 4**

1. Observe and record data from MSA plates. Remember, MSA is a selective and a

Microbiological Procedures 16
differential medium. The 7.5% NaCl favors the growth of *Staphylococcus* (although some strains of *Streptococcus* can grow at high salt concentrations). *S. aureus* can ferment mannitol, producing acid that turns phenol red in the medium from red (neutral pH) to yellow (acidic pH). *S. epidermidis* does not ferment mannitol.

2. Observe and record data from the Blood Agar plates with bacitracin disks. If cells grew adjacent to the disk they are not sensitive to bacitracin and the organism is probably not a beta hemolytic *Streptococcus* in Group A. However, if there is a halo of no growth around the bacitracin disk, the organism is sensitive to the antibiotic and is most likely a Group A *Streptococcus*.

**Results**

Record your observations at each stage of this exercise. Would a table or plot help summarize any of the data?

**Conclusion**

Interpret your data. What do the results suggest in regard to the identities of your unknowns? Explain your thought process. Would diagrams help explain your reasoning? Are you confident of your conclusions? Would other tests help?
4 MOCK STOOL

Background

Bacteria in the gastrointestinal tract carry out a wide variety of metabolic reactions, including degradation of macromolecules, vitamin synthesis, and absorption of bile acids. A balanced microbial ecosystem also inhibits the colonization and growth of pathogenic bacteria. As food passes through the gastrointestinal tract, water is removed from digested material, gradually concentrating it as it's converted to feces. Bacteria make up approximately one-third to one-half of the weight of fecal matter. Solid retention time in the gastrointestinal tract is about 24 hours, so bacteria in the lumen double about one or twice each day.

Bacterial counts of stomach contents are low due to the high acid condition (pH 2), although the lining is often heavily colonized by acid-tolerant lactobacilli and staphylococci. The small intestine is divided into two parts, with the duodenum resembling the stomach in terms of microbiology. The ileum is less acidic and has a richer population of bacteria, with concentrations of $10^5$ - $10^7$ per gram.

Most gastrointestinal bacteria are present in the large intestine. The normal flora are predominantly gram positive, obligate anaerobes, e.g. Bacteroides, Peptostreptococcus, and clostridia, with counts of $10^{10}$ - $10^{13}$ cells per gram of contents. Another component of the population are the enterobacteria, gram negative, facultative anaerobes, e.g. Escherichia coli, Klebsiella, Enterobacter. They are present at much lower concentrations, generally less than $10^5$ per gram. However, some of the Enterobacteriaceae are pathogenic and can be isolated from the blood, urine, or fecal material of infected individuals. The best known pathogens within this group are Salmonella and Shigella. Salmonella are the most common agent of gastroenteritis in the United States. The main symptoms of Salmonella food poisoning are watery diarrhea, sometimes bloody, accompanied with low grade fever. Similar symptoms occur with Shigella infections. The presence of pathogens within this group has led to the development of specific media and biochemical tests to differentiate pathogenic organisms from the normal gut flora.

Biochemical Tests

Triple Sugar Iron (TSI) Agar
TSI is used to differentiate members of the Enterobacteriaceae based on their ability to ferment glucose, lactose, and/or sucrose, and to reduce sulfur to hydrogen sulfide. The medium contains phenol red as the pH indicator; sulfur is included as ferrous sulfate and sodium thiosulfate. The medium contains a small concentration of glucose, which all of the Enterobacteriaceae can ferment, and 10X greater concentrations of lactose and sucrose. After 24 hours at 37°C, fermentation of only the glucose will cause the butt of the slant to turn yellow. (The slant will remain red.) If the lactose and sucrose are fermented, both the butt and slant will turn yellow. Gas production will produce bubbles in the butt and may actually split the medium. Any hydrogen sulfide produced will react with iron in the medium to form ferrous sulfide, a black precipitate. Tubes are inoculated by stabbing almost to the base with the inoculating needle and drawing the needle across the slant on the way out. Results must be read within 18-24 hours of incubation at 37°C to be accurate.

**SIM Agar**

SIM agar is used to differentiate members of the Enterobacteriaceae on the basis of motility, sulfide production, and indole production. Indole is produced as a byproduct of tryptophan catabolism (Figure 5.1). Tubes of medium are inoculated by stabbing 3/4 to the base with an inoculating needle. Check results after incubating at 37°C for about 24 hours. Sulfide production (from cysteine hydrolysis) appears as a black precipitate (FeS) in the agar. Motility is demonstrated by turbidity extending outwards from the stab but may be difficult to observe due to sulfide production. Read the results again after 48 hours because decarboxylases are inducible and are only produced in an acidic environment in the presence of their substrate. Indole production is determined after 48 hours incubation by adding a few drops of Kovacs Reagent to the tube. A pink color is a positive result for indole.

![Indole formation](image)

**Figure 5.1.** Indole production.

**Urea Agar**

Urea media contain urea and phenol red. Hydrolysis of urea to ammonia and carbon dioxide by the enzyme urease causes an increase in pH, causing the medium to change from yellow to pink. Medium is inoculated by streaking across the surface of the slant. Interpretation of this test should be performed 24 hours after incubating at 37°C. A pink color throughout the medium indicates rapid activity, typical of Proteus, Morganella, and Providencia. Pink color only in the slant indicates slower expression of urease activity, typical of Citrobacter, Enterobacter, and Klebsiella.

![Urea hydrolysis](image)

**Figure 5.2.** Urease activity.

**Methyl Red-Voges Proskauer (MR-VP) Broth**
This test is used to differentiate the between enteric bacteria based on the products from glucose fermentation. Some bacteria, for example *E. coli*, produce acidic end products (pH < 4.4) and will cause methyl red to change from yellow (neutral) to red (acidic). Other bacteria within this group, for example *Enterobacter*, produce neutral end products including acetoin. In the Voges Proskauer portion of the test, acetoin is oxidized by KOH in the presence of oxygen to diacetyl. In the presence of α-naphthol, diacetyl forms an intense red color. This test is conducted by inoculating two tubes of MR-VP broth and after incubating at least 24 hours at 37°C adding methyl red to one tube and KOH plus α-naphthol to the other.

**Simmons Citrate Agar**

The ability to utilize citrate is useful for differentiating members of the *Enterobacteriaceae*. Organisms that can utilize citrate (Figure 5.3) increase the pH of the medium. The pH indicator, Bromthymol blue, changes color from green (neutral) to blue (alkaline). Tubes are inoculated by streaking across the surface of the slant. Results are read after incubation at 37°C for at least 24 hours.

**Media**

**MacConkey Agar**

MacConkey agar is a selective and differential medium used to isolate and differentiate gram negative enteric bacteria that can ferment lactose (coliforms) from those that cannot ferment lactose. The medium contains crystal violet and bile salts that inhibit the growth of gram positive organisms. Neutral red, a pH indicator, is colorless above pH 6.8 and red below that pH. Thus, coliforms (e.g., *E. coli*, *Enterobacter*) will form red colonies while organisms unable to ferment lactose (e.g., *Salmonella*, *Shigella*, *Proteus*) will be a pale pink, the color of the medium.

**Eosin Methylene Blue (EMB) Agar**

EMB agar is another selective and differential medium used to isolate and differentiate members of the *Enterobacteriaceae*. Growth of gram positive bacteria is inhibited by methylene blue and eosin. Acid produced from lactose fermentation turns precipitates these dies, producing colonies that are metallic green (typical for *E. coli*) or black. Colonies will be pink if only a small amount of acid is produced. Nonfermenting enteric bacteria form colonies that are colorless or the color of the medium.

**GN Broth, Hajna**

This is an enrichment medium for enteric bacteria, especially *Salmonella* and *Shigella*. This is a fairly rich medium but it contains sodium citrate and sodium deoxycholate which inhibit the growth of gram positive bacteria and coliforms. The relatively high concentration of mannitol, compared to glucose, favors the growth of mannitol-fermenters like *Salmonella* and *Shigella* over the growth of mannitol non-fermenters like *Proteus*.

**Salmonella-Shigella (SS) Agar**

SS agar is a selective and differential medium used to isolate and differentiate enteric bacteria that can ferment lactose from those that cannot. Brilliant green dye and bile salts inhibit the growth of gram positive bacteria. The pH indicator neutral red is used to differentiate colonies of lactose fermenters (pink color) from colonies of lactose nonfermenters (colorless). Furthermore, thiosulfate in the medium can be reduced to sulfide by *Salmonella*, showing up as
the black precipitate ferrous sulfide in the center of colonies. \textit{(Proteus also produces sulfide but ferments lactose, albeit slowly.)} There are other media available which are recommended for isolation and differentiation of \textit{Shigella} (Hektoen and XLD agars).

\textbf{Materials}

\begin{itemize}
  \item \textit{Escherichia coli}
  \item \textit{Enterobacter aerogenes}
  \item \textit{Proteus mirabilis}
  \item \textit{Salmonella enteritidis}
  \item \textit{Shigella sonnei}
  \item \textit{Bacillus cereus}
  \item \textit{Enterococcus faecalis}
  \item MacConkey agar plates
  \item EMB agar plates
  \item GN broth tubes
  \item Salmonella-Shigella agar plates
  \item Triple Sugar Iron (TSI) slants
  \item Sulfide Indole Motility (SIM) agar deeps
  \item Methyl Red-Voges Proskauer (MR-VP) broth
  \item Citrate slants
  \item Urea slants
\end{itemize}

\textbf{Procedure}

\textbf{Note: Plates should be interpreted after 24 h incubation then stored in a refrigerator.}

Day 1

1. Each \textbf{pair} will receive a mixture of four unknown organisms. Record the number of your culture. Each \textbf{person} should make two quadrant streaks of the mixture on a MacConkey agar plate.

2. Each \textbf{person} should inoculate a tube of GN broth with the unknown mixture.

3. In \textbf{groups of four}, perform test streaks of the known organisms on MacConkey agar, using one-third of a plate for each organism (two plates of each medium per set).

4. Each \textbf{person} must make a gram stain of the mixture. Each \textbf{pair} is responsible for performing gram stains with each of the knowns. Record reactions and morphologies of each specimen.

5. Incubate cultures at 37°C overnight.
Day 2

1. Record your observations of colonies on the MacConkey agar plates, comparing those from the unknown mix with the control organisms. Select three colonies with different morphologies (there should only be three different morphologies – why?).

2. Streak the colonies onto EMB and MacConkey agar plates to get pure cultures. Inoculate media with the known cultures as a control. Incubate overnight at 37°C.

3. Perform a quadrant streak on an SS plate and a MacConkey or EMB plate using the GN broth for inocula. Incubate overnight at 37°C.

Day 3

1. Record your observations of the isolates growing on the different media. Compare the appearance of each colony against the known colonies on the same medium. Record a presumptive identification. Select three different isolates from the same type of medium, if possible. **If you aren’t sure you have three different isolates, restreak them on the same medium before moving onto the next step.** (You may want to use the MacConkey plate because it will inhibit swarming by *Proteus.*) The SS plate and MacConkey (or EMB) plate inoculated from the GN broth may be the best source colonies of *Salmonella* or *Shigella*.

2. Once you are sure you have three different organisms isolated, streak each of them on Tryptic Soy Agar (TSA) plates as another purification step. After incubation at 37°C, plates may be stored in the refrigerator.

3. Conduct a gram stain on each isolate from the unknown mixed culture.

Day 4

1. Inoculate three 6-tube setups with the three colonies isolated from the mixture, one isolate per setup. For inocula, touch the top of a colony with an inoculating needle. Each 6-tube setup contains a TSI slant, a SIM agar deep, two MR-VP broths, a citrate slant, and a urea slant.

2. In a group of four, inoculate 6-tube setups with the known organisms.

3. Incubate all cultures at 37°C.

Day 5

1. Record results from media inoculated on Day 4.

2. Based on your results, identify the four organisms that were in your unknown mixture.
**Results**

Generate your own data table in the format of Table 5.1.

**Table 5.1. Metabolic Reactions of Selected Bacteria.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gram Reaction</th>
<th>Lactose fermented</th>
<th>TSI slant/ butt</th>
<th>Gas</th>
<th>H₂S</th>
<th>Urease</th>
<th>Motility</th>
<th>I</th>
<th>M</th>
<th>Vi</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>–</td>
<td>+</td>
<td>A/A</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>–</td>
<td>+</td>
<td>A/A</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>–</td>
<td>–</td>
<td>K/A</td>
<td>w</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>Shigella sonnei</em></td>
<td>–</td>
<td>–</td>
<td>K/A</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>–</td>
<td>–</td>
<td>K/A</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>w</td>
<td>–</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: – = negative, + = positive, K = alkaline, A= acid, w = weak positive, d = delayed positive
I = indole test, M= methyl red, Vi= Voges Proskauer, C = citrate

**Conclusion**

Interpret your results. Identify the four different species of bacteria in your unknown mix, **elucidating the logic of your process**. A flow diagram of your decision-making process would be a good summary to accompany your narrative.
5 STATISTICS

What Are Statistics and Why Do We Use Them?

Statistics is a branch of mathematics that is used to analyze incomplete information in order to decrease the level of uncertainty about a conclusion. In other words, statistics allows us to make a decision based on a sample of a population and to have a specific level of confidence that our decision is correct. Since samples are only a subset of all possible observations, it is critical to evaluate accuracy. Furthermore, there is always some variation between observations and these differences need to be examined to determine if they are real or an artifact of the observation method. Statistical methods are designed to analyze data to help address these difficulties, to provide additional information, and to determine the limitation of the results. Specifically, statistics allow investigators to:

1) use sample sets to make inferences about population parameters,
2) reduce large amounts of data to comprehensible levels summarized by statistical descriptors,
3) determine the level of confidence that supports a particular conclusion.

The use of statistics for data analysis is greatly simplified by selecting the proper tests when designing the experiment.

The two major areas of statistics are descriptive and inferential. In descriptive statistics, data are collected, organized, summarized, and presented. We focus on summarizing the data in a manner that allows us to determine the type of data we are dealing with and to see any underlying patterns that may exist. The two basic methods available are numerical and graphical. Numerical summaries, like the mean, mode, median, or standard deviation can give us a precise, objective description. Graphical methods allow us to construct a plot, for example a histogram, leaf and stem plot, or box plot. Patterns in the data are more obvious with graphical summaries than with numerical summaries.

Inferential statistics allow us to draw a conclusion about a population from a sample of that population. For example, consider if students who took MICRO 2054 last semester needed an average of four hours to complete the Identibacter interacticus assignment and students who took MICRO 2054 prior to last semester required an average of seven hours. Is the difference real or due to chance? Is the magnitude of the time difference accurate or would it change with a larger sample (another class)? These are the types of questions that could be addressed with inferential statistics. The two main methods used in inferential statistics are estimation and hypothesis testing. In estimation, the data are used to estimate a parameter (e.g., the mean) and to construct a confidence interval around the parameter. In hypothesis testing, a null hypothesis (hypothesis of no difference) is stated and the data are examined to see if the null hypothesis can be rejected.

Descriptive Statistics

Data can be presented in a number of ways, including tables, graphs, and numerical summaries. The appropriateness of a presentation method is determined for each situation, depending on the purpose and the kind of data being examined.

Measurement Scales
Characteristics that are measurable can be classified in one of four measurement scales: the nominal scale, the ordinal scale, the interval scale, or the ratio scale. The nominal scale classifies objects or events into mutually exclusive categories. No intermediate conditions can exist, so the object is either “equal to” or “not equal to” the conditions for each category. For example, we might be testing different media with one particular organism and recording “growth” or “no growth.” All trials will fall into only one category and an intermediate state is impossible.

The ordinal scale separates objects into categories, like the nominal scale, but in addition, it permits a ranking of “greater than” or “less than” to be performed. The ranking is qualitative, so it is not possible to determine how much two objects differ. An example of an ordinal scale would be a poll of student satisfaction with a class. Although a student who is “very satisfied” is easy to distinguish from one who is “not satisfied at all,” it is impossible to provide an actual measurement of their difference. Typically, ordinal data is scored from lowest to highest. In the case of bacterial growth, comparative values might be: 0 = no growth, 1 = some growth, 2 = good growth, and 3 = dense growth.

The interval scale and ratio scale both use defined units of measurement. Not only are qualitative differences noted, but the magnitude of the difference can be measured. Values can be discrete and have integer values (e.g., the number of women who became pregnant last month) or continuous (e.g., time). To continue with the example of bacterial growth, measurements of actual cell concentrations would use the interval or ratio scale. The main difference between the two scales is that the ratio scale sets zero equal to zero, while the interval scale uses an arbitrary value for zero. Temperature uses an interval scale, with the freezing point of water used to arbitrarily set 0°C. The ratio scale is used for more tangible physical properties, like mass or time. Since most of the variables we are measuring have continuous and discrete values, we usually use the ratio or interval scales.

Central Tendency

If two die are rolled, the frequency of the values obtained approaches a distribution that can be predicted from the number of combinations possible. That is, there is only one way to roll 2 or 12, so these values occur most infrequently. On the other hand, 7 can arise through six different combinations and will occur most frequently. If the frequency of values are plotted, it forms a triangular distribution with 7 at the peak and 1 and 12 at the base. If more die are used, the pattern is repeated but the distribution begins to form a smoother curve, the shape of which is described as “bell-shaped.” This curve also describes the normal distribution. Although other distributions exist, most of the data we collect will be distributed normally or can be transformed into a normal distribution. Most of the more common statistical calculations require that data be normally distributed. Non-parametric statistics are used for data that don’t occur with a normal distribution.

One of the most useful ways to describe normally distributed data is based on where the peak occurs, which ideally is in the center of the distribution. The central value around which the data values fall is termed the central tendency of the distribution. Although the most common central value we use is the mean, calculated by dividing the sum of all data points by the number of data points (Equation 1).
One shortcoming of the mean is that it is sensitive to extreme values. A single outlying data point can skew the distribution so that the mean no longer represents the peak of the curve. Because of this, other descriptors of the central tendency may actually be more useful. The median is the middle point of the distribution, with an equal number of points above and below it. The median is most often used with ordinal data. The mode is the data value that occurs most frequently in a data set. It is the only measure of central tendency possible with data in a nominal scale. In a perfect normal distribution, all three descriptors are the same. The choice of which to use should depend on the actual shape of the distribution. Sometimes the curve will be skewed and will require mathematical transformation to better approximate the normal distribution.

**Variability**

Although most data points will be clustered around a central value, some will be widely dispersed. The wider the dispersion, the more difficult it is to have confidence in the data interpretations and comparisons. A quantitative measure of the spread is critical to test hypotheses. Variance is the average of the squared deviations, calculated as the sum of squared deviation from the mean divided by the sample size minus one (degrees of freedom) (Equation 2).

\[
\sigma^2 = \frac{\sum(x-x)²}{n-1}
\]

where:  
\( \sigma^2 \) = variance of the data  
\( \Sigma \) = the sum of  
\( x \) = the numerical value of a data point  
\( \overline{x} \) = the mean of the data  
\( n \) = the number of data points

The variance does not provide a very useful description of variation within the data set. The positive square root of the variance is known as the standard deviation (Equation 3), which quantitatively describes the data spread.
The standard deviation represents the average amount by which each observation in a data set differs from the mean. A small standard deviation indicates that all values in the data set are very close to the mean. One standard deviation on each side of the central point is equal to 68.26% of the area under the normal curve. Two standard deviations are equal to 95.44%, three are 99.74%.

If means are calculated from several sample data sets taken from the same population, there will be distribution around the true population mean. The distribution of these means is described by the standard error (Equation 4).

$$SE = \frac{s}{\sqrt{n}}$$

where: $SE$ = the standard error of the mean
$s$ = the standard deviation
$n$ = the number of data points

The standard error describes the variability of the means around the true population mean.

The central limit theorem states that sample means drawn from an arbitrarily distributed population tends to be normally distributed.

Graphs

Graphs are pictorial representations that summarize numerical data. There are many different formats but in all cases a well-designed graph will immediately display patterns in the data. Graphs should be simple, self-explanatory, and with clearly labeled variables including units of measure. Independent variables are plotted along the X-axis; dependent variables are plotted along the Y-axis.

Histograms

Histograms display a frequency distribution for discrete or continuous data. The X-axis displays intervals and the Y-axis displays the frequency of observations within each interval. For example, exam grades may be summarized with possible grades as the independent variable and the number of students earning each grade as the dependent variable. Bar charts are similar to histograms but are used with nominal or ordinal data.

Frequency Polygons

Frequency polygons are very similar to histograms but use a continuous line instead of bars to represent the data. The major advantage of a frequency polygon over a histogram is the ability to

$$s = \sqrt{s^2}$$

where: $s$ = the standard deviation
$s^2$ = the variance
view and compare several data sets on the same graph.

**Box Plots**

*Box plots* require only a single axis and may be presented in a horizontal or vertical manner. A box plot appears to be a bisected box with perpendicular lines extending from two opposite sides of the box. The lower (or left) side of the box represents the 25th percentile (or quartile). The higher (or right) side of the box represents the 75th percentile. The line that bisects the box represents the median or 50th percentile. The lines projecting out from the box extend to the extreme observations and indicate the range of observations.

**Scatter Plots and Line Graphs**

*Scatter plots* and *line graphs* display the relationship between two continuous variables. The primary difference between the two is that intervals on the independent axis are scaled to actual values in the scatter plot while the intervals in line graphs may not all have the same numerical value.

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**Hypothesis Testing**

The basis of hypothesis testing is the comparison of observed results with expected results. Statistics allow us to determine if an observed result is significantly different from an expected result. The most common question is whether the means two groups are significantly different. In statistics, *significant* is a very specific term, used to denote statistical determinations. Stating that two means are significantly different means that the difference is greater than could be attributed to the natural variability of the population or the method.

In order to determine if a hypothesis should be accepted or rejected, it is necessary to select a level of significance ($\alpha$). The most common level in biological sciences is $\alpha = 0.05$. At that level, 5% of the time a true null hypothesis will be rejected. Another way of considering this is that the investigator can be 95% confident that a true null hypothesis will not be rejected. Rejecting a correct null hypothesis is called a *type I error*. Accepting a false null hypothesis is called a *type II error*. Decreasing the $\alpha$ lowers the probability of a type I error but raises the probability of a type II error.

The selection of the appropriate statistical test and proper experimental design requires several steps. The investigator must be clear on the problem being addressed and the type of data (measurement scale and distribution) expected. This will allow selection of the appropriate statistic, which in turn allows the hypotheses to be stated. Common statistical procedures are summarized in Figure 6.1. Once it is known how the data will be used, the experiment can be designed and performed. Analysis of the data will determine if the null hypothesis or the alternative hypothesis will be accepted.

**Parametric Tests**

Parametric tests of significance are used with data that are normally distributed. Data that cannot be assumed to follow a normal distribution need to be analyzed with non-parametric tests. Parametric tests are more powerful, that is they are less likely to result in a type II error.

**The Student t Test**

The Student t test examines whether the means of two groups are the same. The $t$
distribution varies as a function of degrees of freedom and is symmetrical about a mean of 0. The more degrees of freedom, the less spread out the distribution. To use the \( t \) test, a \( t \) value calculated from the data is compared to a critical value based on the \( t \) distribution with degrees of freedom determined from the sample sizes. The \( t \) value is calculated by dividing the difference of the two means by the standard error of the difference of the means. The formula used depends on whether the population means are identical, which is the usual assumption (Equation 5).

\[
\frac{t_A - t_B}{\text{standard error of difference}} = t
\]

where:
- \( t \) = \( t \) value
- \( x_A \) = mean of group A
- \( x_B \) = mean of group B
- \( \sum(x_a - x_A)^2 \) = sum of squares for group A
- \( \sum(x_a - x_B)^2 \) = sum of squares for group B
- \( n_A \) = number of data points in group A
- \( n_B \) = number of data points in group B

The null hypothesis is accepted if the calculated \( t \) value is equal to or less than the tabulated \( t \) value.

The Student’s \( t \) test can only be used to compare two means. A variation uses paired data, but this increases the chance for a type I error. The analysis of variance procedure with multiple range tests may be a better choice for use with multiple groups.

**Analysis of Variance**

Hypothesis testing by comparing multiple groups is best done with an analysis of variance (ANOVA). The \( F \) statistic is calculated and compared with tabulated values from an \( F \) distribution. Usually a control group is compared to several experimental groups. The results only indicate if significant differences exist and not the magnitude or direction of differences.

Two independent variances are calculated, between-group variance and within-group variance. The \( F \) statistic is the ratio of between-group divided by within-group variance (Equation 6).

\[
F = \frac{\sum x_i (x_i - \overline{x}_i)^2 / (k - 1))}{\sum(x_i - x)^2 / (n - k)}
\]

where:
- \( F \) = the \( F \) ratio
- \( x_{ij} \) = the \( j \)th observation in Group \( i \)

\[
t = \frac{(\overline{x}_A - \overline{x}_B)}{\sqrt{\frac{\sum(x_a - \overline{x})^2 + \sum(x_b - \overline{x})^2}{(n_A + n_B - 2)} \left( \frac{1}{n_A} + \frac{1}{n_B} \right)}}
\]

where:
- \( \overline{x}_i \) = the mean of Group \( i \)
- \( \overline{x} \) = the overall mean
Each variance has its own degrees of freedom. The degrees of freedom of between groups is the number of groups minus one. The number of degrees of freedom of the within groups is the total sample size minus the number of groups. If the $F$ ratio is greater than the critical value from the table, the null hypothesis is rejected.

**Nonparametric Tests**

If the data cannot be assumed to be normally distributed and cannot be transformed into a normal distribution, nonparametric tests must be used. These tests are not as powerful as parametric tests but are more conservative and require less assumptions. Some of the more common tests are listed in Figure 6.1. The rank sum or Mann-Whitney test is analogous to the $t$ test. The nonparametric alternatives to the paired $t$ test are the sign test, for when the magnitude of differences cannot be determined, or the Wilcoxon matched-pairs test when the differences can be ranked by the magnitude of the difference. The Kruskal-Wallis test replaces ANOVA with independent samples. Related samples are examined with the Friedman test.

**Correlations and Regressions**

This statistic calculates the degree of relatedness between two parameters of a population. When plotted, a positive correlation displays a positive slope. A negative correlation (as one parameter increases the other decreases proportionally) displays a negative slope. If the two parameters are unrelated, the plot is a collection of random points without an obvious pattern. The strength of the relationship between two variables is reported as the correlation coefficient, symbolized as $r$. Correlation coefficients can be calculated in a number of ways but most calculators and spreadsheets use Pearson’s method. The value can vary between -1 and +1, corresponding to the slope displayed by the relationship.

Regression analysis defines a mathematical relationship between two parameters that permits prediction of one parameter if the other is known. Once data is plotted, the investigator tries to determine the type of relationship that the data fit. A regression line is constructed so as to minimize the variance of the data about the line. The most common form is linear regression, where a straight line is described by the equation $y = mx + b$, where $m$ is the slope and $b$ is the $y$-intercept. Nonlinear regressions are more difficult to calculate and may require a fairly powerful curve fitting program. The equation for the regression line allows the investigator to calculate corresponding values given $x$ or $y$.

Regressions have two sources of variance. In addition to the variance of the dependent variable, there is also an error variance that arises from the relationship between the independent and dependent variable. Without an error variance, all observations would fall on the regression line. The coefficient of determination, symbolized by $r^2$, is the measurement of the variance due to the relationship of the dependent variable to the independent variable. A value of 1 indicates that the value of the dependent variable completely depends on the independent value.
6 USING A DISK DIFFUSION ASSAY TO INTRODUCE STATISTICAL METHODS

Background

Antibiotic resistance is becoming an ever-increasing concern. Thus, the ability to accurately assess the level of resistance of clinical isolates is of great importance, ultimately affecting patient outcome. Antibiotic resistance testing using the disc diffusion technique was developed in the 1940s soon after the discovery of the first antibiotics. In 1966, Bauer et al. (1) published their paper which helped standardize this protocol. Today, NCCLS provides periodic updates of this protocol (6) and tables (5) so when performing this assay results are reproducible from day to day and from lab to lab if using the same isolate and the same antibiotics. That is, if the directions are followed, the same results will always be obtained with the same isolate, regardless of where or when the analysis is performed.

In this exercise we modify the NCCLS disc diffusion assay so that various concentrations of the inoculum are used in addition to the standard inoculum concentration. After incubation for each inoculum, the diameter of the zone of inhibition of each antibiotic disc is measured. Comparisons are made of the effect of inocula concentration on the diameter of the zone of inhibition. By testing this variable (i.e. different concentrations of inocula) we hope to show the importance of following a standardized protocol and to illustrate the use and the value of statistical analysis in microbiology. The null hypothesis, that is the hypothesis of no difference, is that the diameters of the zones of inhibition will be the same regardless of E. coli concentration in the inocula. The hypothesis is tested using the \( t \)-test. In addition, each student in the group measures the zones of inhibitions and their results are compared. In this instance, the null hypothesis, that there is no difference between their measurements, is tested with ANOVA.

Students should read the protocol and organize their groups before the start of the experiment. In preparation for this exercise, we recommend that the instructor have them address the following points:

- Draw a flow diagram of the experiment noting how you would make the dilutions and then perform the plate counts.
- This experiment investigates the effect of bacterial concentration in the disk diffusion assay. What other variables could be investigated and which statistical tools would be appropriate for analysis?
- What are the modes of action of the antibiotics used? Would they be expected to act against gram positive, gram negative organisms or both?
- Do you think that it would make a difference if more than one person inoculated the plates for antibiotic testing? What statistical test would you use to test your hypothesis?
- How is resistance or sensitivity to an antibiotic determined using the disc
diffusion assay? Does this have clinical relevance? What is the measurement that is used to determine this? Would the a measurement of the same magnitude imply resistance (or sensitivity) if the organism tested was a different species?

- Would you expect bacteria in the stationary phase of growth to be more sensitive or resistant to an antibiotic as compared to the same species in the exponential phase? (Hint: Would pre-incubation of plates with the antibiotic disks in place affect the size of the zone around the disk?)
- List some ways in which antibiotic resistance can be transferred from one bacterium to another. Briefly describe each mechanism.

**Purpose**

At the completion of this activity, students will
1. understand the importance of using standardized methods, *i.e.*, antibiotic resistance analysis using the disc diffusion method, and recognize important variables involved in antibiotic resistance analysis
2. better understand the scientific method and its application to experimental investigations
3. be more familiar with hypothesis testing and the use of inferential statistics
4. be able to use spreadsheets for simple statistical functions
5. have improved various lab skills, such as aseptic technique, preparing dilutions, making viable counts, inoculating spread plates, and using pipettes

**Materials (per 4 students)**

32 Mueller Hinton agar plates  
sterile cotton swabs  
one ml sterile pipettes  
sterile tubes  
test tube racks  
overnight culture of *E. coli* on TSA  
*E. coli* culture adjusted to 0.5 McFarland Standard  
tubes of sterile saline for dilutions (4 x 9.9 ml, 6 x 9.0 ml, plus some for adjusting culture to McFarland standards)  
McFarland standards (0.5, 1, 2,4)  
gentamicin antibiotic disks (GM10)  
sulfamethoxazole/trimethoprim antibiotic disks (SXT)  
forceps  
95% ethanol in a beaker  
Bunsen burner  
vortex  
ruler or caliper  
magnifier
sterile bent plastic rods... “hockey sticks”

ice in small buckets or beakers

**Procedure**

**Day 1 (Inoculations)**

1. **Divide into groups of 4 students each.**
2. Each group will be given a culture diluted to 0.5 McFarland standard. This is the cell concentration recommended in the NCCLS protocol and will be considered the control culture in this experiment. You will also be assigned another cell concentration based on the McFarland standard (1, 2, or 4) and will need to make the appropriate suspension from an *E. coli* plate culture using sterile saline. Aseptically transfer colonies from the plate into a tube of sterile saline, vortexing after each addition until the colony is completely dispersed. Continue adding cells until the turbidity of your suspension matches the appropriate McFarland standard. (Hint: Looking at a line of print through the standard and the cell suspension is a good way to compare the density.) The cell suspension you make will be considered the experimental culture. Keep both cultures on ice to inhibit growth and keep cell concentrations from changing during the course of the experiment.
3. Each group will need two tubes of 9.9 ml sterile saline and three tubes of 9 ml sterile saline to enumerate each of the two cell cultures. (Four tubes of 9.9 ml and six tubes of 9 ml saline, total.) Label each series of tubes with the culture density (0.5 McFarland and your other density). Label the first 9.9 ml tube $10^{-2}$ and the second 9.9 ml tube $10^{-4}$. Label the three 9 ml tubes $10^{-5}$, $10^{-6}$, and $10^{-7}$. These tubes will be used to dilute the control and experimental culture to make inocula for spread plates.
4. Plate counts should be performed by diluting the two cultures with the saline dilution blanks (step 3, above). Start by transferring 0.1 ml from the control culture to the tube marked “$10^{-2}$” using good aseptic technique, then vortex that dilution. Using a new, sterile pipette, transfer 0.1 ml from the $10^{-2}$ dilution to the tube marked “$10^{-4}$” and vortex that tube. Transfer 1 ml from the “$10^{-4}$” tube to the tube labeled “$10^{-5}$.” Continue in this manner, inoculating the last two dilutions. Repeat this entire process with the experimental culture.
5. Use your dilutions to inoculate spread plates in order to determine the viable cell count in your cultures. For the control culture and experimental cultures adjusted to the 1 McFarland standard, inoculate duplicate plates each with 0.1 ml from the $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions. With the experimental cultures adjusted to the 2 or 4 McFarland standard, inoculate duplicate plates each with 0.1 ml from the $10^{-5}$, $10^{-6}$, and $10^{-7}$ dilutions. Use a sterile hockey stick to spread the inoculum over the entire surface of the plate, using a fresh hockey stick for each dilution with each culture.
6. For the disk diffusion assay, each group should swab ten Mueller-Hinton agar plates from the control culture. Repeat this, inoculating ten more Mueller-Hinton
agar plates from the experimental culture. Swabbing should be done by following the NCCLS protocol (2, 6):

A. Dip a sterile cotton swab into the appropriate liquid culture.
B. Press the swab against the inside of the tube to squeeze out excess fluid.
C. Lightly rub the swab over the entire surface of the Mueller-Hinton plate.
D. Rotate the plate 60° and swab the surface a second time.
E. Rotate the plate 60° and swab the surface a third and final time.
F. Roll the swab on the agar around the inside edge of the plate.

You can use the same swab to inoculate every plate in the series but you must dip it in the liquid culture before swabbing each plate.

7. After five minutes, but no more than 15 minutes, the two different antibiotic discs should be placed on the agar surface of each of the ten plates from each culture, at least 24 mm from each other and 10 mm from the edge of the plates, and tapped down with sterile forceps. (Placement may be done using an automatic dispenser or by using loose disks in a sterile petri plate, placing the disks with sterile forceps.) Sterilize forceps by dipping the tips in 95% ethanol and igniting the ethanol by rapid passage through a flame. (Keep the tip pointed down, away from the ethanol reservoir, and do not hold the forceps in the flame!) Invert the plates and incubate at 37°C for 18 hours.

**Day 2 (Record Data)**

1. After 18 hours, the plates with the antibiotic disks should be removed from the incubator and the diameters of the zone of inhibition should be measured in mm by each student in the group (i.e., four sets of measurements for the control culture and four sets of measurements for the experimental culture). The use of a magnifier, such as those on colony counters, greatly improves accuracy. Record the results in your lab notebook and share your data with the other members of your group.

2. Count the colonies on the enumeration plates, recording your counts. Calculate the viable cell concentration in the two McFarland cultures and determine if their ratio matches the ratio between the McFarland values for the cultures.

**Day 3 (Analyze Data)**

1. Determine if the sizes of the zones of inhibition with the two McFarland cultures are the same or different for each antibiotic using the t-test. Typical student data is shown in Table 1. This can be done manually using a calculator or with a spreadsheet (recommended, see Appendix). The null hypothesis (no difference between the diameters of the zones of inhibition for different concentrations of *E. coli* in the inocula) is accepted if the calculated *t*-value is less than the critical *t*-value with an alpha of 0.5 and 18 degrees of freedom. A one-tailed test is
appropriate since a higher concentration of *E. coli* may produce a zone of inhibition that is equal to or smaller than a zone resulting with a lower concentration of cells. Thus the critical value of *t* is 1.734.

2. Determine if the individual measurements of the zones of inhibition made by each group member are the same or different for each antibiotic using ANOVA. Student data for a control culture with trimethoprim/sulfamethoxazole is shown in Table 2. The null hypothesis that there is no significant difference between measurements of the diameter of the zones of inhibition made by different students is accepted if the calculated *F*-value is less than the critical *F*-value with an alpha value of 0.5 and 3 degrees of freedom, that is 2.866.

### Results

In addition to recording data in lab notebooks, use a spreadsheet for analyses and use those data sheets as the basis of a report. The report should include the raw data, results of statistical analyses, and interpretations of the statistical analyses. Describe, in your own words, the importance of a standardized antibiotic testing protocol.

A tutorial on using Microsoft® Excel for this experiment is available online on the instructor’s site ([http://faculty.weber.edu/wlorowitz/micro_procedures.htm](http://faculty.weber.edu/wlorowitz/micro_procedures.htm)). Samples of data are shown in Tables 1 and 2. Table 1 displays typical student data for diameters of zones of inhibition from gentamicin for *E. coli* cultures adjusted to 0.5 and 1 McFarland standards. Results of the *t*-test, performed using Microsoft® Excel, indicate that the zones with the greater cell density were significantly smaller than the zones with the less dense culture. Table 2

### Table 1. Comparison of zones of inhibition produced from gentamicin with *E. coli* cultures adjusted to 0.5 and 1 McFarland standard turbidities. The *t*-test was used to examine the effect of cell density on antibiotic inhibition.

<table>
<thead>
<tr>
<th>Disk Diffusion Assay</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GM10, 0.5</td>
<td>GM10, 1</td>
<td>t-Test: Two-Sample Assuming Equal Variances</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
<td></td>
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<tr>
<td>25</td>
<td>26</td>
<td></td>
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<tr>
<td>24</td>
<td>25</td>
<td>Mean</td>
</tr>
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<td>25</td>
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<td>Variance</td>
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<td>25</td>
<td>Observations</td>
</tr>
<tr>
<td>24</td>
<td>26</td>
<td>Pooled Variance</td>
</tr>
<tr>
<td>24</td>
<td>25</td>
<td>Hypothesized Mean Difference</td>
</tr>
<tr>
<td>25</td>
<td>26</td>
<td>df</td>
</tr>
<tr>
<td>24</td>
<td>25</td>
<td>t Stat</td>
</tr>
<tr>
<td>24</td>
<td>25</td>
<td>P((T\leq t)) one-tail</td>
</tr>
<tr>
<td>24.4</td>
<td>25.1</td>
<td>t Critical one-tail</td>
</tr>
<tr>
<td>0.516398</td>
<td>0.737865</td>
<td>= s.d.</td>
</tr>
<tr>
<td>2.100924</td>
<td></td>
<td>t Critical two-tail</td>
</tr>
</tbody>
</table>
displays typical student data for measurements of the diameters of zones of inhibition from gentamicin for an *E. coli* culture adjusted to a 0.5 McFarland standard, made separately by four different students. ANOVA (using a spreadsheet) suggests no significant difference between the sets of measurements.

**Table 2.** Measurements of the diameter of zones of inhibition made with the same ten samples by each of the four group members. Data were compared using ANOVA.

<table>
<thead>
<tr>
<th>Disk Diffusion</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Anova: Single Factor</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>- student</td>
</tr>
<tr>
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<td>29</td>
<td>30</td>
<td>30</td>
<td>31</td>
<td>SUMMARY</td>
</tr>
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<td>29</td>
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<td>31</td>
<td>A</td>
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<td>D</td>
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<td>29</td>
<td>28</td>
<td>29</td>
<td>30</td>
<td>31</td>
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<tr>
<td>Source of Variation</td>
<td>SS</td>
<td>df</td>
<td>MS</td>
<td>F</td>
<td>P-value</td>
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<td>Between Groups</td>
<td>2.7</td>
<td>3</td>
<td>0.9</td>
<td>0.716014</td>
<td>0.542441</td>
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<tr>
<td>Within Groups</td>
<td>45.2</td>
<td>36</td>
<td>1.295596</td>
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<td></td>
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<tr>
<td>Total</td>
<td>47.9</td>
<td>39</td>
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</table>

<table>
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<th>B</th>
<th>C</th>
<th>D</th>
<th>- student</th>
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<td>28</td>
<td>29</td>
<td>30</td>
<td>31</td>
<td>ANOVA</td>
</tr>
</tbody>
</table>

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7 ENUMERATION

Determining the number of cells is important in a wide range of applications, e.g., determining optimum growth parameters, measuring efficiency of antimicrobials, or environmental monitoring. Counts can be direct, where the cells themselves are counted, or indirect, where a property of the cells is measured. In addition, enumeration can involve all cells in a total count or only the live cells in a viable count.

Direct Counts

Total Counts

Microorganisms can be enumerated by using a microscope to actually count the number of bacteria contained within a standard, known volume. In general, a suspension of microorganisms is added to a specially designed and precisely manufactured microscope slide that is etched with a grid of known dimensions and uses a rigid coverslip to provide a uniform volume. Several types of counting chambers exist; the Petroff-Hauss er counting chamber is the most common one used in bacteriology while hemocytometers are used to make blood cell counts. By counting the cells within the grid (a known area) and multiplying that by the distance between the slide and coverslip, it is possible to calculate the concentration of cells per known volume. Prior to counting cells, it is almost always necessary to dilute the original sample.

Direct microscopic counts can also be made by staining cells with a dye and putting the sample through a membrane filter. Fluorescent dyes make this a very useful technique with environmental or other types of “dirty” samples.

The most common automated method for making total counts is with a Coulter counter. This instrument sips a set volume of a cell suspension and passes it through a narrow chamber with two electrodes that are used to measure the electrical resistance of the fluid flowing past. Resistance increases when a cell passes between the electrodes. Enumeration with a Coulter counter is fast and accurate as long as the medium does not contain non-cellular particles.

Viable Counts

Viable counts are especially useful when trying to determine the effectiveness of an antimicrobial, for example a disinfectant. Although fluorescent dyes are available that differentially stain metabolizing (live) and non-metabolizing (probably dead) cells, the most common way to obtain viable counts is by counting colonies on spread or pour plates. Plates used for viable counts must have between 30 and 300 colonies (150 mm diameter plate) for proper determinations. The lower limit is set on the basis of statistical accuracy and the upper limit is set on the basis of competition and overcrowding. As with microscopic counts, samples must be diluted.

Indirect Counts

Total Counts

Total cell numbers can be estimated from a property of cells, for example the concentration of protein, the dry weight, or the amount of light absorbed. In all cases, a standard curve must be
created to accurately associate the measured parameter with cell number.

Although cell features like dry weight or protein concentration can provide very accurate estimates of cell concentrations, the methods to obtain data are somewhat tedious and, like viable counts, may require quite a long time before the data is available. However, these methods can provide additional data, for example dry weights can be used to help determine growth yields.

The most common way to monitor growth is by measuring the amount of light absorbed by a culture. A culture tube is inserted in a spectrophotometer set at a specific wavelength (usually around 600 nm) and the absorbance is measured. As the cells grow, the culture becomes proportionally more turbid, providing an excellent relative measure of growth. This method is simple to perform, provides data within seconds, and is noninvasive.

**Viable Counts**

The most common method to indirectly measure viable cells is with the Most Probable Number (MPN) technique. The MPN technique estimates the number of viable cells in a sample using mathematical inference based on the number of tubes inoculated with different sample dilutions that show growth. Typically, MPN measurements are made with sets of three, five, or ten replicates at each dilution due to the availability of prepared interpretation tables.

**Dilutions**

In many situations the concentration of microorganisms are too high to perform direct counts. When this occurs, samples are diluted to workable levels. Dilutions are usually made in factors of 2 or 10, usually through a series. For most lab applications, diluents are dispensed in tubes so that the final volume (diluent + sample) is 10 ml. For example, 5 ml of *diluent* would be used for 1:2 dilutions, whereas 9 ml diluent and 9.9 ml diluent would be used for 1:10 and 1:100 dilutions, respectively. There isn’t anything magic about using 10 ml volumes, they’re just a convenient size to store and use. Besides using good aseptic technique, it’s important to use precise volumes with dilutions since even a small aberration can be multiplied several-fold during a serial dilution.

The mathematics of dilutions is straightforward but can be confusing if you just try to “fudge” your way through. Remember to keep track of your units (dimensional analysis) and learn the relationships involved in making dilutions. First, consider how *concentration* is defined:

$$\text{Concentration} = \frac{\text{Particles}}{\text{Volume}}$$

or,

$$C = \frac{P}{V}$$  \hspace{1cm} (7.1)  

For example, if 0.1 ml of culture is used for a spread plate and 250 colonies are counted:

$$C = 250 \text{ CFU}/0.1 \text{ ml} = 2500 \text{ CFU/ml}$$

Where:  
\(C\) = the concentration of colony forming units (CFU) per ml in the culture plated  
\(P\) = the number of CFU on the plate  
\(V\) = the volume plated

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However, what if the aliquot plated was from a 1/100 dilution of the culture? Of course, the concentration of CFU in the original culture would be 100X higher than the value calculated above, or 150,000 CFU/ml. This is an obvious relationship and seems somewhat intuitive but we can define the relationship mathematically.

When a suspension is diluted, the number of particles doesn't change:

$$P_1 = P_2$$  \hspace{1cm} (7.2)

but, since the volume increases, the concentration (P/V) decreases (the whole purpose of performing dilutions). By rearranging the equation for concentration (Eqn. 7.1) above:

$$P = CV$$  \hspace{1cm} (7.3)

Since the number of particles is the same in each sample, the concentration in each sample (expressed, for example, as CFU/ml) times the volume (ml) would be the same.

$$C_1V_1 = C_2V_2$$  \hspace{1cm} (7.4)

To calculate dilutions:

$$Dilution = \frac{Volume\ added}{Total\ volume} = \frac{Volume\ added}{Volume\ of\ diluent + Volume\ added}$$  \hspace{1cm} (7.5)

For example, if a culture contains $1 \times 10^9$ cells/ml and 0.1 ml is added to 9.9 ml of diluent,

$$Dilution = \frac{0.1\ ml}{(9.9\ ml + 0.1\ ml)} = \frac{0.1\ ml}{10\ ml} = 0.01 \text{ or } 1/100$$

The Dilution Factor (DF) is the reciprocal of the dilution, or $1/dilution$.

The Total Dilution is the product of all dilutions:

$$D_{total} = D_1 \times D_2 \times D_3 \ldots$$

What does all this mean for spread plates and pour plates? The initial concentration, $C_1$ (that is, the concentration of cells in the undiluted culture) is equal to $C_2$ (the number of colonies/volume plated, or $P_2/V_2$) times the Dilution Factor, or:

$$C_1 = C_2DF = \left(\frac{P_2}{V_2}\right)DF$$  \hspace{1cm} (7.6)

where

- $C_1$ = initial concentration (or concentration in the stock culture)
- $C_2$ = CFU/ml (or the concentration of CFU on the plate)
- $P_2$ = CFU on the plate
- $V_2$ = Volume plated (ml)
- DF = Dilution Factor
Optical Density Measurements

Background
Microbial concentrations can be measured indirectly in a number of ways, the most common relying on light scattering by a suspension of cells. Somewhat simply defined, light scattering is what occurs when light energy strikes an object and is not absorbed. The light must be re-radiated and can emerge in any direction. The light scattered from a small particle depends inversely on the fourth power of the wavelength of light. Therefore, small particles scatter blue light more strongly and transmit red light more efficiently. (That's why the sky is blue except when the sun is below the horizon.) Microbes are large enough so that light scattering most nearly approximates an inverse second power, and therefore appear whitish (less separation of color).

The most common instrument from measuring optical density is a spectrophotometer. Essentially, light of a specific wavelength is directed through a microbial suspension and a photocell measures the amount of light that comes through. The intensity of the light striking the photocell is measured by a galvanometer connected to a display (analog or digital), with a result we interpret as absorbance (A) or optical density (OD). Most colorimeter and spectrophotometers also have a scale for the amount of light transmitted (%T). Although this can be a useful unit at high turbidities (converting the values to absorbance), microbiologists tend to use absorbance since this is directly proportional to the cell density of a suspension. In colorimeters the wavelength of light is set using filters, whereas spectrophotometers use prisms or defraction gratings.

Absorbance is defined as the logarithm of the quantity expressed by dividing the incident light by the transmitted light. That is,

\[ A = \log \left( \frac{I}{I_o} \right) \]

where: \( I_o = \) the incident light
\( I = \) the transmitted light.

This relationship comes from Lambert's law, which states that the proportion of incident light absorbed by a medium is independent of its intensity and that each successive unit layer of the medium absorbs an equal fraction of the light passing through it. Thus,

\[ A = \log \left( \frac{I}{I_o} \right) = \frac{C_o}{C} \]

where: \( C_o = \) concentration of light absorbing material in a standard
\( C = \) concentration of light absorbing material in a sample

What this means to us, is that the relationship between absorbance and cell concentration is linear! That is, if you double the number of cells, you double the absorbance. An important point to remember is that in reality, this relationship is not true at high concentrations of cells and that the absorbance becomes increasingly less than what the formula predicts. This is due to more light getting through than expected because light scattered from one bacterium is rescattered by another bacterium so that the light is redirected back into the phototube. Furthermore, there is intercellular interference with Brownian motion, resulting in a more evenly distributed cell suspension which scatters less light away from the beam.

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As stated above, turbidometry is an indirect method for microbial enumeration. Indirect refers to the fact that it is not microbial cells that are being enumerated directly, but their ability to scatter light. In order to determine the actual number of microbes, a standard curve must be generated in which microbial concentrations are measured directly (as with a counting chamber) and plotted against the absorbance of the culture from which the sample was counted. It follows, therefore, that absorbance is a measure of total cell concentration.

**Purpose**
To become familiar with the principles and use of a spectrophotometer

**Procedure**
Using different concentrations of dyes, read absorbance at different wavelengths of light. Note the linear relationship of concentration to absorbance and that the spectrophotometer needs to be re-zeroed for each change in wavelength.

---

**Direct Microscopic Counts with a Hemocytometer**

**Background**
Microorganisms can be enumerated by actually counting the number of bacteria contained within a standard, known volume. Several types of counting chambers exist; the Petroff-Hausser counting chamber is the most common one used in bacteriology. For this exercise, we will use hemocytometers, which are similar to, but contain a larger volume than Petroff-Hausser chambers. This method of quantitation produces a total cell count (as opposed to a viable cell count). A total cell count includes viable (living) and non-viable organisms. Therefore, viable cell counts are either equal to or less than total cell counts.

Counting chambers are carefully constructed slides which have a grid etched into an area contained within a small enclosure. With the addition of a flat, rigid coverslip, a sealed volume is contained above the grid. It is assumed (although it is often not the case) that the volume is exact. By counting the cells within the grid, one is able to calculate (by knowing the grid and chamber dimensions) the number of cells/ml. Hemocytometers we use are 0.1mm deep and divided into 9 squares of 1 mm². These squares are further divided into 25 smaller squares, which are further divided into 16 squares. Therefore, there are 400 (25 x 16) small squares/mm² (i.e., each small square is 1/400 mm²).

The major advantages of this method are:

1. It's easy and relatively inexpensive.
2. It provides additional information (e.g. morphology)

Major disadvantages are:

1. It is difficult to reproducibly fill the counting chamber
2. Bacteria can adsorb to coverslips, pipettes, etc.
3. Motile cells can be difficult to count and lead to errors.

**Purpose**
To make direct counts of a *Saccharomyces* culture using a hemocytometer.

**Materials and Methods**

**Saccharomyces cerevisiae**

hemocytometer

1. Suspend yeast cells and make appropriate dilutions (probably 1/10 and 1/100).

2. Place coverslip on hemocytometer and use pasteur pipet to fill both chambers at introduction point.

3. Focus on central square at 100X. Count cells at 400X in at least five (5) secondary squares (1/25 mm²), *i.e.*, the 4 corners and the center squares. *For cells on lines, count left and top, not right and bottom. Buds of equal size count as 2, not 1 cell.*

4. Calculate the concentration:

   \[
   \text{(# cells in 5 squares) x 5 = # cells/mm}^2 \text{ surface area (include DF, if necessary)}
   \]

   \[
   \text{(# cells/mm}^2\text{) x 10 = # cells/mm}^3 \text{ (volume, since chamber is 0.1 mm deep)}
   \]

   \[
   \text{(# cells/mm}^3\text{) x 1000 = # cells/cm}^3 \text{ or # cells/ml}
   \]

Putting it all together:

\[
\text{# cells/ml = (# cells in 5 squares) x (5 x 10^4) x DF}
\]

**Results**

Values for the total concentration of yeast cells.

**Viable Counts of *Saccharomyces cerevisiae***

**Background**

By definition, *viable cells* are able to divide, and thus give rise to a *colony* when cultivated under suitable conditions. Therefore, the number of colonies arising from a specific culture volume can be used to calculate the concentration of viable cells within the culture. Use of selective media allows enumeration of specific groups of bacteria. The two most common methods of performing viable counts are the **spread plate** and the **pour plate**.

For pour plates, the culture aliquot is added directly to molten agar (45-50°C), mixed, and poured into an empty petri dish. Colonies arise throughout the medium, so growth of aerobes can be limited. For spread plates, a small volume of cell suspension is dispensed on an agar plate and spread with a sterile glass rod.

It must be recognized that these methods assume that colonies are well-separated and growth conditions are good. Since less than optimal conditions (*e.g.*, cells clumping or inhibition of growth) can skew viable counts, results are expressed as **colony forming units/ml** (CFU/ml), rather than as cells/ml. For statistical accuracy, only plates with between 30 and 300 colonies are counted. An additional limitation of spread plates is that no greater than 0.2 ml is spread on a
plate or the surface will be too wet, causing colonies to run into each other. To meet these requirements, cultures often need to be diluted.

**Purpose**
To perform viable counts of a yeast culture, using pour and spread plates.

**Materials and Methods**

*Saccharomyces cerevisiae*

empty petri plates
sterile 1 ml pipettes
diluent tubes (9 ml 0.7% saline)
tubes of molten PDA agar (9 ml)
PDA plates

1. Dilute yeast cultures 1/10 and 1/100 (9 ml blanks)
2. **Spread Plates**: Dispense 0.1 ml of each dilution to a PDA plate and spread with sterile glass "hockey stick".
3. **Pour Plates**: Add 1 ml of each dilution to 9 ml molten agar, mix, pour into empty petri dishes. Pour and mix in an additional 9 ml molten agar.
4. Incubate plates at 30°C.

**Results**
Colonies on each plate are to be counted. Spread plates represent $10^{-2}$ and $10^{-3}$ dilutions, while pour plates are $10^1$ and $10^2$ dilutions.
8 PIPETTING AND SPECTROPHOTOMETRY

Background

The pipet is a fundamental tool in biological and chemical laboratories and its proper use is an important basic skill. Although the principle behind using pipets is simple, it is critical that sufficient practice be performed to develop reproducibility. Serological pipets, the most common pipets we use, are easy to read by holding the pipet vertically and observing the graduation lined up with the bottom of the meniscus. Although that appears simple, there are a number of factors that need to be considered. For example, what is the volume of the pipet? Although 1, 5, and 10 ml pipets are most common, other sizes, larger and smaller, can be purchased. Knowing the volume of the pipet being used is related to observing what volumes are represented by the graduations. Another point to consider is if the pipet is “to deliver” (marked T.D. or has a double ring at the top) or “to contain” (marked T.C. at the top). Pipets that are T.D. deliver the desired volume when fluid is allowed to flow freely from the pipet; T.C. pipets release the chosen volume when the fluid is released to a certain point. (In other words, T.D. pipets include the volume in the tip while T.C. pipets do not.)

Micropipets are small hand pumps that allow the user to set a volume (typically 1-1000 µl) and have disposable tips. Depressing a spring-loaded plunger evacuates the pump, allowing the preset volume to be sucked into the pipet tip. Depressing the plunger once more dispenses the fluid. In order to totally evacuate the pipet tip, the plunger is actually depressed past the point used to fill the pump. Although simple to use, variations between samples can occur due to slight differences in the amount the plunger is depressed or sloppiness in setting the volume. (Dropping the micropipet can also wreak havoc with its calibration.) The more practice one has with a micropipet, the more reproducible the volume transferred.

There are many other pipets used in the lab, for example glass Pasteur pipets and disposable plastic pipets with an integrated bulb (often sterile and packaged individually). These types of pipets are useful for transferring solutions or even inoculating cultures but do not provide the quantitative precision of serological or micropipets.

In this exercise, pipetting precision and reproducibility will be investigated by performing replicate dilutions of methylene blue and measuring their absorbance with a spectrophotometer. Furthermore, dilutions of a yeast suspension will be used to observe the relationship between cell density and absorbance.

Materials

serological pipets (1 and 10 ml)
micropipets (100 µl)
16 x 150 mm test tubes
spectrophotometers
microscopes
hemocytometers
methylene blue solution (1 mg/ml), 25 ml per group of 4 students
dense yeast suspension in 5% NaCl, 100 ml per group of 4 students
Procedure

**Determination of Variability in Pipetting**

1. Dilute the methylene blue solution 1/400 using micropipets and 1 ml serological pipets with solutions containing dye and 10 ml serological pipets for the diluent (use water). Each person should do this at least five (5) times. You may use any dilution scheme desired but each person in the group should use the same scheme.
2. Make five more 1/400 dilutions of the methylene blue dye using only the 10 ml serological pipets. Each person should make at least five dilutions this way.
3. Read the absorbance of each 1/400 dilution at 600 nm and record the results.

**Determining the Relationship Between Cell Density and Absorbance**

1. Each group needs to dilute the yeast suspension in water to 10%, 20%, 30%, 40%, 50%, 60%, 70%, and 80% of the stock.
2. Measure the absorbance of each dilution at 660 nm. Use the same tube for each reading. What fluid will you use in your blank?
3. Measure the absorbance of each dilution at 440 nm.
4. Plot the data and if a linear portion is not apparent, perform additional dilutions and measure their absorbance.
5. Use a hemocytometer to make a total cell count of the stock yeast suspension. (See Chapter 7.)

**Results**

1. Record your data.
2. Analyze the dilution data to see if there are any differences between the absorbencies resulting from using different pipets. Perform ANOVA on your data (three sets of five measurements) and the group data (three sets of 20 measurements).
3. Calculate the means and standard deviation of your data. (See Chapter 6.)
4. Plot the data (A$_{660}$ vs. Relative Density) from the yeast dilutions and determine the range of cell density where absorbance is linear. Do the same with A$_{440}$ data.

**Conclusions**

1. Are dilutions made using different pipets the same? Explain how you made your decision.
2. If you answer to question 1 was “no,” which dilution method appears most accurate and precise?
3. At what absorbance did cell density become nonlinear? Was there a difference due to the wavelength used?
4. Using the total cell count, can you establish a correlation between cell density and absorbance?
APPENDIX

Colony Morphology

Isolated colonies should be described using common terms relating to six different features. These features are:

- **Size**
  - diameter (mm) after specific incubation time
  - pinpoint, small, medium, or large (not as useful)

- **Colony surface**
  - smooth or rough
  - shiny or dull
  - firm, viscous, or dry

- **Pigmentation**
  - any color, soluble or insoluble
  - opaque, translucent, iridescent, pr opalescent

- **Shape**
  - circular, irregular, rhizoid, or lenticular (lenticular usually applies only to embedded colonies)

- **Edge or Margin**
  - entire, lobate, undulate, or filamentous

- **Elevation**
  - raised, flat, convex, or umbonate

![Diagram of colony shapes, margins, and elevations]