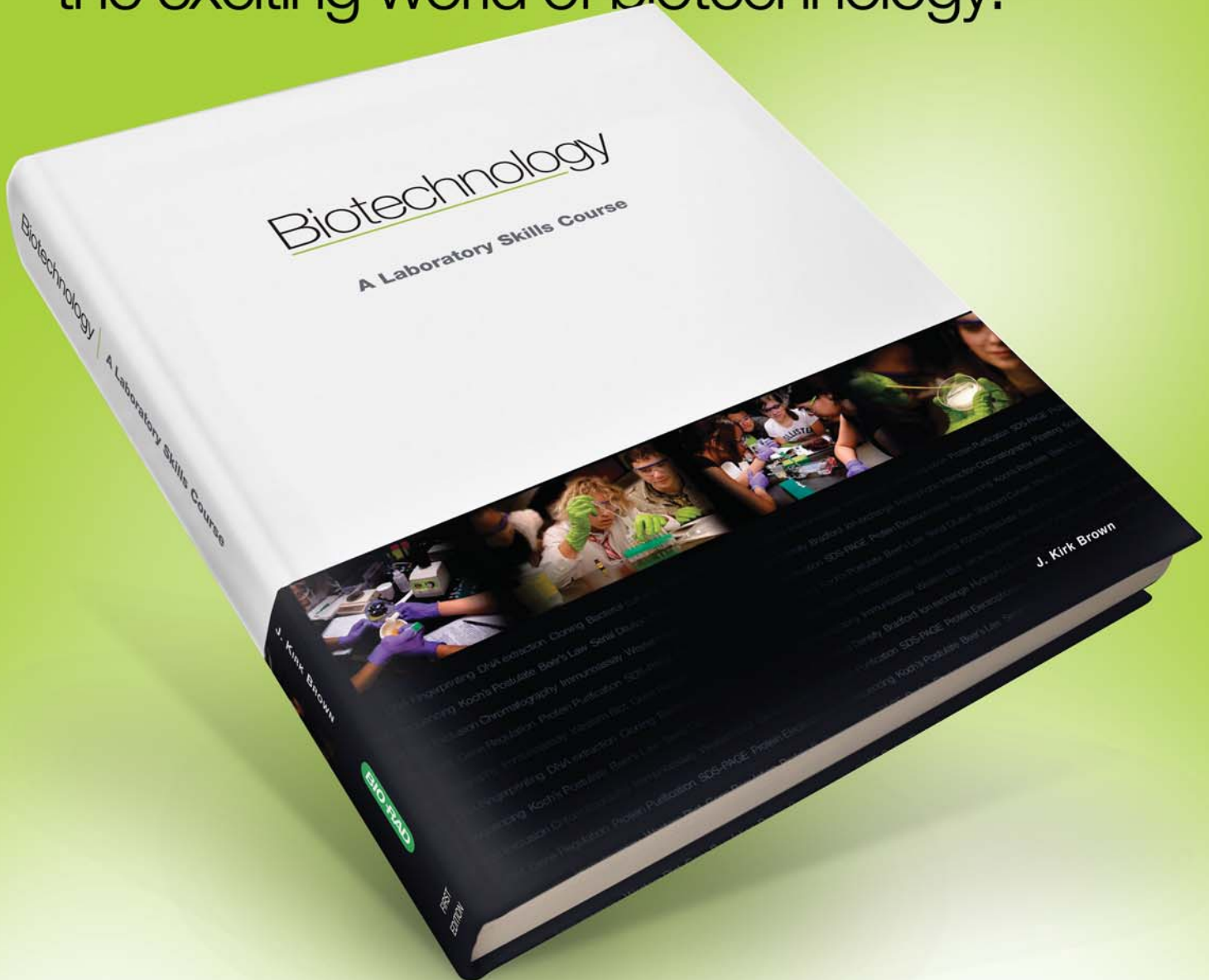


Biotechnology: A Laboratory Skills Course

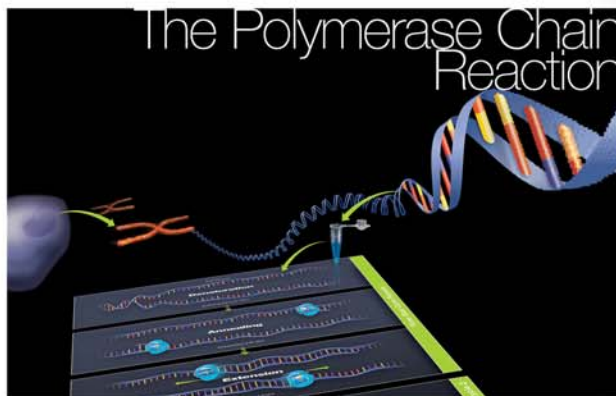
will guide you and your students through
the exciting world of biotechnology!



BIO-RAD

Student Textbook Content

Essay questions follow the background section for each chapter and act as starting points for independent literature research beyond the textbook.



Chapter 6: Overview

- ✓ Invention of PCR
- ✓ What is PCR?
- ✓ Thermal Cycler
- ✓ Types of PCR
- ✓ PCR Optimization
- ✓ Techniques Based on PCR
- ✓ Real World Application of PCR
- ✓ Laboratory Activities
- Activity 6.1 STR PCR Analysis
- Activity 6.2 GMO Detection by PCR
- Activity 6.3 Detection of the Human PV92 Alu Insertion

Summary

The polymer chain reaction (PCR) has revolutionized the study of living things. Invented by Kary Mullis in 1983, PCR has been a springboard for molecular biology research. It is the basis of the Human Genome Project, modern forensic analysis, and genetic engineering. Using PCR, a small sequence of DNA of a few hundred base pairs can be found within a genome of billions of base pairs. Billions of copies of the sequence are then generated, making the DNA sequences available for study. PCR has been used to improve the accuracy of DNA fingerprinting, to identify genetically modified crops. Even more recently, cows and goats have been genetically engineered to produce pharmaceutical drugs in their milk, creating a new industry called pharming. PCR has made forensic analysis cheap, fast, and extremely accurate. Today's DNA profiles have less than a one in a trillion chance of matching another random individual, providing law enforcement with a powerful tool to fight crime. PCR has also been used to compare Neanderthal and human DNA. The insights this has had on the population of humans thousands of years ago are astounding. In this chapter use PCR to investigate DNA profiling, to detect genetic modifications in food, and to study human ancestry.

Invention of PCR

was on a stretch of highway 128 in California at mile marker 46.58 in April 1983 that Kary Mullis (see Figure 6.1) had an epiphany. He actually pulled over and sketched out the concept that would later be known as the polymerase chain reaction (PCR). He had envisioned the use of primers to bracket and replicate a section of DNA. Mullis was a chemist working at Cetus, one of the first biotech companies in the U.S. (Cetus was acquired by Chiron Corporation in 1991, and Chiron was later acquired by Novartis International AG in 2006). Mullis ran a laboratory that made oligonucleotides (short, single strands of DNA) and was interested in methods to sequence single nucleotide polymorphisms (SNPs). After reporting his theory to the company, he was placed on the project full time. In December 1983, Mullis got the process to work by using a mixture of primers to amplify a target DNA. He generated millions of copies of the target DNA sequence. Mullis was given a \$10,000 bonus at the time of his discovery. He left Cetus in 1986 and won the Nobel Prize in 1993 in chemistry for his invention. After much controversy regarding the patents for PCR, they were sold to Hoffman-LaRoche for \$300 million in 1992.

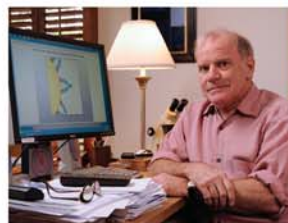


Figure 6.1. **Kary Mullis.** Mullis won the Nobel Prize in 1993 for the development of PCR.

The Nobel Prize was given to Mullis because of the impact PCR has had on the world. PCR revolutionized molecular biology and impacted research in almost all fields of biology and beyond. PCR made gene cloning and DNA fingerprinting accessible and affordable to most research laboratories, where these technologies could previously be performed only by specialists at great expense and effort. Even more importantly, PCR paved the way for brand new technologies such as automated sequencing, which allowed the Human Genome Project to be completed.

What is PCR?

PCR is a simplified version of bacterial DNA replication that copies a specific sequence of DNA (the target sequence) again and again, so that the target sequence is amplified. The target sequence is then amplified exponentially to make millions or billions of copies. The final product of PCR is called the PCR product or amplicon.

The strength of PCR lies in its ability to specifically target a section of DNA within a much larger mass of DNA such as a whole genome. The sequence is targeted with short, single strands of DNA, called primers, which are designed to match and bind (anneal) each end of the target sequence. The first primer, called the forward primer, anneals at the beginning of the targeted region of DNA, and the second primer, called the reverse primer, is designed to bind at the end of the targeted region (see Figure 6.2). Primers provide the specificity of PCR, selecting the region to be amplified.

Primer binding creates a double-stranded region of DNA necessary for the DNA polymerase to bind and start replicating the DNA. Special DNA polymerases are used in PCR. They are heat

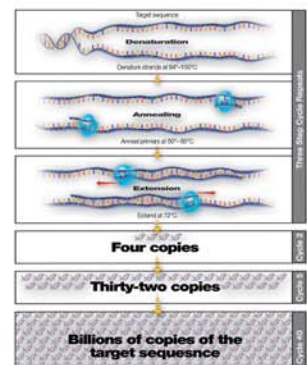


Figure 6.2. The polymerase chain reaction. One cycle of PCR consists of separation of strands (denaturation), binding of primers to the single stranded DNA in a specific location to bracket the target sequence (annealing), and extension of the primer by DNA polymerase, which reads the sequence from the template DNA strand and adds complementary nucleotides to the 3' end of the primer (extension). These three steps are repeated and double the number of copies of the target sequence each cycle. By cycle 5 there are 32 copies and by cycle 40 there are billions of copies.

Chapter overview gives a roadmap of subject matter covered in the book.

Four types of vignettes – show how biotechnology concepts covered in the chapter play a role in our daily lives. Vignette topics include discussions about bioethics, careers, spotlights on key skills, and real-life case studies.

Bioethics

Personal Genetic Information

On May 27, 2008, President Bush signed into law the Genetic Information Nondiscrimination Act (GINA), which prohibits U.S. insurance companies and employers from discriminating on the basis of information derived from genetic tests, which includes reduced coverage or altered pricing, and prohibits employers from making adverse employment decisions based on a person's genetic code. In addition, insurers and employers are not allowed under the law to request or demand a genetic test.

In the fall of 2010, the University of California-Berkeley offered incoming freshmen genetic testing as part of an introductory program to stimulate student interest in broad-reaching issues, such as personalized medicine and genetic testing. When this program was announced, there was clamor from much of the campus community and others. The program was eventually modified such that students would not receive their own results. What impact would the test results have had on students' lifestyles had students received their genetic information? Would you submit your DNA for testing?

Direct-to-consumer personal genetic tests are now available to purchase online. These tests allow consumers to send saliva samples to a commercial laboratory that tests for genetic risk factors for Alzheimer's disease, breast cancer, diabetes, heart disease, lung cancer, and multiple sclerosis. Consider how different people would react to the news that they are at higher risk of a disease. How do consumers interpret disease results? Do consumers understand how a positive result relates to the probability that they will acquire the disease? If a woman finds that she is at higher risk of breast cancer, should she take radical action, such as a double mastectomy to eliminate the risk of breast cancer, or would more frequent mammograms be a better course of action?



Careers In Biotech

Charles Lo, PhD, MBA
Commercial Rotation Development
Program Associate, Genentech
South San Francisco, CA



research in an ecology laboratory and decided to continue a career research by pursuing a PhD at the University of California, San Francisco, followed by a post-doctoral fellowship at the University of Chicago, IL, and the University of California, San Diego. While completing her training as a scientist, Charles learned his early lived science was not what interested in the business side of science. So, he went back to New York to get an MBA from Cornell Business School. Upon returning to the US, he got accepted into a 3-month intensive training program in analysis and marketing at the University of California, San Diego. He then worked for 10 years as a senior manager in a large pharmaceutical company. He then quit his job to become a full-time entrepreneur. Charles called himself approximately 125 physicians, which gave him a sense of perspective that he never felt before about how science impacts the lives of individuals. The connection between scientific research, people in the real world, and business became clear to him. He was now in a second circle at Genentech headquarters in South San Francisco CA, working in a marketing department, helping experienced doctors at the field, combined with his rich background in science, his great Charles passed right into the business world and was given a lot of freedom to be a leader that he wanted to be. He was now in the third circle. His experience as a research scientist gave growth in the field provided for a career in science and marketing.

Chapter 4

How To...

Set Up a Restriction Digest

To set up a restriction digest, the components and quantities need to be determined. First, calculate the necessary volume of DNA. This depends on the concentration of the DNA sample. For example, if 1 µg of DNA is to be digested in a 20 µl reaction volume and the stock is 0.1 µg/µl then 10 µl of DNA should be used.

Second, calculate the amount of restriction enzyme required. This depends on the concentration of the enzyme which is typically printed on the enzyme tube label. In a digest, 10 U of enzyme are commonly used per μg of DNA. For example, if the enzyme concentration is 15,000 U/mL, then 1 μL of enzyme contains 15 U and, 1 μL of enzyme should be added to the reaction.

Third, determine the type and volume of restriction-digestion buffer. Most restriction buffers are provided at a 10x concentration and are generally packaged with the enzyme. If a digestion using two enzymes is to be performed, look up the manufacturer's recommendation to determine the appropriate buffer for your application.

Finally, calculate the amount of water required to bring reaction up to the final volume.

To set up the reaction, add the calculated volume of the components to a microcentrifuge tube in the following order: water, buffer, DNA, and lastly, enzymes. Mix the components by pipetting up and down, and pulse-spin the tube in a microcentrifuge to collect the contents at the bottom. Incubate the reactions at 22°C in a water bath or dry bath for 30–60 min. Place the tube at 4°C until analysis on an agarose gel.

Concentration	Quantity	Volume in Digestion Reaction
DNA (stock 10 ng/ μ l)	1 μ g	10 μ l
10 \times restriction buffer	1 \times	2.0 μ l
Restriction 100 U/ μ l	5 U	0.5 μ l
Molecular biology grade water	q.t. for 20 μ l (up to 10-15 μ l added)	7.5 μ l
	Total	20 μl

Chapter 4

Biotech In The Real World

Did the Baker Do It?

The rights to a method for making the extrastreamline, isotachic, katalytic first use of DNA fingerprinting in a criminal case are said to accrue to a scientist. In 1986, Alec Jeffreys received a call from the Leicestershire police who were investigating the rape and murder of two schoolgirls, Linda Mann and Dawn Ashworth, who lived in the village near Leicester, UK. A local man, Richard Buckland, had confessed to the murder of Dawn Ashworth but refused to confess to the killing of Linda Mann. Dr. Jeffreys had been conducting tests using DNA fingerprinting to differentiate sources of DNA using RFLP analysis. The police wondered if it was possible to test Buckland's DNA against the DNA in the samples of semen collected from the girls' bodies. Results of the DNA fingerprints showed that the semen from both girls came from the same man but did not from Buckland. The DNA was completely different. He was not the murderer. The results were unexpected, and the facts were repeated and then confirmed by a different laboratory. In the end, the police accepted that Buckland was innocent, and he was cleared of the girls' murders on November 21, 1986.

Crushed by this new technology, the Los Angeles police decided to narrow down the suspect pool. In January 1996, the police requested that all of the local men between the ages of 17 and 34 submit DNA samples for testing to eliminate themselves from the investigation. By September 1996, samples from over 4,000 men had been tested without success. A village was in the local pool one day and admitted to the friends that he had provided a CFA sample on behalf of Cole Pitchfork, a local baker. One of his friends later told the police, and Pitchfork was arrested in September 1996. Pitchfork's DNA was tested by Jefferys and shown to match that of the samples from the bodies of the two girls. On January 23, 1998, Pitchfork was sentenced to life for the murders of Linda Mason and Dawn Amato.



Activities implement the techniques described in the background information. Early activities focus on building basic skills, while later activities use those basic skills as a foundation for more advanced techniques.

Laboratory skills are acquired by performing the activity. The requirements necessary to claim proficiency in those skills are described in the Laboratory Skills Assessment Rubric in Appendix E.

Graphics illustrate the hands-on activities to help students learn techniques.

Step-by-step protocols lead students through procedures and provide guidance on results analysis.

Activity 3.4 Gram Staining

Overview

In 1882, a technique to discriminate between the two types of bacterial cell walls was invented by the Danish scientist Hans Christian Gram. This technique utilizes a four-step staining procedure using two different dyes and is still one of the first tests used when trying to identify unknown bacteria. Gram-positive bacteria have a very thick layer of peptidoglycan composed of layers of carbohydrates cross-linked with polypeptides. Crystal violet stain binds peptidoglycan very tightly and makes the bacteria a deep purple color. Gram-negative bacteria have a very thin layer of peptidoglycan in between two layers of phospholipid membrane, and the crystal violet stain does not bind well and is washed out by decolorizer (alcohol). Safranin, which is used as a counterstain, makes gram-negative bacteria appear pink (see Figure 3.32).

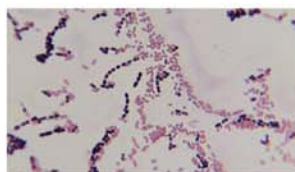


Figure 3.32 A mixture of *E. coli* and bacteria from yogurt. The mixture was Gram stained and viewed under an oil immersion lens at 1,000x magnification.

In this activity, you will perform Gram staining of *E. coli* HB101 and yogurt bacteria. You will observe the stained bacteria using a microscope and determine the shape of the bacteria and assess whether they are gram-positive or negative.

Tips and Notes

Wear gloves to avoid staining your fingers. Have multiple beakers of water available for washing the stain from the slides during the Gram staining procedure. Wooden clothes-pegs can be used to hold slides while staining and flaming; these clothes-pegs can lower the chance of getting the stain on your fingers.

Safety Reminder: Review the MSDS of all the stains used in this activity. Before placing any stains on the benchtop, ensure that flaming of slides is complete and Bunsen burners are turned off. The decolorizer contains a high percentage of alcohol and is very flammable. Wear appropriate PPE.

Research Questions

- Are bacteria found in yogurt and *E. coli* HB101 bacteria gram-positive or gram-negative?

- What is the size and shape of *E. coli* HB101 and bacteria from yogurt?

Objectives

- Mount bacteria on a microscope slide using aseptic technique
- Perform Gram staining of bacteria
- Determine gram status of bacteria
- Determine cell shape of bacteria
- Determine size of bacteria

Skills to Master

- Perform Gram staining of bacteria
- Heat fix bacteria to a slide
- Observe bacteria using a microscope
- Differentiate gram-positive bacteria from gram-negative bacteria
- Identify bacteria from cell shape
- Estimate the size of cells using a microscope

Student Workstation Materials

Items	Quantity
Microscope and optional accessories, including immersion oil and lens cleaning tissue	1
Stage micrometer (optional)	1
Microscope slide	1
Bunsen burner	1
Inoculation loop*	1-2
Wooden clothes peg (optional)	1
Tissue or paper towel	1
Wax pencil	1
Microbial waste container	1
Sterile water	1 ml
Beakers of tap water	3
Crystal violet stain	1
Gram's iodine	1
Decolorizer (alcohol)	1
Safranin stain	1
LBS agar plate with yogurt bacterial colonies (from Activity 3.3) or fresh yogurt	1
Agar plate with <i>E. coli</i> HB101 (from Activity 3.3)	1

* If a metal inoculation loop is available, one loop is sufficient and must be sterilized by flaming with a Bunsen burner. Alternatively, two disposable plastic loops can be used.

Prelab Focus Questions

- What part of the bacterium does the Gram staining procedure stain?
- Describe the three basic shapes of bacteria, and give the scientific terms used to describe these shapes.
- How do you determine the magnification when using a microscope?

Research questions and objectives outline the experiments.

Prelab focus questions ensure students' understanding of the activity, and postlab focus questions help students analyze their results and generate conclusions.

Assessment rubrics help students understand what is expected of them and how to proficiently complete a task.

Activity 3.4 Gram Staining Protocol

Activity Protocol

Part 1: Heat Fix Bacteria to the Slide

- Using a wax pencil, draw two circles about 1 cm in diameter at one end of a microscope slide. Label the left circle **Yogurt** and the right ***E. coli***.
- If using a metal inoculation loop, sterilize it by flaming. Sterile plastic loops should not be flamed.
- Using aseptic technique, dip the loop in sterile water so that a film appears across the loop. Transfer the water into the circle drawn by the wax pencil. Flame the metal inoculation loop again or use a new sterile plastic loop, and transfer water into the second circle.
- Flame the metal inoculation loop again or obtain a new sterile plastic loop. Use the loop to very lightly touch a yogurt colony from the **yogurt** agar plate or touch the liquid on top of the fresh yogurt. Make sure to touch the bacterial colony or yogurt very lightly to avoid transferring too many bacteria.

Appendix E: Laboratory Skills Assessment Rubric

Activity	Skill	Novice	Developing	Proficient
2.1 2.2 2.3 2.4 2.5 and all activities	Follow laboratory protocols	Student may not understand the importance of following proper laboratory procedures. Procedure is performed out of order or is missing steps, or the methods recorded in the laboratory notebook are incomplete.	Student understands the importance of following proper laboratory procedures. Procedure is performed in the appropriate order but one or more procedural steps are missing. The methods recorded in the laboratory notebook are missing one or two steps.	Student understands the importance of following proper laboratory procedures. Procedure is performed in the appropriate order with no steps missed. The methods are clearly and completely recorded in the laboratory notebook.
2.1 and all activities	Select and wear proper PPE	Student may not understand the purpose of PPE. Student may need to be reminded to wear PPE, is missing critical PPE, or does not verify with the instructor that the PPE is appropriate.	Student understands the purpose of PPE. Student remembers to wear the most critical PPE but may be missing PPE that protects clothing. Student may not have verified with the instructor that the PPE is appropriate.	Student understands the purpose of PPE. Student wears PPE appropriate to the task and PPE is worn correctly. Student asked the instructor for information on appropriate PPE when unsure.
2.1	Extract DNA from cells	Student may not understand the purpose behind DNA extraction. Student performs the procedure incorrectly, misses steps, or performs steps out of order, resulting in no visible DNA.	Student understands the purpose behind DNA extraction. Student performs procedure but performs one or more steps incorrectly. DNA is visible but may be broken up (flocculent) or present in small quantities, making collection difficult.	Student understands the purpose behind DNA extraction and follows procedures correctly. DNA is easily visible and may be present in strands or clumps that can be transferred to another container.
2.1	Precipitate DNA	Student may not understand the principles of DNA precipitation. Student may perform the protocol incorrectly and DNA is not visible.	Student understands the principles of DNA precipitation. Student may handle the sample roughly, leading to DNA that is broken up or flocculent.	Student understands the principles of DNA precipitation, performs the protocol carefully, and obtains white, thread-like pieces of DNA.
2.1 2.2 2.3 2.4 2.5 2.6 and all activities	Maintain a laboratory notebook	See Laboratory Notebook Rubric (Appendix F).	See Laboratory Notebook Rubric (Appendix F).	See Laboratory Notebook Rubric (Appendix F).
2.2 2.3	Use a serological pipet with a pipet pump or filler	Student may not demonstrate the ability to use a serological pipet correctly. Student may not use a pump or filler, or inserts the pipet loosely into the pump or filler such that liquid does not remain in the pipet and pours out upon transfer. The cotton plug becomes wet or volumes transferred are inaccurate.	Student demonstrates the ability to use a serological pipet correctly. Student inserts the pipet into the pump or filler correctly but liquid leaks. Student does not read the volume from the bottom of the meniscus or transfers a slightly inaccurate volume.	Student demonstrates the ability to use a serological pipet correctly. Student inserts the pipet into the pump or filler correctly and no liquid escapes. Student reads the volume from the bottom of the meniscus and transfers an accurate volume.

Microbiology and Cell Biology

Cell biology is the study of cells. Microorganisms are comprised of single cells or small clusters of cells; in contrast, human tissues are made of millions of cells working in concert. Cells are either prokaryotic or eukaryotic. Eukaryotic cells have a nucleus and other membrane-bound organelles. Prokaryotic cells do not have a nucleus or membrane-bound organelles and are much smaller than eukaryotic cells (see Figure 3.1).

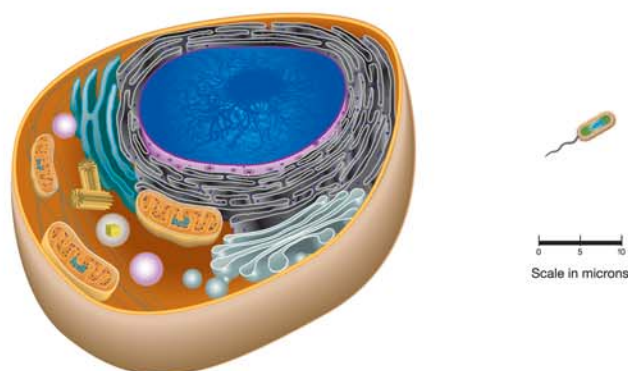


Figure 3.1. Size comparison between prokaryotic and eukaryotic cells.

Part of cell biology, **microbiology** is the study of microorganisms and their effects on other living organisms. The terms **microbe** and **microorganism** refer to organisms that must be viewed at the microscopic level such as bacteria, yeast, algae, protozoa, fungi, and viruses. (Note: There is no consensus on whether viruses are living organisms; however, since viruses can be viewed with a microscope, they are often included under the umbrella of microbiology.) The majority of naturally occurring microbes are harmless, and many are used in biotechnology to benefit mankind. However, harmful microbes cause many types of disease affecting people, animals and plants.

Understanding cell biology and microbiology is necessary in biotechnology because cells do much of the work of biotechnology, such as making **recombinant DNA** and proteins and because cells are the targets of many biotechnological products. For example, drugs are engineered to target specific proteins on the plasma membranes of human cells.

Three Domains of Life

Life on earth is organized into three domains: Archaea, Bacteria, and Eukarya (see Figure 3.2). Archaea and Bacteria are prokaryotes. Eukarya include yeast, algae, and humans. There are many more prokaryote species than eukaryote species on earth, and there are more Bacteria than Archaea.

Archaea were not recognized as a separate domain until the 1970s. Currently, there are no known human diseases caused by Archaeans. Archaea are found in most habitats on earth including the human digestive system. Many Archaea live in very harsh environments such as deep ocean volcanic vents or salt lakes and

are called **extremophiles**. Archaea are being investigated as sources of enzymes that could be used in harsh manufacturing or experimental conditions (for example, enzymes used to process food at high temperatures). Bacteria and eukaryotic cells are described in detail in this chapter.

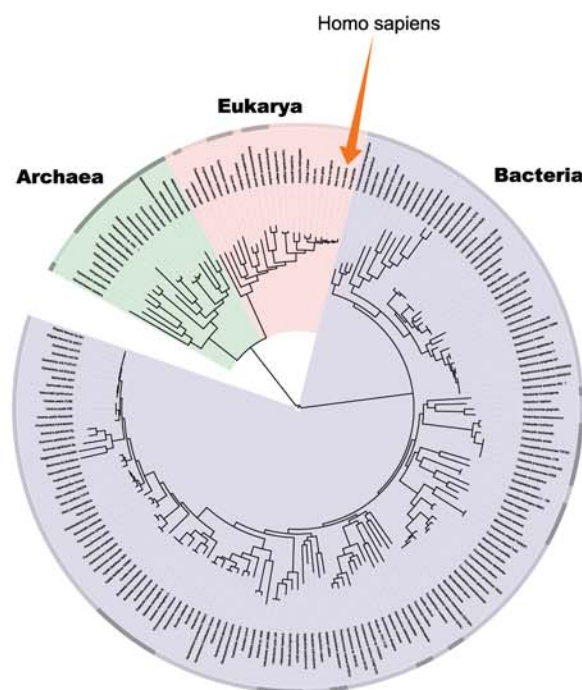


Figure 3.2. The three domains of life. This is a phylogenetic tree based on the genetic similarities between organisms that have had their genomes sequenced. Three domains can be seen: Archaea (in green), Bacteria (in blue), and Eukarya (in pink). The position of humans (*Homo sapiens*) is shown. This image was generated using Interactive Tree Of Life (iTOL), an online phylogenetic tree viewer. iTOL (2006) <http://itol.embl.de/>. Accessed October 2, 2010.

Microorganisms and History

Throughout history, man has been afflicted by diseases. The impact of diseases such as malaria, anthrax, and plague has changed the course of history. Analyses of the remains of King Tutankhamun in Egypt (see Figure 3.3) indicate that malaria, which is caused by a single-celled eukaryotic protozoan from the genus *Plasmodium*, contributed to the king's death more than 3,000 years ago. Malaria also had a role in to the decline of the Roman Empire 1,500 years ago. Anthrax is caused by the *Bacillus anthracis* bacterium whose spores are common in soil. Anthrax is thought to be the source of the fifth plague of Egypt mentioned in the Book of Exodus in the Bible that devastated Egyptian livestock. Plague, which is caused by the bacterium *Yersinia pestis*, is believed to be the source of the Black Death that killed millions of people during the Middle Ages, and resulted in the loss of 30–60% of Europe's population.

Today, malaria kills around 1 million people each year, most of whom are children in sub-Saharan Africa. A major effort is under way by the World Health Organization (WHO) to reduce the number of people killed by malaria. Plague infects thousands of people worldwide each year, but advances in antibiotics and other treatments, have dramatically reduced fatalities. Anthrax rarely infects humans (see Figure 3.4) but remains a threat to society because it can be used as

Excerpt of background section from student textbook



Figure 2.10. Adjustable-volume micropipets.

Burettes

A burette is a long, glass, graduated tube that is similar to a graduated pipet; however, it is filled by pouring a solution into the top and emptied via a stopcock at the base (see Figure 2.11). A burette is usually held upright with a stand and clamp. This glass tube is commonly used during titration when the volume of liquid to be dispensed is variable and needs to be measured after dispensing. A measurement is taken before and after dispensing the liquid, and the volume dispensed is then calculated.

$$V_{\text{Final}} - V_{\text{Initial}} = V_{\text{Delivered}}, \text{ where } V = \text{volume}$$

Burettes are usually used to accurately measure volumes between 10–100 ml; for example, a 25 ml burette can have an accuracy of ± 0.06 ml.



Figure 2.11. A burette.

Liquid Containers

Erlenmeyer Flasks

An Erlenmeyer flask has slanted sides and a narrow opening that facilitate swirling and mixing of the contents without spilling. (see Figure 2.14). Erlenmeyer flasks are typically not used for measuring, although the graduations on the flasks provide a rough guide for measuring liquids within approximately 5% of the target volume. Erlenmeyer flasks with the capacity to handle from 25 ml to 4 L are commonly found in a laboratory. Erlenmeyer flasks with fire-polished tops are primarily used for mixing and moving solutions from one location to another; these flasks can be used for short-term storage when covered with Parafilm or another impermeable cover. Erlenmeyer flasks with a culture top or screw cap top are used to grow liquid cultures of microorganisms. Such flasks may have baffles added to the bottom to aid in the agitation and oxygenation of the liquid medium as it is swirled by the shaking incubator. Erlenmeyer flasks may also have ground glass stoppers that enable the contents to be stored temporarily.

How To...

Use an Adjustable-Volume Micropipet

The volume of a micropipet is changed by twisting either a ridged cylinder on the micropipet handle or the top of the plunger, depending on the style of micropipet. When the volume is changed, the new volume is displayed on the readout dial. Figure 2.12 demonstrates how to read the volume on three common micropipet sizes. A micropipet is always used with a pipet tip. The end of the micropipet is inserted into the open end of the tip and tapped gently while the tip is in the box. This method ensures that the end of the tip is not touched. Tips are removed by pressing the tip ejector button.



Figure 2.12. Dial readout from adjustable-volume micropipets. From left to right, 2–20 µl, 20–200 µl, and 200–1,000 µl micropipets reading 15.16 µl, 127.4 µl, and 758 µl, respectively.

Once the volume is set and a pipet tip is installed, depress the micropipet plunger. The micropipet stops naturally as the target volume is reached — this is called the first, or soft, stop (see Figure 2.13). Place the tip into the liquid, and gently release the plunger. Once the set volume is drawn into the tip, move the pipet to the desired location and depress the plunger to deliver the contents. Depressing the plunger further to the second, or hard, stop blows out any residual contents. Remove the tip from the liquid before releasing the plunger to avoid sucking the liquid back into the pipet. Discard the tip by ejecting.

Always watch the liquid going into and out of the pipet tip so that you will notice if the tip is loose or blocked. Never use the hard stop except to blow out contents.



Figure 2.13. Plunger positions of micropipets. From left to right, plunger at rest, at soft stop, and at hard stop.

Excerpt of background section from student textbook with “How To...” vignette.

Activity 4.5 Forensic DNA Fingerprinting Protocol

Activity Protocol

Part 1: Setting Up the Digestion Reactions

1. Label colored microcentrifuge tubes:

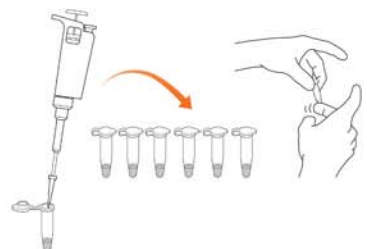
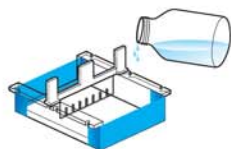
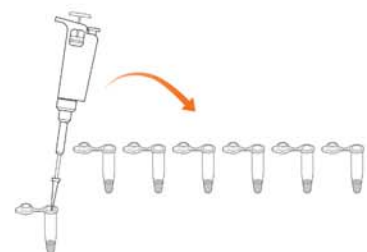
green tube	CS (crime scene)
blue tube	S1 (suspect 1)
orange tube	S2 (suspect 2)
violet tube	S3 (suspect 3)
pink tube	S4 (suspect 4)
yellow tube	S5 (suspect 5)

Label all the tubes with your initials and date, and place them in the microcentrifuge tube rack.

2. Using a fresh tip for each sample, pipet 10 μ l of each DNA sample into the matching colored microcentrifuge tube. Make sure each sample is transferred to the bottom of the tube.
3. Using a fresh tip each time, pipet 10 μ l of enzyme mix (ENZ) into the bottom of each tube. Pipet up and down carefully to mix.
4. Tightly cap the tubes, and mix the components by gently flicking the tubes with your finger. Collect the samples at the bottom of the tubes by tapping the tubes gently on the table or by pulse-spinning them in a microcentrifuge.
5. Incubate the digestion reactions for 45 min at 37°C or overnight at room temperature.
6. After the incubation, store the samples at 4°C until the next laboratory period. Samples can be stored for 1 month at 4°C. If there is sufficient time, proceed to running the gel.
7. 1x TAE electrophoresis buffer and a 1% TAE agarose gel is required for the next part of the activity. If necessary, prepare 1x TAE (refer to part 1 of Activity 2.4) and a 1% TAE agarose gel (refer to Activity 4.2).

Part 2: Running the Gel

1. If condensation has collected on the lids of the tubes, collect the samples at the bottom of the tubes by tapping the tubes gently on the bench or by pulse-spinning them in a microcentrifuge.
2. Using a fresh tip for each sample, pipet 5 μ l of the 5x sample loading buffer (SLB) into each tube. Cap the tubes and mix by gently flicking the tubes with your finger. Collect the samples at the bottom of the tubes by tapping them gently on the bench or by pulse-spinning them in a centrifuge.



Excerpt of activity protocol from student textbook

Teacher Supplement:

Step-by-step activity preparation

The teacher supplement is a full-size bound book with more than 200 pages to help you prepare and teach the activities.

Activity 3.5 Quantifying Bacterial Numbers

Quantifying Bacterial Numbers

In this activity, students will use aseptic technique, learn how to perform serial dilutions, and then use serial dilutions to quantify bacterial cell number. This activity uses an *E. coli* HB101 liquid culture, but the same technique could be used with yogurt. Each sample is diluted by a factor of 10; therefore, the culture is diluted 10x, 100x, 1,000x, and so on. (The amount that the culture is diluted by is called the dilution factor.) Another way to describe the dilution is that the sample is one-tenth, one-hundredth, and one-thousandth the concentration of the original culture. Students can conceptualize the dilution factor as a decimal point moving to the right. (If students consider the change to the concentration rather than dilution, the decimal point would move one place to the left.) This is great opportunity to revisit scientific notation.

Activity Summary

In this activity, students will make seven tenfold dilutions from a confluent *E. coli* HB101 liquid culture. In other words, the culture will be diluted 10,000,000 times, or by a factor of 10^7 . A sample from each dilution will be spread onto LB agar plates. Each individual bacterium spread on the plate will grow into a colony and is referred to as a colony forming unit (CFU). Once grown, the number of CFU per plate will be determined. The number of bacteria per ml in the original culture will then be calculated by multiplying the number of CFU on the plate by the dilution factor.

Students require seven 60 mm LB agar plates poured in Activity 3.1.

Safety

Students should use aseptic technique and dispose of microorganisms properly by autoclaving or soaking in 10% bleach. Have a beaker of 10% bleach available at each workstation for disposal of contaminated pipet tips. Students should wear appropriate PPE.

Stopping Points

Serial dilutions must be spread on agar plates immediately since bacteria will continue to divide in the diluted samples. Once colonies have grown, the plates can be wrapped in Parafilm and stored at 4°C for up to a week until analysis can be performed.

Tips

Remind students to thoroughly mix or vortex samples before pipetting into the next dilution to ensure that the bacteria are evenly distributed.

Remind students to use an inoculation loop or bacterial spreader to spread the bacteria all over the plate; the streak plate technique should not be used for this activity.

Activity Timeline

Tasks	Time	Notes/Reminders
Review the activity/lecture	45 min	Review the concept of serial dilution and practice calculations from hypothetical results.
Perform serial dilutions and plate results	45 min	This activity requires an overnight incubation.
Count colonies and perform calculations	45 min	Encourage students to do the calculations by themselves.

Anticipated Results

Plates should show a tenfold decrease in the number of colonies formed across the dilution series. So if the last plate has 45 colonies, the previous plate should have approximately 450, and the plate before that should have around 4,500, and so on. The first three or four plates will likely have a bacterial lawn with too many colonies that are to count. The final three or four plates should be counted, and the last plate may have no colonies at all.

Analysis of Results

Students should begin by recording their results and counting the plates that have approximately 30–200 colonies. Plates with fewer than 30 colonies have large room for error. When plates have too many colonies to count, teach students to use the phrase “too many to count,” or TMC. When there are too many colonies to count, students should learn to differentiate a bacterial lawn (where individual colonies cannot be distinguished) from the scenario where individual colonies can be distinguished but there are just too many colonies to count. Students do not always see this distinction.

Once students have counted the number of CFU on the plate, they should calculate the number of bacteria in the original culture by multiplying the CFU by the dilution factor. Alternatively, the CFU can be divided by the concentration of the sample to calculate the number of bacteria in the original culture.

Students will also need to adjust for the fraction of bacteria plated, since only 100 μ l of the 1 ml sample (that is, one-tenth of the total culture) was spread on the plate. Therefore, the actual number of CFU would have been 10 times higher if the entire 1 ml had been plated.

Activity 3.5 Quantifying Bacterial Numbers

$$\text{Bacteria per ml in the original culture} = \# \text{ of CFU} \times \text{dilution factor} \times \frac{1,000 \mu\text{l}}{100 \mu\text{l}}$$

$$\text{or} \quad \text{Bacteria per ml in the original culture} = \frac{\# \text{ of CFU}}{\text{Concentration of sample}} \times \frac{1,000 \mu\text{l}}{100 \mu\text{l}}$$

If there were 40 CFU on the plate with a dilution factor of 10^7 (which is the same as a concentration of 10^{-7} or one-millionth the original culture), then:

$$\text{Bacteria per ml in the original culture} = \frac{40 \times 10^7 \times 1,000}{100} = 4 \times 10^9 \text{ CFU/ml}$$

$$\text{or} \quad \text{Bacteria per ml in the original culture} = \frac{40 \times 1,000}{10^{-7} \times 100} = 4 \times 10^9 \text{ CFU/ml}$$

Students may obtain different results since they may have two or three plates they can count. This could lead to a discussion of experimental variance and standard errors. Ask students how they could control for this variance and its potential impact on experiments. If a single culture was used for all student groups, compare and analyze class data and calculate the variance.

Assessment

Assess students formatively by observing their aseptic technique, and ensure that they are using safe practices. Once colonies have grown, have students lay the plates in a line from most to least concentrated and assess each dilution series visually. For summative assessment, check students' calculations and evaluate their understanding of dilutions, concentrations, and scientific notation.

Advance Preparation Procedures for the Instructor

Advance Preparation Tasks

Take an inventory (see Inventory Table) at least 1 week prior to the activity to ensure all the required materials are available. Preparations for this activity should take 15–30 min. The activity requires fifty-six 60 mm LB agar plates (7 per team) and 100 ml of LB broth made in Activity 3.1. If the LB broth and LB agar plates are not available, they should be prepared 2–3 days prior to the activity. It will take 1–2 hr to prepare them, not including the autoclave time. The *E. coli* HB101 cultures require an overnight incubation.

1. Ensure that 100 ml of LB broth (prepared in part 1 of Activity 3.1) and sixty 60 mm LB agar plates (prepared in parts 3 and 4 of Activity 3.1) are available. If they are not available, see Bio-Rad bulletin 5891, Large Class/Multiple Class Preparation Guide, for protocols on how to prepare LB agar plates for multiple workstations. Store plates wrapped in plastic at 4°C for up to 3 months and LB broth at 4°C for up to 1 year.
2. One day prior to the activity, add 0.25 ml of LB broth to rehydrate the vial of *E. coli* HB101 bacteria. Label eight 15 ml culture tubes *E. coli* and add 2 ml of LB broth to each tube. Transfer 25 μ l of the rehydrated bacteria into each culture tube of LB broth (one per team). Incubate the tubes overnight at 37°C with shaking or rolling. If a shaking incubator, water bath, or roller is not available, manually shake the tubes as often as possible and incubate them at overnight 37°C. Once grown, the cultures can be stored at 4°C for up to 5 days.
3. Just prior to the activity, set up each workstation with the materials listed in the student materials list. Consider making stations responsible for collecting and returning common laboratory equipment.

Answers to Prelab and Postlab Focus Questions

Prelab Focus Questions

1. Why is it necessary to perform a serial dilution of the bacterial culture before plating each diluted sample on an LB agar plate?

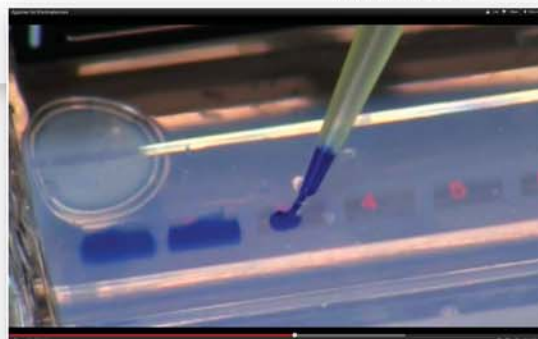
Answer: The number of bacteria would be too large to count if the original culture were plated without dilution.

2. Why is it important to thoroughly mix each dilution before pipetting it into the next dilution and before plating it?

Answer: It is important to thoroughly mix the diluted sample before pipetting it into the next dilution and before plating to ensure that the bacteria are evenly distributed since bacteria will sink to the bottom of the tube. The sample needs to be evenly distributed to ensure the portion removed is representative of the whole sample.

3. If 60 colonies formed on an LB agar plate, how many bacteria were plated? How many CFU are there?

Answer: 60 bacteria, 60 CFU.



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- Demonstrations of key techniques
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- Tips
- Safety
- Activity timelines
- Stopping points
- Anticipated and analysis of results
- Assessment
- Inventory table
- Advanced preparation procedures
- Answers to prelab and postlab questions



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The Author

J. Kirk Brown taught at Tracy High School in Tracy, CA, for more than 20 years. Integrating his teaching with inspiring hands-on laboratory experiences in his International Baccalaureate Biology and Biotechnology courses, he's helped hundreds of students develop into savvy biologists. Kirk also helped to found the Agricultural/Scientific Academy at Tracy High School, which allows students to apply science learned in the classroom to the community they live in. Currently Kirk serves as the Director of Science and Special Projects for the San Joaquin County Office of Education in Stockton, CA.

Kirk's passion for education extends into the postsecondary level through his work as an adjunct associate professor at San Joaquin Delta College, where he teaches courses in core biology and fundamentals of biotechnology. His collaborations with the California Department of Education, Lawrence Livermore National Laboratory (LLNL), San Joaquin County Office of Education, Access Excellence (Genentech), and the Exploratorium, and his ongoing partnership with Bio-Rad Laboratories, have led to significant teacher and student advancements.



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