Killer Proteins!
A Bactericidal Assay to Study the Role of Complement in Killing Bacteria

John F. Long
johlong@dallasisd.org
Woodrow Wilson High School
100 S. Glasgow Drive
Dallas, Texas 75214

Funded by the American Association of Immunologists –
John H. Wallace High School Teachers Summer Research Program

Mentor:

Dr. J. David Farrar
Associate Professor, Department of Immunology
University of Texas Southwestern Medical Center, Dallas, Texas

TABLE OF CONTENTS

TEACHER GUIDE

The Texas Essential Knowledge and Skills (TEKS) that are addressed throughout this unit are as follows:

Chapter 112. Texas Essential Knowledge and Skills for Science
Subchapter C. High School

§ 112.43 Biology
Introduction.

(1) In Biology, students conduct field and laboratory investigations, use scientific methods during investigations, and make informed decisions using critical-thinking and scientific problem-solving. Students in Biology study a variety of topics that include: structures and functions of cells and viruses; growth and development of organisms; cells, tissues, and organs; nucleic acids and genetics; biological evolution; taxonomy; metabolism and energy transfers in living organisms; living systems; homeostasis; ecosystems; and plants and the environment.

(2) Nature of Science. Science, as defined by the National Academy of Sciences, is the "use of evidence to construct testable explanations and predictions of natural phenomena, as well as the knowledge generated through this process." This vast body of changing and increasing knowledge is described by physical, mathematical, and conceptual models. Students should know that some questions are outside the realm of science because they deal with phenomena that are not scientifically testable.

(3) Scientific inquiry. Scientific inquiry is the planned and deliberate investigation of the natural world. Scientific methods of investigation can be experimental, descriptive, or comparative. The method chosen should be appropriate to the question being asked.

(4) Science and social ethics. Scientific decision making is a way of answering questions about the natural world. Students should be able to distinguish between scientific decision-making methods and ethical and social decisions that involve the application of scientific information.

(5) Science, systems, and models. A system is a collection of cycles, structures, and processes that interact. All systems have basic properties that can be described in terms of space, time, energy, and matter. Change and constancy occur in systems as patterns and can be observed, measured, and modeled. These patterns help to make predictions that can be scientifically tested. Students should analyze a system in terms of its components and how these components relate to each other, to the whole, and to the external environment.

Science Concepts

1. The student uses scientific methods and equipment during laboratory and filed investigations.
2. The student uses critical thinking, scientific reasoning, and problem solving to make informed decisions within and outside of the classroom.
3. The student knows that cells are the basic structure of all living things with specialized parts that perform specific functions and that viruses are different from cells.
4. The student knows that biological systems work to achieve and maintain balance.

Student Expectations (based on TEKS)

(1) Scientific processes. The student, for at least 40% of instructional time, conducts laboratory and field investigations using safe, environmentally appropriate, and ethical practices. The student is expected to:

(A) Demonstrate safe practices during laboratory and field investigations.
(B) Demonstrate an understanding of the use and conservation of resources and the proper disposal or recycling of materials.

(2) Scientific processes. The student uses scientific methods and equipment during laboratory and field investigations. The student is expected to:

(A) Know the definition of science and understand that it has limitations, as specified in subsection (b)(2) of this section.
(B) Know that hypotheses are tentative and testable statements that must be capable of being supported or not supported by observational evidence. Hypotheses of durable explanatory power which have been tested over a wide variety of conditions are incorporated into theories.
(C) Know scientific theories are based on natural and physical phenomena and are capable of being tested by multiple independent researchers. Unlike hypotheses, scientific theories are well-established and highly-reliable explanations, but they may be subject to change as new areas of science and new technologies are developed.
(D) Distinguish between scientific hypotheses and scientific theories.
(E) Plan and implement descriptive, comparative, and experimental investigations, including asking questions, formulating testable hypotheses, and selecting equipment and technology.
(F) Collect and organize qualitative and quantitative data and make measurements with accuracy and precision using tools such as calculators, spreadsheet software, data-collecting probes, computers, standard laboratory glassware, microscopes, various prepared slides, stereoscopes, metric rulers, electronic balances, gel electrophoresis apparatuses, micropipettors, hand lenses, Celsius thermometers, hot plates, lab notebooks or journals, timing devices, cameras, Petri dishes, lab

incubators, dissection equipment, meter sticks, and models, diagrams, or samples of biological specimens or structures.
(G) Analyze, evaluate, make inferences, and predict trends from data.
(H) Communicate valid conclusions supported by the data through methods such as lab reports, labeled drawings, graphic organizers, journals, summaries, oral reports, and technology-based reports.

(3) Scientific processes. The student uses critical thinking, scientific reasoning, and problem solving to make informed decisions within and outside the classroom. The student is expected to:

(A) In all fields of science, analyze, evaluate, and critique scientific explanations by using empirical evidence, logical reasoning, and experimental and observational testing, including examining all sides of scientific evidence of those scientific explanations, so as to encourage critical thinking by the student.
(B) Communicate and apply scientific information extracted from various sources such as current events, news reports, published journal articles, and marketing materials.
(C) Draw inferences based on data related to promotional materials for products and services.
(D) Evaluate the impact of scientific research on society and the environment;
(E) Evaluate models according to their limitations in representing biological objects or events.
(F) Research and describe the history of biology and contributions of scientists.

(4) Science concepts. The student knows that cells are the basic structures of all living things with specialized parts that perform specific functions and that viruses are different from cells. The student is expected to:
(A) Compares and contrast prokaryotic and eukaryotic cells.
(B) Investigate and explain cellular processes, including homeostasis, energy conversions, transport of molecules, and synthesis of new molecules; and
(C) Compare the structures of viruses to cells, describe viral reproduction, and describe the role of viruses in causing diseases such as Human Immunodeficiency Virus (HIV) and influenza.

(5) Science concepts. The student knows how an organism grows and the importance of cell differentiation. The student is expected to:

(A) Describe the stages of the cell cycle, including deoxyribonucleic acid (DNA) replication and mitosis, and the importance of the cell cycle to the growth of organisms.
(B) Examine specialized cells, including roots, stems, and leaves of plants; and animal cells such as blood, muscle, and epithelium.
(C) Describe the roles of DNA, ribonucleic acid (RNA), and environmental factors in cell differentiation.
(D) Recognize that disruptions of the cell cycle lead to diseases such as cancer.

(6) Science concepts. The student knows the mechanisms of genetics, including the role of nucleic acids and the principles of Mendelian Genetics. The student is expected to:

(A) Identify components of DNA, and describe how information for specifying the traits of an organism is carried in the DNA.
(B) Recognize that components that make up the genetic code are common to all organisms.
(C) Explain the purpose and process of transcription and translation using models of DNA and RNA.
(D) Recognize that gene expression is a regulated process.
(E) Identify and illustrate changes in DNA and evaluate the significance of thesechanges.
(F) Predict possible outcomes of various genetic combinations such as monohybrid crosses, dihybrid crosses and non-Mendelian inheritance.
(G) Recognize the significance of meiosis to sexual reproduction.
(H) Describe how techniques such as DNA fingerprinting, genetic modifications and chromosomal analysis are used to study the genomes of organisms.

(7) Science concepts. The student knows evolutionary theory is a scientific explanation for the unity and diversity of life. The student is expected to:

(A) Analyze and evaluate how evidence of common ancestry among groups is provided by the fossil record, biogeography, and homologies, including anatomical, molecular, and developmental.
(B) Analyze and evaluate scientific explanations concerning any data of sudden appearance, stasis, and sequential nature of groups in the fossil record.
(C) Analyze and evaluate how natural selection produces change in populations, not individuals.
(D) Analyze and evaluate how the elements of natural selection, including inherited variation, the potential of a population to produce more offspring than can survive and a finite supply of environmental resources, result in differential reproductive success.
(E) Analyze and evaluate the relationship of natural selection to adaptation and to the development of diversity in and among species.
(F) Analyze and evaluate the effects of other evolutionary mechanisms, including genetic drift, gene flow, mutation, and recombination.
(G) Analyze and evaluate scientific explanations concerning the complexity of the cell.

(8) Science concepts. The student knows that taxonomy is a branching classification based on the shared characteristics of organisms and can change as new discoveries are made. The student is expected to:

(A) Define taxonomy and recognize the importance of a standardized taxonomic system to the scientific community.

(B) Categorize organisms using a hierarchical classification system based on similarities and differences shared among groups.
(C) Compare characteristics of taxonomic groups, including archaea, bacteria, protists, fungi, plants, and animals.

(9) Science concepts. The student knows the significance of various molecules involved in metabolic processes and energy conversions that occur in living organisms. The student is expected to:

(A) Compare the structures and functions of different types of biomolecules, including carbohydrates, lipids, proteins, and nucleic acids.
(B) Compare the reactants and products of photosynthesis and cellular respiration in terms of energy and matter.
(C) Identify and investigate the role of enzymes.
(D) Analyze and evaluate the evidence regarding formation of simple organic molecules and their organization into long complex molecules having information such as the DNA molecule for self-replicating life.

(10) Science concepts. The student knows that biological systems are composed of multiple levels. The student is expected to:

(A) Describe the interactions that occur among systems that perform the functions of regulation, nutrient absorption, reproduction, and defense from injury or illness in animals.
(B) Describe the interactions that occur among systems that perform the functions of transport, reproduction, and response in plants.
(C) Analyze the levels of organization in biological systems and relate the levels to each other and to the whole system.

(11) Science concepts. The student knows that biological systems work to achieve and maintain balance. The student is expected to:

(A) Describe the role of internal feedback mechanisms in the maintenance of homeostasis.
(B) Investigate and analyze how organisms, populations, and communities respond to external factors.
(C) Summarize the role of microorganisms in both maintaining and disrupting the health of both organisms and ecosystems.
(D) Describe how events and processes that occur during ecological succession can change populations and species diversity.

(12) Science concepts. The student knows that interdependence and interactions occur within an environmental system. The student is expected to:

(A) Interpret relationships, including predation, parasitism, commensalism, mutualism, and competition among organisms.

(B) Compare variations and adaptations of organisms in different ecosystems.
(C) Analyze the flow of matter and energy through trophic levels using various models, including food chains, food webs, and ecological pyramids.
(D) Recognize that long-term survival of species is dependent on changing resource bases that are limited.
(E) Describe the flow of matter through the carbon and nitrogen cycles and explain the consequences of disrupting these cycles.
(F) Describe how environmental change can impact ecosystem stability.

Source: The provisions of this §112.34 adopted to be effective August 4, 2009, 34 TexReg 5063

I. Overview

This unit will focus primarily on the concept of complement. As a secondary concept, antigen-antibody reactions and enzymes will also be utilized. Complement, as well as immunology as a whole, is a topic that is seldom discussed or reviewed in detail in the high school classroom. Furthermore, actual analysis of immunological interactions will be studied in this lesson.

The goals of the laboratory exercises developed for this unit are to help students to become familiar with immunological assays, basic cell/bacterial culture, and basic laboratory procedures and data analysis. Students will also be utilizing safe laboratory practices, procedures, and relation of prior learned knowledge to new concepts. The labs themselves should introduce the student to assays and procedures that are commonly utilized in high-level academic research and development centers of learning.

Although some of the concepts are of higher level of detail than may be expected outside of an Advanced Placement (AP) class, this unit can still be used in different areas of biology. It can be used during immune system study, disease study, biochemistry, or enzymes unit. The labs are geared mostly for AP biology, Pre-AP biology, or general biology.

Students will run a technical serum bactericidal assay (colony counting method) to demonstrate the innate response to bacteria via the complement system. They will analyze the effectiveness of the complement system in clearing bacterial infections without the use of antibiotics. Students will evaluate the effectiveness of the bactericidal assay through data analysis.

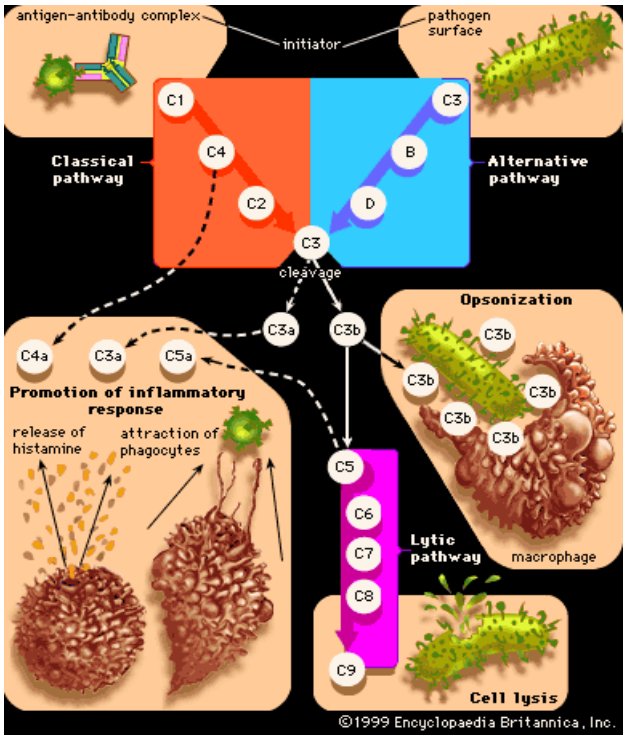
Through both background lecture and lab activity, students should learn the importance of the immune system as a whole. Specifically, students should gain an understanding that there are components of their own blood that have the ability to clear bacterial infections without the use of antibiotics. Students should gain a greater appreciation and understanding of how infection and disease are combated. The labs should offer a glimpse into the “everyday” world of academic research and development, and should introduce the students to another avenue of career development. Students will see how

such research leads to a better understanding on immunology, disease control, and research.

II. Science Background

Complement was discovered by Jules Bordet in the 1890's when he showed that sheep antiserum to *Vibrio cholerea* caused lysis of the bacteria and that heating the antiserum allowed the bacteria to survive. Over the years, it was discovered that the bactericidal (or lytic) action of complement was due to the interactions of a complex of proteins. It was also shown that complement plays a key role in both innate and adaptive immunity (these terms will be defines below).
After activation, complement carries out a wide variety of functions including the lysis or breaking down of cells, bacteria, and viruses (by antibody-dependent and independent ways), opsonization (uptake of antigens), activation of inflammatory responses, and clearance of immune complexes.

Complement makes up roughly 5% of serum. Most of the proteins of the complement system are produced by liver hepatocytes and circulate in the serum in inactive forms (or zymogens) until the inhibitory fragment is removed allowing for activation. Activation can occur by one of three pathways: the classical, the alternative, or lectin pathway (see diagram). Ultimate activation involves the development of the membrane attack complex (or MAC) which cause cell lysis.



(http://media-2.web.britannica.com/eb-media/03/20903-004-379E4AF3.gif)

Innate immunity, also called natural immunity, is active from birth before one is exposed to microbes or foreign substances. It is a first line, immediate form of immune response and protection. Innate immunity is, however, non-specific, and cannot adapt to microbes changing (or mutating) to evade clearance.

Adaptive immunity is activated after exposure to a certain antigen. There is an initial delay between the exposure and activation, however, adaptive immunity is highly specific to particular antigens and makes it difficult for microbes to evade (even with mutation) due to the specificity.

III. Student Outcomes

This unit will cover innate and adaptive immunity as background to introducing complement and its role as an antimicrobial agent. It allows students to correlate through a bacteria colony counting assay how complement is able to "kill" bacteria at differing concentrations. It also introduces the student to valuable laboratory techniques, data analysis, and the importance of laboratory protocol and attention to detail.

IV. Learning Objectives

- Students will learn how the immune systems works, specifically innate versus acquired immunity.
- Students will learn the importance of serum proteins and the complement system as an anti-microbial agent.
- Students will learn to perform basic laboratory actions: micropipetting, tissue culture, sterile technique, plating, and serial dilution.
- Students will learn to analyze data, graph data via PC or MAC, and present findings in a lab report format.

V. Time Requirements

The total time needed for this unit is 5 days. The initial 1-3 days are set aside for background lecture over innate and adaptive immunity, and complement.

The laboratory investigation can be run in two subsequent 90 minute class periods. The initial stock of bacteria will need a 24 hours period to be in good log-growth for use in this laboratory. This culture can be started at the end of the third class period. The fourth class period will be needed for the actual set-up and running of the assay, followed by the fifth class period which can be used for plate counting and discussion (along with data analysis).

VI. Advanced Preparation

Preparation of reagents used in the laboratory exercise can be made prior to the laboratory and stored. LB-Agar plates are the easiest to prepare and store at 4^{o}C.

Bacteria cultures can be frozen at - 80^{o}C and a small loop inoculation taken or a loop streak plate can be made and stored for a week. Data suggests that bacterial colonies older than a week on plates do not yield as good of results as "fresh" bacteria.

LB-Agar Plates (per 500 ml)	Alternative LB-Agar (per 500 ml)
5 g Nacl	10 g DIFCO® LB
5 g Tryptone	10 Agar
2.5 g yeast	Bring to 500ml in dH_2O (de-ionized water)
10 g Agar	Autoclave for 45 minutes at 121^{o}C
Bring to 500ml in dH_2O (de-ionized water)	
Autoclave for 45 minutes at 121^{o}C	

VII. Materials and Equipment

Materials

LB-Agar plates (see Section VI for receipe)

Petri-dishes (for LB-Agar Plates)
VWR Scientific, Catalog # 25384-302 500/$174.04

Rabbit Complement
PelFreeze, Catalog # 31060-1 100ml/$75.00

Assay Buffer
Dublecco's phosphate-buffer saline (PBS) containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$, pH7.4, (Invitrogen, catalog # 14080055) with 0.1% glucose (Sigma, catalog # G7528). Filter sterilized (0.22 µM) and stored at +4^{o}C

PBS (10x)	500 ml/$31.20
0.1% glucose	10mg/$18.00

NUNC 96 well Plates
VWR Scientific, Catalog # 62409-310 case of 60/$282.59

Test Tubes – polypropylene
VWR Scientific, Catalog # 82051-626 case of 500/$213.05

Microipetes and tips capable of measuring 10 µL – 1000 µL volumes
37^{o}C incubator
Shaker (preferably a floor shaker)

E. coli culture (Strain DH5α, Promega)
LB broth
Containers with ice
Bunsen burner
Distilled or de-ionized water
Spreaders
Erlenmeyer flask
Alcohol

VIII. Student Prior Knowledge and Skills

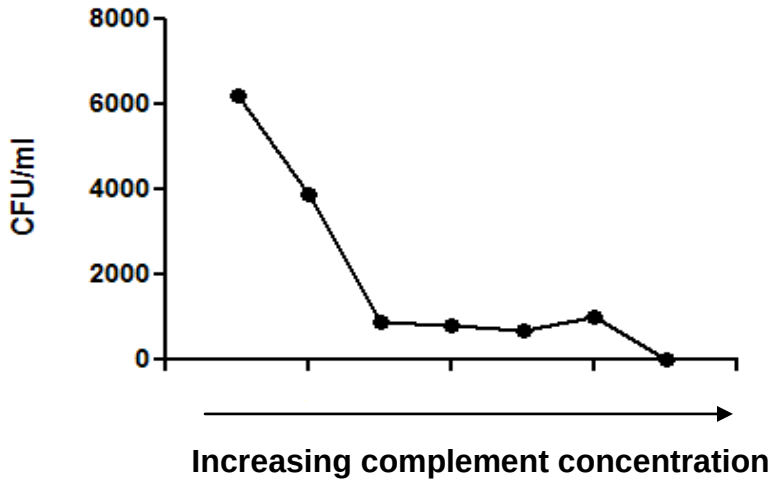
Students should have prior knowledge of basic immunological reactions. Students should also have previous experience and/or exposure in bacterial growth, pipette function, serial dilutions, plating of bacteria, and basic use of a PC or MAC with graphing program.

IX. What is Expected from Students

Students will be expected to present their findings (data, etc.) in a formal lab report. This lab report should include an analysis of their data with supporting calculations and graphs. Students should convert the number of counted colonies on their plates to CFU (colony forming units) per milliliter (CFU/ml) for graphing.

X. Anticipated Results

If done correctly, students should see a decrease in the number of formed colonies on each plate as the concentration of rabbit complement increases. Furthermore, it is always best to run the assay in duplicate in order to have better results. One way to do this is to have several groups of students and combine data as a class set.



XI. Classroom Discussion

DAYS 1 and 2

Questions for Discussion

1. What does immunity mean to you?
2. What are the two types of immunity?
   Innate and Adapative
3. What are two parts of Innate Immunity?
   Skin, mucous membranes, and the subsurface parts (cells, proteins (complement))
4. What are the two types of adaptive immunity?
   Humoral and cellular
5. How do innate and adaptive immunity differ?
   Innate is the first line of immunity; it is and non-specific and immediate. Adaptive is the next line of immunity; it is acquired and takes time.

DAY 3

1. What is complement?
   A series of proteins found in serum that are capable of killing microbes
2. What form are complement proteins found?
   Zymogens
3. What are the three pathways of complement activation?
   Classical, alternative, and lectin
4. What are the four ways complement can clear microbes?
   Lysis, opsinization, inflammatory response, clearing of immune complexes
5. What is the ultimate "goal" of complement proteins?
   Formation of the MAC (membrane attack complex)

DAYS 4 and 5

1. Why is necessary to have the bacterial culture in log growth?
   It is necessary to insure that the bacteria are actively growing and that there are enough total bacteria to produce enough colonies upon dilution and plating.
2. What is a serial dilution?
   A step wise dilution making the same dilution step over and over, using the previous dilution as the input for the next dilution.
3. How do you determine the colony forming units given the amount of bacteria plated?
   You are plating 10 µL of the overall sample. The starting concentration of bacterial on the assay was 10^{-5}. By counting the number of colonies and dividing this by 0.1 ml then multiplying this answer by 10^5 and the dilution in the 96 well plate will give you cfu/ml.
4. What type of graph would be useful to construction for your experiment? Why?
   A line graph showing error bars (if class data is combined for each point) would be the best. A line graph show a simple way to put logarithmic data points that you will have for the dilution concentrations.

5. Where there any “errors” with your data? Why? What would have caused them?

There may be errors in several places: incorrect dilutions, incorrect calculations, incorrect pipetting technique, overheated spreader that caused bacteria to be killed, etc. Most of these errors can be attributed to “rushing” through the experiment or by not paying careful attention to the procedure.

XII. Assessment

The culminating assessment for this exercise will be the laboratory report for the exercise. There is no set rubric for a formal lab write-up since each educator differs in what they feel is necessary for the report.

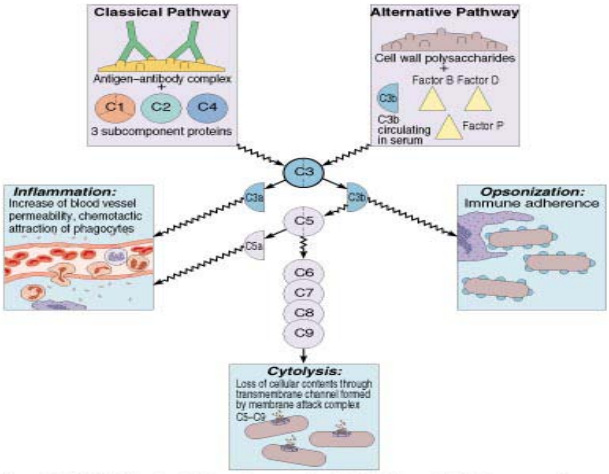
STUDENT GUIDE

I. Rationale

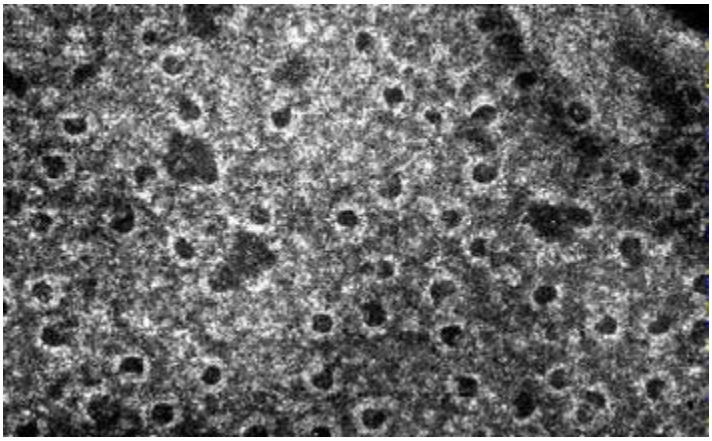
Bacteria, viruses, and other nasty pathogens are everywhere you are. Many of these microbes are capable of making you very sick and maybe even killing you. We are constantly bombarded by pathogens since we offer an ideal environment for growth, reproduction, protection, and transportation for these pathogens. But why don't more of us simply get sick and die? The answer is found in our immune system.

Our first line of defense is a barrier, such as our skin. It provides a fairly good obstacle for pathogens to not get into the body. However, we are not 100% able to fend of these invaders simply because we have to do things like breath and eat. Thus, our bodies have developed two internal defense systems for identifying and eliminating pathogens. The first defense system is called *innate immunity*. Innate immunity is found in all animals and is a rapid response to a pathogen whether or not your body has seen that pathogen or not. The second defense system is the *acquired immunity* system. This system is found only in vertebrates and develops more slowly due to the fact that you have to have previous exposure to the pathogen to obtain a response (i.e. like when you get a vaccine).

Parts of both systems of defense are made up of proteins. Proteins play a crucial role in many body systems and none more so then the immune system. One of the main sets of proteins (about 30 or so) is the *complement system*. Complement carries out a number of functions (see figure below), all with one goal in mind – getting rid of pathogens!



Most complement proteins circulate in the serum of blood in an inactive form. These are called *zymogens* until they are activated by the removal of an inhibitory fragment. Once activated though, the complement cascade is a highly effective killer protein machine. The proteins that make up complement ultimately form a membrane-attack complex (or MAC) which causes microbial lysis.



[Lesions on the membrane of a red blood cell resulting from membrane-attack complexes. *J. Humphrey and R. Dourmashkin, 1969,* Advances in Immunology ***11****:75*]

In this exercise, you will be examining the effects of different concentrations of purified complement on the growth of bacteria (*E.coli).* You will also be utilizing many of the same laboratory techniques and data analysis that is used in academic research and development throughout the world. You are responsible for the careful following of laboratory protocol crucial to the correct outcome of this exercise.

II. Materials

As with most laboratory exercises, safety is the key to success. Please be sure to wear the proper safety equipment when doing this exercise (goggles, aprons, and gloves).

Materials that will be needed per group:

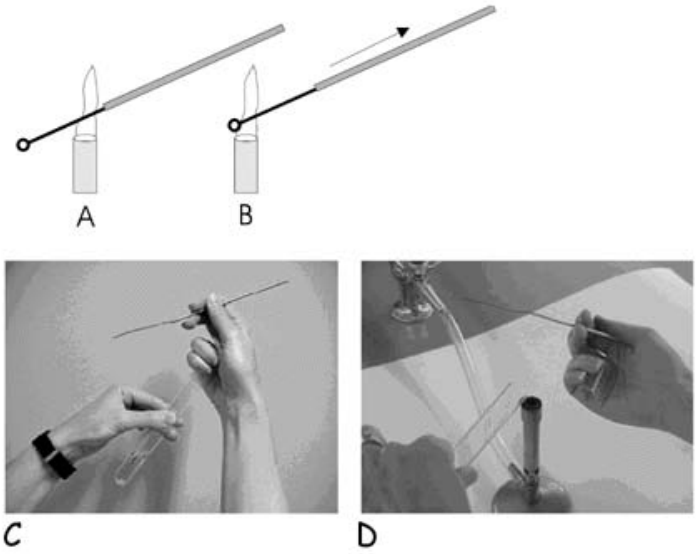
- Assay Buffer (1x PBS with 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, pH7.4, and 0.1% glucose – about 60 ml
- 1 NUNC 96-well plate
- Micropipettes and tips (P-10, P-20, P-100, and P-1000)
- Test tubes (about 5 for serial dilutions)
- LB-Agar Plates (about 20)
- Rabbit complement (about 50 µL)
- Ice bucket with ice
- Bactria spreader
- Alcohol
- Bunsen Burner
- Bacteria culture (*E.coli)* in 10ml of LB Broth grown for 24 hours in test tube
- Permanent Marker

III. Procedure

Day 1

Preparation of bacterial overnight culture

1. Using either a frozen bacteria stock or a previous streak plate, inoculate about 10 ml of LB broth for overnight culture.
2. To inoculate, heat the entire length of the wire portion to red hot, and then pass part of the handle through the flame to burn off any dust. In any and all cases, the entire length that will go into the tube must be flamed.
3. Insert the wire end and be sure that it is cool. Then, touch the circular loop to the streak of bacterial growth. You do not need to get great big globs of it. Just mere touching will bring up millions of bacteria. Remove the loop from the tube trying not to touch the insides of the end of the tube (that is where contamination is most likely to be if there is any at all). If using a streak plate, carefully pick off one single colony from the plate.
4. Carefully flame the lip of the test tube containing the LB broth.
5. Insert the inoculating loop into the broth.
6. Re-flame the lip and close the tube.
7. Label your tube with group name, date, and bacterial strain.
8. Again heat the entire length of the wire portion to red hot.



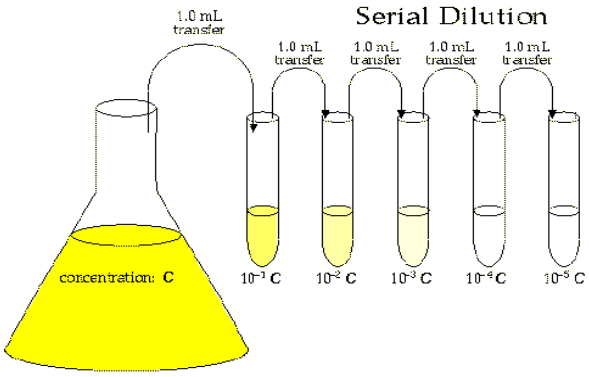
(figure from http://abacus.bates.edu/~ganderso/biology/resources/sterile.html)

9. Place test tube in floor shaker (or something like it) for 24 hours at 37^{o}C.
10. Return streak plate or vial to appropriate location (+4^{o}C for plate or -80^{o}C for stock vial).
11. Clean up area, put away safety materials, and wash hands.

Day 2

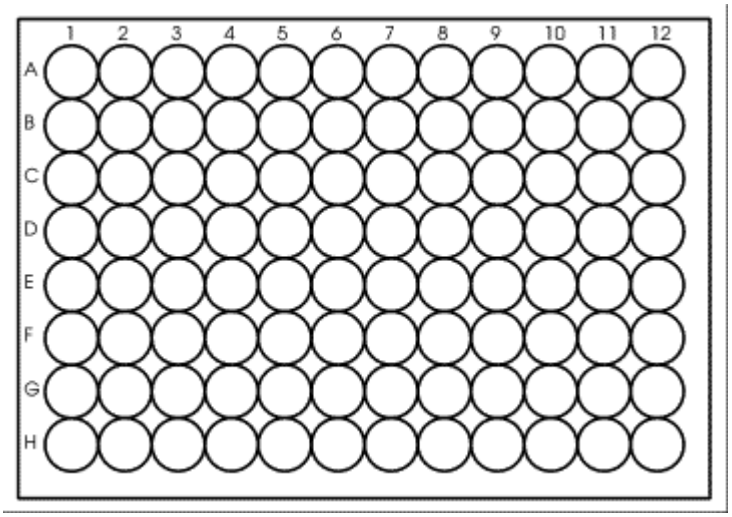
1. Obtain the following materials:
   1 NUNC 96-well plate
   Micropipettes and tips (P-10, P-20, P-100, and P-1000)
   Test tubes (about 5 for serial dilutions)
   LB-Agar Plates (about 24)
   Rabbit complement (about 50 µL)
   Ice bucket with ice
   Bactria spreader
   Alcohol
   Bunsen Burner
   Bacteria culture (*E.coli)* in 10ml of LB Broth grown for 24 hours in test tube
   Permanent Marker
   Assay Buffer (1x PBS with 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, pH7.4, and 0.1% glucose – about 60 ml

   2. Place complement and assay buffer in ice bucket (the complement needs to stay cold!).
   3. Label 5 test tubes 10^{-1}, $10^{-2,}$ $10^{-3,}$ $10^{-4,}$ and 10^{-5}. These will be used to dilute your overnight bacterial culture. The final dilution of 10^{-5} will be used in the actual assay.
   4. Perform serial dilution of overnight culture using 9 ml of assay buffer per 1 ml of previous dilution to get a final volume of 10 ml.



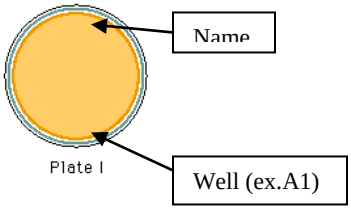
(figure from http://www.mysciencefairprojects.com/bio/microbiology/Minimizing_Bacteria_Meat.aspx)

5. Place diluted bacteria tubes on ice until needed.
6. To the 96 well plate, add the following amount of assay buffer to the following wells using a P-100 pipette:



A1/B1: 30 µL	A2/B2: 30 µL	A3/B3: 30 µL
A4/B4: 30 µL	A5/B5: 30 µL	A6/B6: 30 µL
A7/B7: 30 µL	A8/B8: 30 µL	A9/B9: 30 µL
A10/B10: 30 µL	A11/B11: 30 µL	**A12/B12: 40 µL**

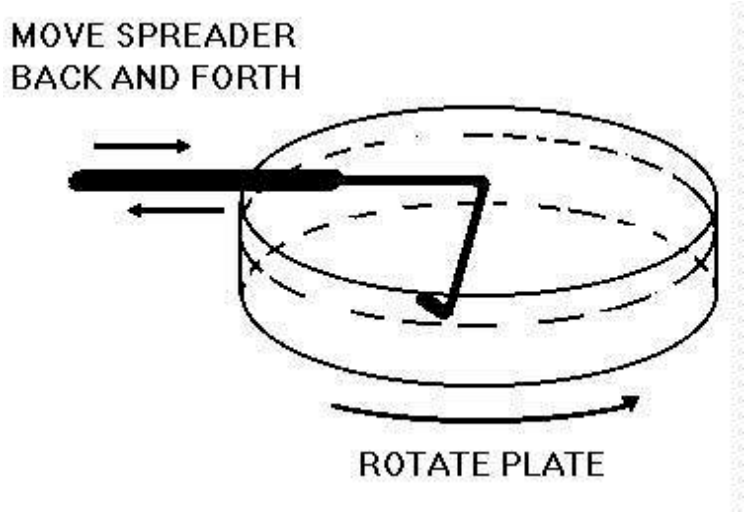
7. Using a P-20 pipette, add 20 µL of rabbit complement to wells A1 and B1. You are now going to perform serial dilutions on the complement **inside** of the wells.
8. After adding 20 µL of complement to wells A1 and B1, using a fresh pipette tip, mix the contents of these wells by taking up and letting out 20 µL of volume 10-15 times within the well. Once you have done that, remove 20 µL form A1 and place it into A2. Repeat this for B1.
9. Repeat Step 8, using a fresh pipette tip between wells, until you reach A9 and B9.
10. At wells A9 and B9, after adding the complement from wells A8 and B8, remove 20 µL from A9 and B9 and discard it. Do not add this 20 µL to the A10 and B10 wells.
11. To wells A11 and B11, add only 10 µL of pure complement to the wells. These wells are one of our controls.
12. Now take your 10^{-5} diluted bacteria culture and using a P-10 pipette, add 10 µL to wells A1-A10 and wells B1-B10. Do not add any bacteria to wells A11, A12, B11, or B12.
13. Incubate plate for 45 minutes at 37^{o}C.
14. During incubation period, begin labeling 24 LB agar plates with the appropriate wells and group name.



15. After 45 minute incubation, remove 96 well plate and place on ice in ice bucket.

16. Position the Bunsen burner, the alcohol, and the spreaders in an area big enough to have room to work. The next few steps are crucial to insure sterile technique when plating your bacteria samples.
17. Using a P-10 pipette, remove a 10 µL aliquot (a sample) from well A1 and place it on the LB Agar plate labeled A1.
18. Dip spreader into alcohol and run across the flame of the Bunsen burner. BE CAREFUL! Do not catch yourself on fire. Allow spreader to cool, then spread bacteria evenly over plate (see diagram).

PLATING OF BACTERIA



(http://www.bch.msu.edu/bchug/web/bch472/472lm2.htm)

19. Again, dip spreader into alcohol and run across the flame of the Bunsen burner.
20. Repeat steps 17-20 for wells A2-A12 and B1-B12.
21. After plating all wells, incubate the plates upside down (to avoid condensation failing on your bacterial colonies) for 45 minutes at 37^{o}C.
22. After 24 hours, count the number of colonies and record your results in the data table.
23. Clean up your area, dispose of plates in a biohazard bag, turn off burner, and disinfect the work area (Lysol works well).

IV. Data Collection

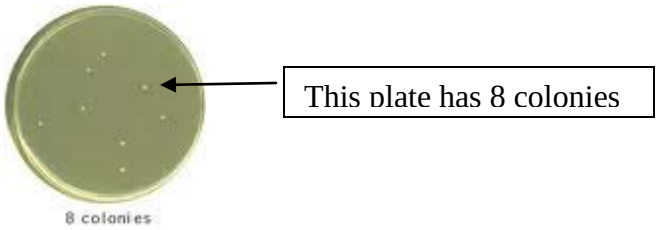
This portion of the exercise will help you with recording the data that you will collect so that you can use it in conjunction with a graphing program.

Day 3

1. After 24 hour incubation of LB-Agar plates, remove them from the incubator and place them in an area that you can easily read them.
2. Carefully count the number of colonies on each plate and record your results in the table.

Well #	# of Colonies	Well #	# of Colonies
A1		B1	
A2		B2	
A3		B3	
A4		B4	
A5		B5	
A6		B6	
A7		B7	
A8		B8	
A9		B9	
A10		B10	
A11		B11	
A12		B12	

An example of a plate that contains colonies would look like this:

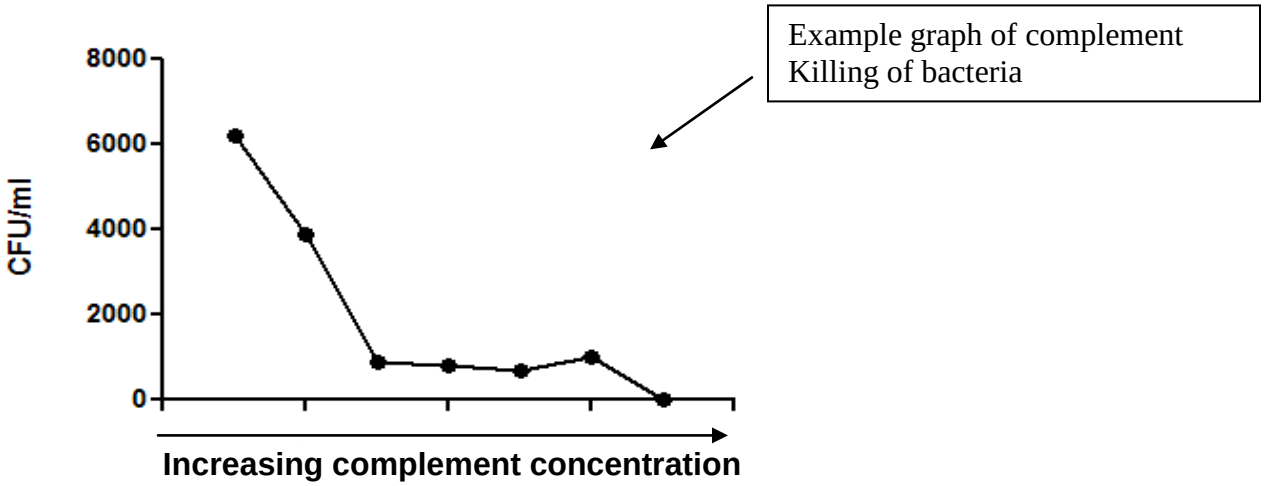


(Image from www-micro.msb.le.ac.uk/LabWork/bact/-6L.jpg)

3. Once all colonies have been counted and recorded, discard plates in a biohazard bag to be disposed of properly by your teacher.
4. Determine the number if colony forming units from the original culture. Remember that 10 µL was plated from the 96 well plate and that the beginning culture of bacteria was diluted to 10^{-5} to start and there was some dilution of that in the wells. You will need to take all of these into consideration when determining CFU's. Show your work in the space below.
5. Determine the concentration of the complement used in each well to get the x-axis of your graph. Show work below and place into chart.

Well #	Complement dilution	Well #	Complement dilution
A1		B1	
A2		B2	
A3		B3	
A4		B4	
A5		B5	
A6		B6	
A7		B7	
A8		B8	
A9		B9	
A10		B10	
A11		B11	
A12		B12	

6. Once the dilutions have been determined, graph your results for both the complement dilutions and the CFU's on the graph. Make sure to label your graph appropriately. Your graph should look similar to the example provided.



V. Discussion/Analysis

Answer the following questions and be prepared to discuss.

1. Why does an increase in complement concentration cause a decrease in the number of living bacterial colonies?

2. Why is it important to ensure that you have good sterile technique when plating your samples? Would this cause any unforeseen differences?

3. Why is it important to dilute your initial bacterial culture for the assay?

4. Why is it important to have a negative control? Positive control?

5. What could be a “next step” in this assay to determine if complement is truly causing the bacterial to “die”?

6. Knowing about how complement has the ability to kill bacteria, why do you think it is not used a normal method, such as an antibiotic that you get from the doctor, for people to use?

VI. References

(1) Barrow, R. and Carlone, G. (2001). Serogroup B and C Serum Bactericidal Assays. *Methods in Molecular Medicine: Meningococcal Vaccines, 66,* 289-304.
(2) Figure page 9: http://media-2.web.britannica.com/eb-media/03/20903-004-379E4AF3.gif)
(3) Figure page 16: Lesions on the membrane of a red blood cell resulting from membrane-attack complexes. *J. Humphrey and R. Dourmashkin, 1969,* Advances in Immunology ***11****:75*
(4) Figure page 17: http://abacus.bates.edu/~ganderso/biology/resources/sterile.html
(5) Figure page 18: http://www.mysciencefairprojects.com/bio/microbiology/Minimizing_Bacteria_Meat.aspx
(6) Figure page 20: http://www.bch.msu.edu/bchug/web/bch472/472lm2.htm
(7) Figure page 22: www-micro.msb.le.ac.uk/LabWork/bact/-6L.jpg