Doctor YOU:
Applying Immunological Methodology to Diagnosis

Rita C. English
rita.english@woodbridge.k12.nj.us
John F. Kennedy Memorial High School
200 Washington Avenue
Iselin, New Jersey 08830

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

Mentored by

Debra L. Laskin, Ph. D.
Chair, Department of Pharmacology and Toxicology
Ernest Mario School of Pharmacy, Rutgers, the State University of New Jersey

Carol R. Gardner, Ph. D.
Associate Research Professor, Department of Pharmacology and Toxicology
Ernest Mario School of Pharmacy, Rutgers, the State University of New Jersey

Laurie B. Joseph, Ph. D.
Assistant Research Professor, Department of Pharmacology and Toxicology
Ernest Mario School of Pharmacy, Rutgers, the State University of New Jersey

Yinglin Liu, Ph. D.
Research Associate, Department of Pharmacology and Toxicology
Ernest Mario School of Pharmacy, Rutgers, the State University of New Jersey

Table of Contents

Teacher Guide

Student Section

TEACHER GUIDE

The following New Jersey Core Curriculum Standards are addressed in this unit:

STANDARD 5.1 (Scientific Processes) All students will develop problem-solving, decision-making and inquiry skills, reflected by formulating usable questions and hypotheses, planning experiments, conducting systematic observations, interpreting and analyzing data, drawing conclusions, and communicating results.

STANDARD 5.2 (Science and Society) All students will develop an understanding of how people of various cultures have contributed to the advancement of science technology, and how major discoveries and events have advanced science and technology.

STANDARD 5.4 (Nature and Process of Technology) All students will understand the interrelationships between science and technology and develop a conceptual understanding of the nature and process of technology.

STANDARD 5.5 (Characteristics of Life) All students will gain an understanding of the structure, characteristics, and basic needs of organisms and will investigate the diversity of life.

STANDARD 5.6 (Chemistry) All students will gain an understanding of the structure and behavior of matter.

I. Overview

Doctor You: Applying Immunological Methodology to Diagnosis is a unit that covers the concepts of protein structure and function as well as the mechanisms of antibody-antigen reactions. Lecture and discussion should be used to introduce students to proteins and the human body immune system. Methods of protein identification are presented in virtual online procedures and authentic laboratory experiences that explore electrophoresis.

The goals of these laboratory exercises include familiarizing students with immunological assays including ELISA and gel electrophoresis while also reinforcing lab safety and procedural skills. Students will run gels using gel electrophoresis techniques and analyze the difference in protein composition of a number of samples. Students will also develop an understanding of antibody-antigen relationships and visualize protein compositions and structures. In addition to comprehending the evaluation techniques involved in immunological studies, students will gain an understanding of how infection and disease research can impact their lives and how those proteins are identified and used as diagnostic tools from serious illness to allergic reactions.

There are three practical components of this guide which can be used in a number of different ways. The first directs students to a virtual lab. The second presents to students a set of prepared samples for analysis. The third section builds on the knowledge of electrophoresis to interpret Western Blot results.

This unit compliments several content areas. It can be incorporated into biochemistry, immune system, or disease units within general biology, anatomy and physiology, advanced placement biology, or chemistry classes. The gel electrophoresis technique may also be used to show evolutionary relationships, nutritional comparisons, or any content involving protein comparisons.

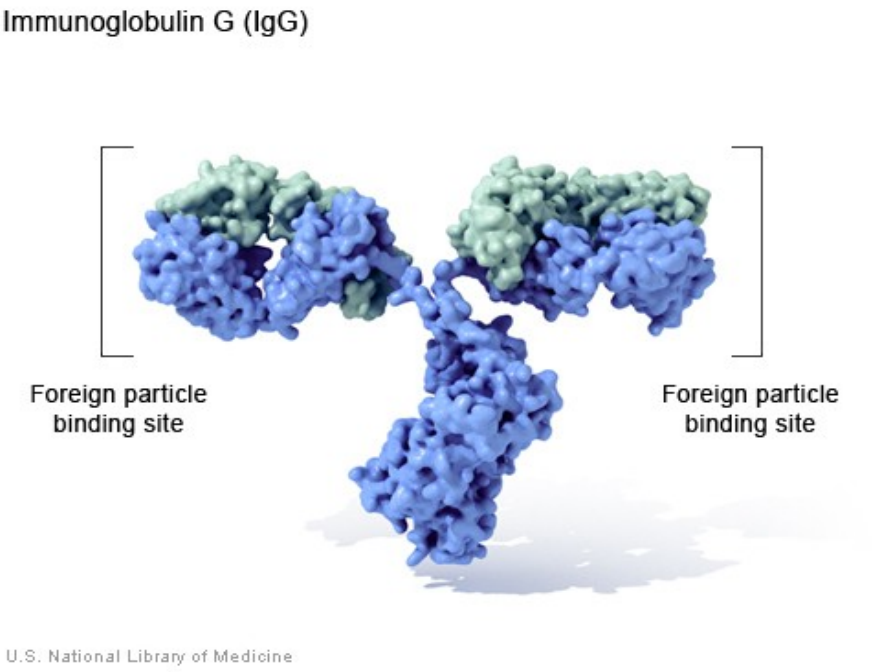
II. Science Background

In his text Biochemistry, Lubert Stryer explains the biochemistry of proteins as extremely important biological molecules which act in a number of life sustaining ways. Essentially, proteins are enzymes that catalyze reactions or make reactions happen at least a million times faster than they normally would. Proteins also transport and store materials, coordinate motion, and provide support. For example, nerves use proteins to relay information between the brain, spinal cord and body allowing us to move, breathe, and respond, the movement of our muscles depends on proteins, and collagen is a protein found in human skin and bone that provides support.

Growth and genetic expression are also regulated by proteins. Hormones and other proteins control the growth of individual cells and organisms. They regulate internal conditions and the expression of our genes as well as protect us from invading materials. Proteins identify and destroy invading viruses, bacteria, and other toxins acting as a vital part of our immune system. However, proteins have complicated structures that are made of amino acids connected by peptide bonds. Many proteins have other components attached to them such as lipids and sugars. Proteins are large molecules that fold up to form compact shapes. Improper folding of proteins can result in disorders such as cystic fibrosis and possibly Alzheimer's disease.

So, why study proteins? Our bodies are composed of different types of proteins, we consume proteins in our food, some of our clothes are made of protein, and our fossil fuels that we burn for energy are composed of protein. Proteins digest our food and give us energy and building materials to stay alive. Many infectious agents are also made of proteins that must be understood before they can be rendered harmless.

In this unit, we will examine the role of proteins in our immune system. Foreign substances such as viruses or bacteria can enter the body in a number of ways, such as through the air we breathe, the skin via a wound, or the digestive track. These invading substances are antigens. We have antibodies to defend our bodies from these invaders. Some proteins, nucleic acids and polysaccharides are antibodies. Protein antibodies are Y-shaped molecules that attach to invaders and act as tags to identify antigens. Other proteins and immune cells then destroy the invaders and protect against infection.



(http://ghr.nlm.nih.gov/handbook/illustrations/proteins?show=igg)

Introduction to Antibodies (Chemicon International, Inc.) describes antigens as foreign substances that bind to specific antibodies. Their "Y" shaped configuration includes two identical copies of "heavy" polypeptide chains (heavy in terms of molecular weight.) They also contain two identical "light" polypeptide chains. Antibodies are divided into classes based on their Y units and their heavy chains. Bonding between antigens and antibodies are generally weak and non-covalent, but some are strong. The bonding of antigens and antibodies is reversible. Chemically checking for the presence of antibodies and antigens can give scientists and doctors a great deal of valuable information.

Antibodies are usually found in our blood protecting us from disease, but they can also be used in the lab to detect functions of the immune system for signs of infection or abnormality. Some of the methods used to evaluate antigens and antibodies include Western Blot, immunohistochemistry, immunocytochemistry, enzyme-linked immunosorbent assay (ELISA), and immunoprecipitation.

The Howard Huges Medical Institute has a virtual immunology lab illustrating the ELISA test at:
http://www.hhmi.org/biointeractive/vlabs/immunology/index.html
This simulation takes approximately thirty minutes to complete. The following questions are for the students to answer as they work through the site.

Questions for the Howard Huges ELISA virtual lab

1. What are the limitations of ELISA?

Answer: A positive result does not necessarily mean the person is sick. If a person is a poor producer of antibody, the antibody may be too low to measure. A positive result may occur if an unrelated antibody reacts with an antigen.

2. What is the purpose of the purple capped vial (without sample) in the centrifuge?
   Answer: Counter balance for the centrifuge
3. Why are the samples centrifuged?
   Answer: To precipitate the blood cells and obtain serum
4. What is the purpose of serial dilutions?
   Answer: To determine the amount of antibody in the sample
5. Why do you discard the pipettor tips between dilutions?
   Answer: To prevent contamination of samples
6. What is a primary antibody?
   Answer: 1st antibody used in an immunoassay to detect the foreign particle
7. Why do you incubate the samples?
   Answer: It helps ensure the antibody in the sample well interacts correctly with the antigen at a temperature similar to body temperature.
8. Why do you remove the liquid from the plate?
   Answer: To remove any antibody that did not react with the SLE antigen
9. Why do you add the rabbit anti-human antibody buffered solution?
   Answer: This does not recognize the SLE antigen. It reacts with the human antibody still present after washing from producing an false positive result.
10. Fill in the following chart by checking the boxes that are colored in the ELISA plate.

A	B	C	+	_
X	X	X	X	
X	X	X	X	
X	X	X	X	

X indicates where students should answer.

11. Which patient is likely to have SLE?
   Answer: A

Antibodies can be used to identify specific proteins through a technique called Western Blotting. The first step is gel electrophoresis. In this method, proteins within a sample are separated based on the molecular weight of their polypeptide chains. Then, a protein sample is placed on a gel (a jelly-like medium that carries the sample) and an electrical current is run through the gel. Proteins contain amino acids which may be positively charged, negatively charged or neutral. Proteins also differ in shape because they are made of long chains that fold in different ways and bond at different sites.

Electrophoresis works by having molecules separate based on their molecular weight as they move along the gel. Proteins are separated based on their molecular weights. Proteins have complicated shapes based on their secondary, tertiary, and quaternary structures. In secondary protein structure, there are hydrogen bonds

connecting amino acids. Additional bonding and interactions among chains give the protein its characteristic shape. To separate proteins by weight they must first be "straightened out" and their charges must be standardized. SDS (sodium dodecyl sulfate) is detergent used in combination with heat and □-mercaptoethanol to prepare the protein sample. Treatment with SDS and heat breaks the hydrogen bonds, renders the protein negative and unfolds the protein. □-mercaptoethanol breaks disulfide bonds. This brings the protein to the linear form used in electrophoresis.

The electrical charge in the gel now causes the different proteins to travel towards the positive electrode. The small molecules (those with lower molecular weight) travel faster as the proteins move along the gel. A stain is added to the finished gel to show the points along the gel at which the proteins stopped. This is used to compare proteins.

Specific proteins can be separated from the gel using a method called Western Blotting. In Western Blotting, the proteins from the electrophoresis are transferred to a nylon membrane and can be reacted with antibodies to detect and determine disease or infection. The lab procedure in this unit will be used to compare proteins from a number of different fish samples using gel electrophoresis. Students may do a pre-lab practice using the instructive electrophoresis animation at:
http://learn.genetics.utah.edu/units/biotech/gel/

The lab included in this unit examines a variety of fish proteins. There are numerous possibilities for protein analysis including comparisons of food sources. Some additional procedures can be found at:
http://www.ncbe.reading.ac.uk/NCBE/PROTOCOLS/protein.html

The following background material comes (with permission) from Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels lab from Carolina Biological Supply Company:

Fish represent a diverse group of organisms that have evolved to live in many different aquatic environments. Fish were chosen as the sample sources because there are many different varieties and because protein sources for many fish species are readily available. Students will compare the proteins of seven different types of fish through gel electrophoresis of the fish protein extracts provided.

The proteins samples provided were extracted from fish muscle tissue. There are a number of proteins that make up muscle tissue. Following is a list of some muscle proteins that may be present as bands in the fish protein fingerprints, along with their approximate molecular weights in kilodaltons: actin (42 kDa), myosin heavy chain (210 kDa), myosin light chains (15, 17, and 24 kDa), titin (3000 kDa), dystrophin (400 kDa), filamin (270 kDa), spectrin (265 kDa), nebulin (107 kDa), α-actinin (100 kDa), gelsolin (90 kDa), fimbrin (68 kDa), tropomyosin (35 kDa), troponin T (30 kDa), thymosin (5 kDa). Since all of the fish protein samples are from muscle tissue, there will be some expected similarities. Nevertheless, difference in the protein banding patterns will also be apparent and these differences can be used to assess evolutionary relatedness.

The buffer that the fish protein extracts are provided in contains both SDS and □-mercaptoethanol to disrupt the structure of the proteins. To ensure that the proteins are fully denatured, the samples should be boiled immediately before being loaded onto the gels, as described in the procedure. To maintain protein denaturation during

electrophoresis, the gels are made with a buffer that contains SDS. The electrophoresis running buffer also contains SDS.

Electrophoresis on Polyacrylamide Gels

The two gel materials most often used in molecular biology applications are agarose and polyacrylamide. Because of its greater resolving power, polyacrylamide is popularly used for protein separations. Polyacrylamide gels can separate molecules that differ in size by as little as 0.2% (1 bp in 500 if working with DNA).

Polyacrylamide is a polymer of the monomer acrylamide. In the presence of free radicals, acrylamide polymerizes into long chains that create a viscous solution of no particular use. To form a rigid gel matrix, acrylamide is polymerized in the presence of a second monomer, N,N'-methylenebisacrylamide (bisacrylamide). The bisacrylamide polymerizes along with the acrylamide and crosslinks the chains to form a rigid meshwork. The *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260) includes four pre-cast, ready-made polyacrylamide gels.

Electrophoresis on Agarose Gels

Although polyacrylamide gels offer greater resolution of protein bands than agarose gels, they are more difficult to use and usually require vertical electrophoresis chambers. As an alternative, gels can be made with fine-sieving agarose. This type of agarose offers better resolution than regular agarose while retaining the ease of use of agarose gels. It is a reasonable compromise for applications in which some resolution can be sacrificed in return for the practical advantages. *The Fish Protein Fingerprinting on Agarose Gels* kit (21-1255) contains enough fine-sieving agarose to run six gels.

III. Student Outcomes

The content of this unit allows students to see and experience the processes of antibody-antigen reactions and understand the biological importance of these mechanisms. Specifically, students will understand the functions of proteins as enzymes, transportation and storage molecules, movement catalysts, supportive agents, neuro-transmitters, growth and expression regulators, and immunological agents.

IV. Learning Objectives

- Students will understand how the immune system works and the importance of proteins in our immune system.
- Students will learn how proteins are identified in samples.
- Students will see the difference in protein composition between related species.
- Students will learn gel electrophoresis techniques.
- Students will perform protein analysis.
- Students will research Western Blot procedure and apply that knowledge in a diagnostic exercise.
- Students will present their information and data to their peers and in a lab report format.

V. Time Requirements

Total time for the unit is approximately 6 hours (more if all research is done in class). The electrophoresis lab is 3 hours. The virtual ELISA is 30 minutes. The Western Blot exercise is 30 minutes in class, with research to follow either in class or at home. Introductory information, including electrophoresis animation, may be presented as a lecture/power point and may take 2 hours. An overnight period is needed to destain gels.

VI. Advanced Preparation

The following material comes from Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels lab from Carolina Biological Supply Company:

The materials needed for one station to perform the activities in this lab is listed below. The materials provided in the *Fish Protein Fingerprinting on Agarose Gels* kit are sufficient for running six agarose gels (6 stations). The materials provided in the *Fish Protein Fingerprinting on Polyacrylamide Gels* kit are sufficient for running four polyacrylamide gels (4 stations). Note: There is enough extra of the fish protein extracts, the stain and destain solutions, and the Tris-glycine-SDS buffer for 6 stations, should you have or choose to purchase additional gels, protein marker, staining trays, and gloves. The protein size standard and seven fish protein samples must be shared among the laboratory workstations in the classroom. Divide your class size accordingly to work at the appropriate number of stations. Prepare as many setups as needed for your class.

For *Fish Protein Fingerprinting on Agarose Gels*, each station will need:
gel casting tray
well-forming comb
masking tape (for sealing gel trays)
35 mL of prepared 4% agarose solution
horizontal gel electrophoresis chamber
350 mL of prepared 1. Tris-glycine-SDS buffer

Time Requirements
Preparation of working solutions 10 min
Preparing agarose gels (if applicable) 20 min
Setting up and loading gels 30 min
Running gels 1 hr 15 min
Staining gels
Agarose: 10 min
Polyacrylamide: 45 min
Destaining gels Overnight

Pre-Lab Preparation
disposable transfer pipet or Pasteur pipet (optional)
gel loading device (e.g., micropipettor and tips)
10 µL of protein size standard
10 µL of each fish sample, on ice

power supply (shared)
staining tray
latex gloves, 1 pair
distilled or deionized water
50–75 mL of Coomassie® stain
100–150 mL of prepared 1. destain solution

For *Fish Protein Fingerprinting on Polyacrylamide Gels*, each station will need:
latex gloves, 2 pairs
pre-cast polyacrylamide gel
vertical electrophoresis chamber
350 mL of prepared 1. Tris-glycine-SDS buffer
disposable transfer pipet or Pasteur pipet (optional)
gel loading device (e.g., micropipettor and tips)
10 µL of protein size standard
10 µL of each fish sample, on ice
power supply (shared)
flathead screwdriver, small (shared)
staining tray
distilled or deionized water
50–75 mL of Coomassie® stain
100–150 mL of prepared 1. destain solution

Preparation of Working Solutions

Tris-glycine-SDS buffer is supplied at a 5X concentration. To prepare the working 1. concentration, dilute the supplied Tris-glycine-SDS buffer 1:5 by adding 400 mL of distilled or deionized water to each 100 mL of 5. concentrate. Each station will need approximately 350 mL of 1. Tris-glycine-SDS buffer. Coomassie® stain is supplied at a 1. working concentration. No dilution is necessary; use as provided. The Coomassie® stain solution can be collected after staining and reused several times. Destain solution is supplied at a 5X concentration. To prepare the working concentration, dilute the supplied destain solution 1:5 by adding 400 mL of water to each 100 mL of 5X concentrate. Each station will need approximately 100–150 mL of 1. destain solution.

Preparation of Agarose Gels for *Fish Protein Fingerprinting on Agarose Gels*

Prepare a 4% solution of fine-sieving agarose by adding 14 g (the entire amount) of powdered fine-sieving agarose to 350 mL of prepared 1. Trisglycine- SDS buffer in a clean 500-mL flask or beaker. Dissolve the agarose by heating the mixture in one of the following ways:

1. Cover the flask or beaker and heat in a boiling water bath until the agarose is completely dissolved. The water level should be just above the level of the agarose mixture. To prevent the agarose from boiling over, swirl the container

2. Heat the uncovered container on a hot plate with magnetic stirring capability.
3. Place a magnetic stir bar in the flask/beaker and stir at a continuous, moderate rate. Heat until the agarose is completely dissolved. Remove from heat at the first sign of vigorous boiling. (Note: The 4% solution of fine-sieving agarose has a tendency to form bubbles while heating. Microwaving increases the number of bubbles produced and is therefore not a recommended means of preparing the agarose solution.)
4. Allow the flask/beaker to cool until it can be held in a bare hand without pain. It should still feel warm and be around 65°C. You can use the agarose immediately or hold it at this temperature in a 65°C-water bath until you are ready to use it.

VII. Materials and Equipment

The following material comes from Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels lab from Carolina Biological Supply Company:

(Note: Upon receipt, store the fish protein extracts in a freezer at approximately –20°C. Store the protein size standard and pre-cast polyacrylamide gels in a refrigerator at approximately 4°C. All other materials may be stored at room temperature at approximately 25°C. Avoid repeated freezing and thawing of fish protein extracts. Keep fish protein extracts on ice while in use.)

Materials
Materials included in *Fish Protein Fingerprinting on Agarose Gels*
kit (21-1255)
fish protein extracts, 7 samples, 150 µL each
protein size standards, 2 tubes, 50 µL each
fine-sieving agarose, 14 g
Tris-glycine-SDS buffer, 5X̃ concentrate, 500 mL
Coomassie® protein stain solution, 500 mL
destain solution, 5X̃ concentrate, 500 mL
latex gloves, 6 pairs
staining trays, 6

Materials not supplied in *Fish Protein Fingerprinting on Agarose Gels* kit (21-1255)
horizontal gel electrophoresis chambers
gel casting trays
well-forming combs
masking tape (for sealing gel trays)
power supplies capable of providing 130 volts
water bath, boiling
micropipets and tips capable of measuring 10 µL volumes, or other
gel-loading device
distilled or deionized water

containers with ice
platform shaker (optional)
water bath, 65°C (optional)
transfer pipets or Pasteur pipets (optional)
white light illuminator (optional)

Materials included in *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260)
fish protein extracts, 7 samples, 150 µL each
protein size standards, 50 µL
pre-cast polyacrylamide gels in Tris-glycine-SDS buffer, 4
Tris-glycine-SDS buffer, 5X concentrate, 500 mL
Coomassie® protein stain solution, 500 mL
destain solution, 5X concentrate, 500 mL
latex gloves, 8 pairs
staining trays, 4

Note: There is enough extra of the fish protein extracts, the stain and destain solutions, and the Tris-glycine-SDS buffer for 6 stations, should you have or choose to purchase additional gels, protein marker, staining trays, and gloves.

Materials not supplied in *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260)
vertical gel electrophoresis chambers for 9.5 10-cm gels
power supplies capable of providing 130 volts
water bath, boiling
micropipets and tips capable of measuring 10 µL volumes, or other
gel-loading device
distilled or deionized water
flathead screwdriver, small
containers with ice
transfer pipets or Pasteur pipets (optional)
platform shaker (optional)
white light illuminator (optional)

Teacher Tips

- Agarose gels may be cast during one lab period and stored up to two days in the electrophoresis chamber covered in 1 Tris-glycine-SDS buffer. Loading the gels with fish protein samples and performing electrophoresis can then be performed on a subsequent day.
- Store the fish protein extracts in a freezer at approximately –20°C and do not thaw them until you are ready to use them. Repeated freezing and thawing can degrade the protein samples and decrease the quality of the protein fingerprints. Keep the extracts on ice during use.

- The fish protein samples must be heated in a boiling water bath before loading on the gel. Heat the water for the boiling water bath while students are setting up their gels, so that the water bath is ready when the loading stage is reached.
- The protein size standard is included in the electrophoresis as a control to help determine whether the gels were run properly. As an extension, you may wish to have your students use this standard to estimate the sizes of particular proteins in their samples.

Safety Tips

- Eye protection is recommended for all procedures associated with this activity.
- Wear gloves when handling the polyacrylamide gels.
- The Tris-glycine-SDS buffer, Coomassie® protein stain, and destain solution are nontoxic and may be disposed of down the drain.
- Coomassie® stain (composed of Coomassie® Brilliant Blue) is an efficient protein stain and therefore easily dyes skin and clothing. Wear gloves when working with this stain; avoid stain contact with skin and clothing.
- Polyacrylamide gels are made of polymers of acrylamide and bisacrylamide. Acrylamide is a dangerous neurotoxin, but it becomes harmless when polymerized with bisacrylamide to form a polyacrylamide gel. However, gels may still contain traces of unpolymerized material and should be handled with gloves. Used gels may be disposed of in the regular trash.
- The pre-cast polyacrylamide gels contain 0.02% sodium azide as a preservative to prevent microbial contamination during refrigerated storage. Sodium azide at this concentration is not known to cause health problems, although high concentrations of sodium azide are harmful (refer to the Materials Safety Data Sheet supplied for these gels by the manufacturer).

Fish protein extracts are provided in:
50 mM Tris-HCl, pH 6.8
5% □-mercaptoethanol
2% sodium dodecyl sulfate
0.1% bromphenol blue
10% glycerol

Protein size standard
The tube(s) of protein standard provided contain approximately 1 mg/mL each of the following proteins:

Protein	Molecular weight (kilodaltons)
myosin	200.0
□-galactosidase	116.3
phosphorylase B	97.4
bovine serum albumin	66.3
glutamic dehydrogenase	55.4
lactate dehydrogenase	36.5

carbonic anhydrase 31.0
trypsin inhibitor 21.5
lysozyme 14.4
aprotinin 6.0
insulin B chain 3.5
insulin A chain 2.5

Tris-glycine-SDS buffer, 5X concentrate
0.125 M Tris base
1.25 M glycine
0.5% SDS (sodium dodecyl sulfate)

Coomassie® Protein Stain
10% acetic acid
10% isopropanol
0.25% Coomassie® Brilliant Blue

Destain Solution, 5X concentrate
50% acetic acid
50% isopropanol"

VIII. Student Prior Knowledge and Skills

Students should understand the structure and function of proteins. They should have knowledge of antibody-antigen reactions. Students should be familiar with lab safety and measurement procedures. The electrophoresis simulation should provide some useful background information.

IX. What Is Expected from Students

Students are expected to learn the importance of proteins in the immune system. They will learn the mechanism of gel electrophoresis and be able to explain the reasons behind the procedures. Students will run gels, prepare samples, analyze results. They will also apply the knowledge to analyze Western Blots and present their results to the class.

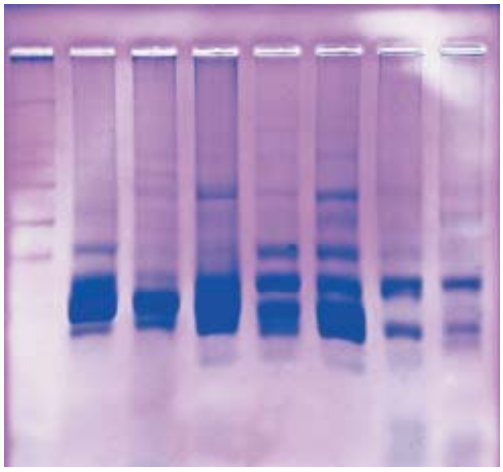
X. Anticipated Results

Answers are included in the science background section to the ELISA online simulation.

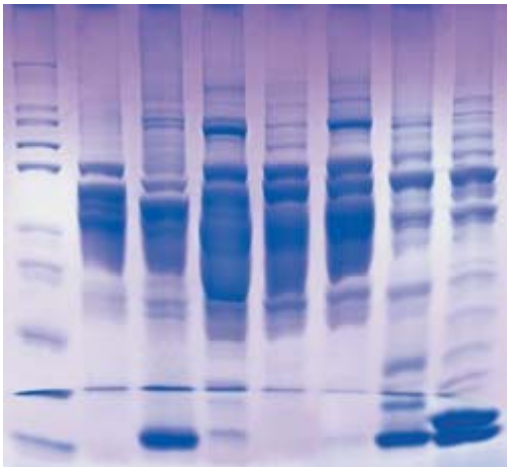
Answers to the Dr. YOU section are:
Patients 1: Ulcers
2: Normal
3: Ulcers & Lymes
4: HIV
5: Lymes
6: Normal

7: HIV & Lymes

Student gels should show similarities and differences in proteins between fish samples. A sample gel should resemble the following:
(from Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels lab from Carolina Biological Supply Company)



Agarose gel



Polyacrylamide gel

Results for the samples you prepare will vary. The bands may not be as distinct, but students should be able to distinguish related foods, and comparative numbers of proteins.

XI. Classroom Discussion

Answers to Lab Questions:

1. What is the function of the gel itself?
   Answer: It is a matrix with pores that molecules travel through and sort by size.

2. Why is an electric current added?
   Answer: It is the force that causes the negatively charged protein molecule to move toward the positive poles.

3. How do you know the proteins are moving along the gel because of the differences in their molecular weights and not the differences in their shapes or charges?
   Answer: You have added SDS, heat, and β-mercaptoethanol to unfold the proteins and standardize the charge.

4. What would happen to the proteins if you forgot to turn the power off?
   Answer: They would run off the end of the gel.

5. What do the bands on your gel represent?
   Answer: Different proteins

6. How many bands are shared between the samples?
   Answer: Check results

7. How many samples have bands that are unique?
   Answer: Check results

8. What have you learned from this lab?
   Answer: Varies

9. List some areas in which the method used in this lab could help people.
   Answer: Varies, but may include - Medicine, Criminal Investigation, Materia Analysis for Industry

10. Using the information from your lab construct a graph showing the relationship between molecular weight and distance traveled by the proteins in each sample.
   Answer: Varies, but should show a linear relationship – as molecule weight increases, distance traveled should increase.

11. Draw your gel results and explain what you have found.
   Answer: Varies

Students should include analysis and conclusion including possible sources of error in their lab report.

XII. Assessment

Students should be required to write a lab report including worksheet answers for the ELISA and gel electrophoresis labs. The results of the unit may be presented by assigning each group a topic.

Group 1 – Parts and Functions of the Immune System
Group 2 – Threats to the Immune System
Group 3 – Antibody-Antigen Reactions
Group 4 – How Vaccines Work
Group 5 – Infectious Diseases and Their Impact on Society
Group 6 – Gel Electrophoresis – How does it work? How is it used?

If you prepare your own samples from food sources, students may include a descriptive analysis of the gels. They can describe the differences between the food materials in terms of the protein content. If the instructor prepares the samples, students may be asked to research the protein content of the materials. Students can be given the samples as unknowns and, by comparing their research and results, they can identify their unknowns.

XIII. References

Berg, J.M., Tymoczko, J.L., and L. Stryer. (2002) *Biochemistry*. 5th Ed. New York: W.H. Freeman

Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels. Carolina Biological Supply Company. Burlington, NC

Introduction to Antibodies. Chemicon International, Inc. Temedcula, CA.

http://ghr.nlm.nih.gov/handbook/illustrations/proteins?show=igg [viewed 8/8/2008]

http://learn.genetics.utah.edu/units/biotech/gel/ [viewed 8/8/2008]

www.jemsekclinic.com/lyme_detail.php?sid=9 [viewed 9/4/2008]

http://www.hhmi.org/biointeractive/vlabs/immunology/index.html [viewed 8/8/2008]

http://www.hivinfosource.org/hivis/hivbasics/results [viewed 8/17/2008]

http://www.ncbe.reading.ac.uk/NCBE/PROTOCOLS/protein.html [viewed 8/7/2008

STUDENT SECTION

I. Rationale

Take a deep breathe, have a meal, shake hands, open a door, or use a phone. Everything we do exposes us to pathogens. Inside our bodies and in our environment, there are pathogens, such as bacteria, viruses, and parasites, capable of infecting and killing us. What keeps us alive? Our healthy bodies do most of the work but medicines, including vaccines, also help us survive. How are they made and what about the diseases that we do not have medications or vaccinations to turn to for help? How do scientists know what to put in a vaccine to keep us safe from the measles, polio, smallpox and so many other diseases? They look inward at our defenders always at work in our immune system.

Pathogens are constantly invading our bodies. These invaders are met by our bodies' defense cells in our immune system. Our immune system includes a number of different cells. They include lymphocytes (B and T) and macrophages. Macrophages engulf foreign particles to begin their breakdown. Proteins identify and destroy invading viruses, bacteria, and other harmful substances to keep us healthy.

You have studied the structure and function of proteins. Scientists use their knowledge of proteins in a number of ways. One important way is to better understand and assist the immune system. You will be examining and performing lab procedures used to identify and compare proteins in a number of organisms.

Introduction

The following material comes from Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels lab from Carolina Biological Supply Company:

The biochemical composition of organisms includes their protein molecules. Thus, the degree of relatedness of two species can be estimated from the amount of similarity between their protein profiles. To compare protein profiles between organisms, scientists separate the mixture of protein molecules in a particular tissue, such as muscle tissue, by gel electrophoresis. This creates a unique pattern of bands for each organism, called a protein fingerprint. The individual bands correspond to different proteins and may vary in intensity between species. In addition, some bands may be visible in one species fingerprint but not in another. In general, protein fingerprint patterns obtained from different species are more similar when the species are more closely related and less similar when they are more distantly related.

In this exercise, you will compare the protein fingerprints of seven different types of fish through gel electrophoresis of the fish protein extracts provided. Fish were chosen as the sample sources because there are many different varieties and because protein sources for many fish species are readily available.

The behavior of a molecule during gel electrophoresis depends on its size, shape, and net charge. Linear DNA molecules have uniformly negatively charged backbones and a shape that normally varies only in its length. Therefore, migration of DNA is directly dependent on the size of the DNA fragment. The migration of proteins, however, is affected by multiple factors involving their structural organization.

There are four levels of structural organization in proteins. The primary structure of a protein is its sequence of amino acids. Amino acids can be positively charged, negatively charged, or neutral. This means that proteins can carry either a net positive, net negative, or neutral charge depending on the combination of amino acids they contain.

The shapes of proteins vary widely. The shape of a protein is created by its secondary, tertiary, and quaternary structure. In secondary protein structure, hydrogen bonds form between adjacent parts of the amino acid chain to form folded, coiled, or twisted shapes, including α-helices and β-sheets. Additional interactions, such as hydrogen bonds, hydrophobic interactions, electrostatic interactions, and/or disulfide bonds lead to the tertiary structure of a protein. At the quaternary structural level, several folded amino acid chains associate in unique ways to form a functional protein with a distinctive shape.

Native conformations of proteins (the form in which they are biologically active) vary widely in charge and shape. As such, the molecular weight of proteins cannot be determined by electrophoresis of native proteins. To make protein migration rates a function of molecular weight, it is necessary to impose a uniform shape and charge on all of the proteins in a mixture. This can be primarily achieved by treating the protein mixture with the negatively charged detergent sodium dodecyl sulfate (SDS) and heat. Treatment with SDS and heat disrupts hydrogen bonds and unfolds the protein structure. SDS also binds to and coats the protein backbone, regardless of the amino acid sequence, and imparts a uniform negative charge to all the molecules. Treating protein samples with a reducing agent such as β-mercaptoethanol breaks disulfide bonds and denatures the proteins into linear chains of amino acids (its primary structure).

Under these conditions and for the purpose of electrophoresis, all of the proteins in a mixture assume the same shape and charge. They differ only in molecular weight. Like DNA, they migrate toward the positive electrode during electrophoresis at a rate inversely proportional to the log10 of their molecular weights. The buffer that the fish protein extracts are provided in contains both SDS and β-mercaptoethanol to disrupt the structure of the proteins. To ensure that the proteins are fully denatured, the samples should be boiled immediately before being loaded onto the gels, as described in the procedure. To maintain protein denaturation during electrophoresis, the gels are made with a buffer that contains SDS. The electrophoresis running buffer also contains SDS.

II. Materials

Computer with Internet Access
Student Worksheet for ELISA

Dr. YOU pictures and questions

The following material comes from Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels lab from Carolina Biological Supply Company:

Materials included in *Fish Protein Fingerprinting on Agarose Gels* kit (21-1255)
fish protein extracts, 7 samples
protein size standards
fine-sieving agarose, 14 g
Tris-glycine-SDS buffer, 5X concentrate
Coomassie® protein stain solution
destain solution, 5X concentrate
latex gloves,
staining trays

Materials not supplied in *Fish Protein Fingerprinting on Agarose Gels* kit (21-1255)
horizontal gel electrophoresis chambers
gel casting trays
well-forming combs
masking tape (for sealing gel trays)
power supplies capable of providing 130 volts
water bath, boiling
micropipets and tips capable of measuring 10 μL volumes, or other
gel-loading device
distilled or deionized water
containers with ice
platform shaker (optional)
water bath, 65°C (optional)
transfer pipets or Pasteur pipets (optional)
white light illuminator (optional)

Materials included in *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260)
fish protein extracts, 7 samples
protein size standards
pre-cast polyacrylamide gels in Tris-glycine-SDS buffer, 4
Tris-glycine-SDS buffer, 5Xconcentrate
Coomassie® protein stain solution
destain solution, 5X concentrate
latex gloves,
staining trays
trays, and gloves.

Materials not supplied in *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260)
vertical gel electrophoresis chambers for 9.5 . 10-cm gels
power supplies capable of providing 130 volts
water bath, boiling
micropipets and tips capable of measuring 10 μL volumes, or other gel-loading device
distilled or deionized water
flathead screwdriver, small
containers with ice
transfer pipets or Pasteur pipets (optional)
platform shaker (optional)
white light illuminator (optional)

III. Procedures

Some of the methods used to evaluate antigens and antibodies are Western Blot, immunohistochemistry, immunocytochemistry, enzyme-linked immunosorbent assay (ELISA), and immunoprecipitation.

The Howard Huges Medical Institute has a virtual immunology lab illustrating the ELISA test at:
http://www.hhmi.org/biointeractive/vlabs/immunology/index.html

Use this simulation to answer the following questions:

Questions for the Howard Huges ELISA virtual lab

1. What are the limitations of ELISA?

2. What is the purpose of the purple capped vial (without sample) in the centrifuge?

3. Why are the samples centrifuged?

4. What is the purpose of serial dilutions?

5. Why do you discard the pipettor tips between dilutions?

6. What is a primary antibody?

7. Why do you incubate the samples?

8. Why do you remove the liquid from the plate?

9. Why do you add the rabbit anti-human antibody buffered solution?

10. Fill in the following chart by checking the boxes that are colored in the ELISA plate.

A	B	C	+	_

11. Which patient is likely to have SLE?

The following material comes from Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels lab from Carolina Biological Supply Company:

Electrophoresis Procedure

1. Seal the open ends of the gel casting tray with masking tape so that no seams or gaps appear.

2. Insert the well-forming comb in the top set of grooves over the black stripe in the casting tray.

3. Carefully, pour a thin layer of the prepared 4% fine-sieving agarose solution into the casting tray until it just covers the bottom of the tray (~35 mL). Thin gels will give more desirable protein electrophoresis results than thick gels.

4. While the agarose is still liquid, move bubbles and debris to the perimeter of the tray with the wellforming comb. Return the comb to its position in the top set of grooves in the casting tray.

5. Allow the gel to sit undisturbed while it solidifies for **10 minutes**. Be careful not to move or jar the casting tray during this time.

6. Once the agarose has solidified, slowly and carefully remove the comb from the gel without tearing the wells.

7. Remove the tape at the ends of the casting tray to unseal the gel.

8. Place the gel and casting tray into the electrophoresis chamber oriented with the red stripe towards the positive (red) end, and the black stripe towards the negative (black) end.

9. Fill the electrophoresis chamber with 1. Tris-glycine-SDS buffer to a level that just covers the surface of the gel.

10. The gel is now ready to load with samples. If you will be loading the gel at another time, cover the electrophoresis tank with the lid to prevent the gel from drying out.

11. Rinse gel debris from the wells of the gel by pipetting the surrounding 1. Tris-glycine-SDS buffer in and out of the wells. If available, a disposable transfer pipet or a Pasteur pipet works well for this purpose.

12. Prepare fish protein extracts for loading by immersing the sample-containing portion of the tubes in a boiling water bath for 3 to 5 minutes. Do not immerse the lids. **Do not boil the protein size standard**. Immediately after the samples have been heated, it is time to carefully begin to load them.

13. Load 10 µL of protein size standard and fish extracts into the wells (also called lanes) from left to right following the order and procedure below.

- ☐ To load the first sample (protein size standard) into the well, draw 10 µL of the sample into a pipet tip.
- ☐ Using your dominant hand, steady the pipet over the well.
- ☐ Rest the elbow of your dominant arm on the lab bench to stabilize your hand.
- ☐ Using your non-dominant hand, guide the pipet tip through the surface of the buffer and position it directly over the well.
- ☐ Slowly expel the sample into the well (see Figure 1).

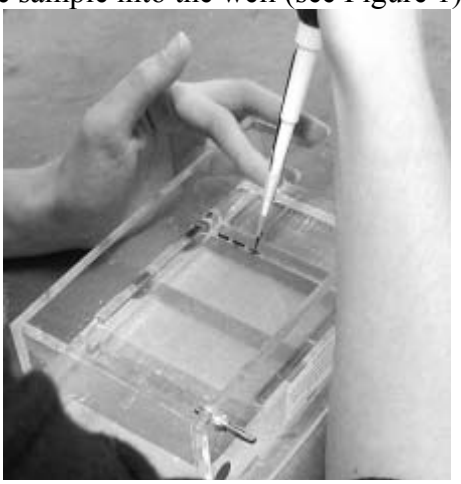


Figure 1. Loading samples into the agarose gel.

- ☐ The sample will sink to the bottom of the well because it has been mixed with glycerol to increase its density.
- ☐ Repeat this process for each sample, continuing from left to right according to the Order of Loading.
- ☐ Use a clean pipet tip for each sample.
- ☐ Be sure to check the label on each tube before you load to ensure that it matches the intended order.

Order of Loading
lane 1 protein size standard
lane 2 shark
lane 3 catfish
lane 4 salmon
lane 5 swordfish
lane 6 tuna
lane 7 flounder
lane 8 orange roughy

14. Connect the electrodes to the power supply [positive lead to positive input (red to red) and negative lead to negative input (black to black)] and run the gel at 130 volts. At this

voltage, the bromphenol blue loading dye in the samples should move through the gel to the bottom in approximately 1 hour and 15 min

.

15. After electrophoresis is complete, turn off the power supply and remove the lid of the electrophoresis chamber.

Staining and Destaining Procedure for Agarose Gels

1. Wearing gloves, place the gel in a staining tray and flood it with Coomassie® stain solution until it is completely covered (~50 to 75 mL). The entire gel will turn blue. Let the gel stain for 10 minutes.

2. After staining is complete, carefully return the Coomassie® stain to its container. The Coomassie® stain solution can be reused several times.

3. Rinse the gel several times with distilled or deionized water by repeatedly flooding the tray with water and pouring the wash down the drain.

4. Flood the gel with destain solution until it is completely immersed (~50 to 75 mL). If possible, gently agitate the gel on a platform shaker while it destains.

5. Allow the gels to destain overnight. The destain solution can be changed during destaining, to facilitate the destaining process. Distinct blue protein banding patterns should become visible in the gel as the blue background lightens. Gels can be stored covered in destain solution for several days.

6. Gels are best viewed when placed on a white light illuminator, if available.

Electrophoresis Procedure for Fish Protein Fingerprinting on Polyacrylamide Gels

These instructions are written for use with the Carolina™ Vertical Gel Electrophoresis Chamber (21-3671). If you do not have this equipment, modify these instructions to suit your apparatus.

1. Wearing gloves, remove the polyacrylamide gel from its aluminum package. Rinse the gel with distilled or deionized water.

2. Remove the comb and rinse the exposed wells with distilled or deionized water.

3. Inspect the bottom of your gel cassette. If the bottom of the gel is exposed, proceed to the next step. If a plastic tab conceals the bottom, snap off the removable lower portion of the plastic cassette from the pre-cast gel (there is an indentation that separates the bottom tab from the main unit). Rest the cassette on the bench top with the bottom tab overhanging the edge. Press downward on the cassette with one hand,

then rotate the detachable tab up and down until it snaps free (see Figure 2). Removing the lower portion of the cassette exposes the bottom of the gel so that it can be in direct contact with the running buffer during electrophoresis.

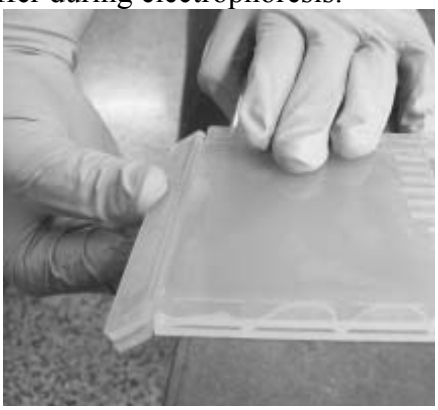


Figure 2. Removing the lower portion of the plastic cassette.

4. Place the pre-cast gel in the lower electrophoresis chamber with the notched plate flush against the side of the upper buffer chamber. The edges of the gel should rest on small plastic platforms that raise the gel about 1 mm off the bottom of the chamber. This will allow the running buffer to contact the bottom of the gel during electrophoresis (see Figure 3).

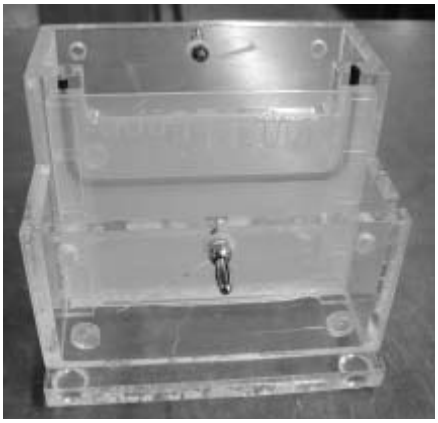


Figure 3. Placement of the polyacryamide gel in the electrophoresis chamber (arrows indicate the raised platforms on which the edges of the gel should rest).

5. Add just enough 1. Tris-glycine-SDS buffer to the lower chamber to cover the bottom of the gel. Make sure no air bubbles are trapped beneath the gel as they will interfere with the electrical current during electrophoresis.

6. Secure the gel in place by sliding the lid (with the red electrical lead) on the lower chamber and tightening the thumbscrews all the way. If the screws are not securely fastened, buffer will leak from the upper chamber to the lower chamber and electrophoresis will be halted.

7. Add 1. Tris-glycine-SDS buffer to the upper chamber until the top of the gel is covered. The buffer layer should remain at the same level. If the buffer level begins to sink below the top of the gel, tighten the thumbscrews and add more buffer to the upper chamber. The top and bottom of the gel must be covered with buffer at all times for an electrical current to be maintained.

8. Slide the lid (with the black electrical lead) onto the upper chamber and tighten the thumbscrews to secure it in place.

9. Rinse gel debris from the wells of the gel by pipetting the surrounding 1. Tris-glycine-SDS buffer in and out of the wells. If available, a disposable transfer pipet or a Pasteur pipet works well for this purpose.

10. Prepare fish protein extracts for loading by immersing the sample-containing portion of the tubes in a boiling water bath for 3 to 5 minutes. Do not immerse the lids. **Do not boil the protein size standard.** Immediately after the samples have been heated, load them according to the following instructions.

11. Load 10 µL of protein size standard and fish extracts into the wells (also called lanes) from left to right.

- ☐ To load the first sample (protein size standard) into the well, draw 10 µL of the sample into a pipet tip.
- ☐ Coming from the upper chamber, place the tip of the loading device against the unnotched gel plate directly over the well to be loaded.
- ☐ Slowly expel the sample into the well (see Figure 4).

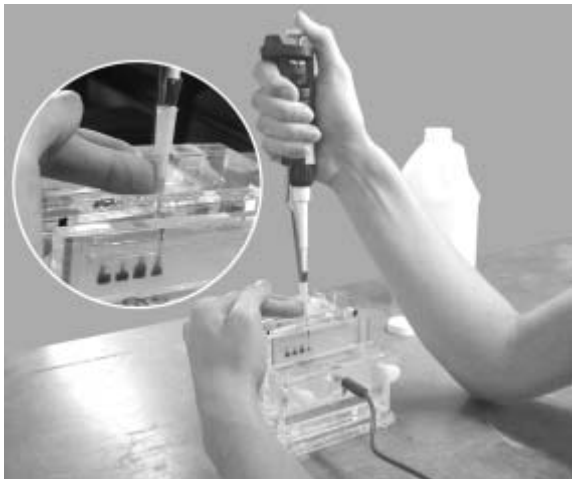


Figure 4. Loading samples into the polyacrylamide gel.

- ☐ The sample will sink to the bottom of the well because it has been mixed with glycerol to increase its density.
- ☐ Repeat this process for each sample, continuing from left to right in the order given below.
- ☐ Use a clean pipet tip for each sample. Be sure to check the label on each tube before you load to ensure that it matches the intended order.

Order of Loading
lane 1 protein size standard
lane 2 shark
lane 3 catfish
lane 4 salmon
lane 5 swordfish
lane 6 tuna
lane 7 flounder
lane 8 orange roughy

12. Connect the electrodes to the power supply [positive lead to positive input (red to red) and negative lead to negative input (black to black)] and run the gel at 130 volts. At this voltage, the bromphenol blue loading dye in the samples should move through the gel to the bottom in approximately 1 hour and 15 min.

13. After electrophoresis is complete, turn off the power supply and remove the lid to the bottom chamber by loosening the thumbscrews and sliding off the lid.

14. Wearing gloves, take the gel cassette out of the chamber. The polyacrylamide gel must now be removed from the plastic cassette. To remove the gel, first place the cassette on a lab bench. Insert the tip of a small flat screwdriver between the plates and twist the screwdriver gently until you hear the seal break (see Figure 5). Move along the edge of the cassette and repeat this action until the entire seal is broken. Break the seal on the other side in the same manner. Gently remove the top plate to access the gel.

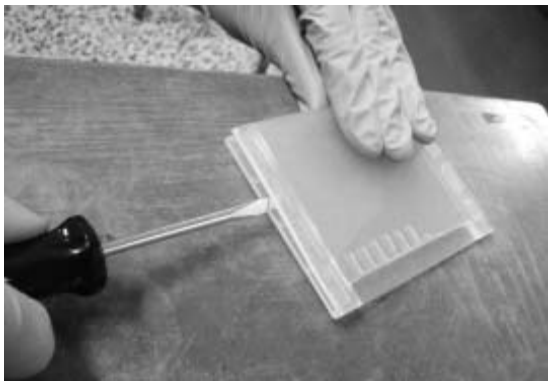


Figure 5. Removing the gel from the plastic cassette.

Staining and Destaining Procedure for Polyacrylamide Gels

1. Wearing gloves, place the gel in a staining tray and flood it with Coomassie® stain solution until it is completely covered (~50 to 75 mL). The entire gel will turn blue. Let the gel stain for 45 minutes.

2. After staining is complete, carefully return the Coomassie® stain to its container. The Coomassie® stain solution can be reused several times.

3. Rinse the gel several times with distilled or deionized water by repeatedly flooding the tray with water and pouring the wash down the drain.

4. Flood the gel with destain solution until it is completely immersed (~50 to 75 mL). If possible, gently agitate the gel on a platform shaker while it destains. Allow the gels to destain overnight. The destain solution can be changed during destaining, to facilitate the destaining process. Distinct blue protein banding patterns should become visible in the gel as the blue background lightens. Gels can be stored covered in destain solution for several days

.

5. Gels are best viewed when placed on a white light illuminator, if available.

IV. Data Collection

Keep track of how long it took to load your gels, include a diagram of your gel before placement of samples, and at 30 minutes intervals along the way. Draw a final diagram of your results. Be sure you have recorded the correct lane orders for your samples.

V. Discussion/Analysis

Include your ELISA worksheet in your discussion. Discuss sources of error. Answer the following questions as part of your analysis:

1. What is the function of the gel itself?

2. Why is an electric current added?

3. How do you know the proteins are moving along the gel because of the differences in their molecular weights and not the differences in their shapes or charges?

4. What would happen to the proteins if you forgot to turn the power off?

5. What do the bands on your gel represent?

6. How many bands are shared between the samples?

7. How many samples have bands that are unique?

8. What have you learned from this lab?

9. List some areas in which the method used in this lab could help people.

10. Using the information from your lab construct a graph showing the relationship between molecular weight and distance traveled of the proteins in each sample.

11. Draw your gel results and explain what you have found.

Dr. YOU

Patients see their doctors because of symptoms, family histories, or possible contact with infectious agents. In the following cases, the doctors have narrowed their findings down to a few disorders. As the doctor, you will diagnosis your patients based on immunological testing results.

You have seven patients who may have one or more of the following disorders:

HIV
Lymes Disease
Ulcers (caused by bacteria)

Examine the Western Blots to determine what disorders are present in which patients.

> Western Blots are immunological tests that do not test for the antigen, the infecting agent. These tests detect the presence of the antibodies our immune system uses to combat the antigen. If there are high levels of particular antibodies, then there were antigens that caused their production. As the doctors, you can use the results of the Western Blot as an indicator of infection.

The pictures are actual Western Blot results. You can see that they are not always easy to read.

http://www.hivinfosource.org/hivis/hivbasics/results/
www.jemsekclinic.com/lyme_detil.php?sid=9
http://www.cdc.gov/NCIDOD/eid/vol6no2/dobosG.htm

HIV
To be HIV positive a Western Blot must have 5 horizontal stripes in common with the control.

Lymes
To be Lyme positive, 5 stripes must match.

Ulcers
To have ulcers caused by *Mycobacterium ulcerans bacteria*, 5 stripes must match.

Report Directions:

- ☐ Examine the seven patients and write a report of your findings for them. The introduction of your report should explain what Western Blotting is and how it is used to diagnose illness.
- ☐ Then, you will explain the particular antigen-antibody reactions in each of the Western Blot tests (HIV, Lymes, and Ulcers).
- ☐ Next, give the particular symptoms and prognosis for each disorder.
- ☐ Finally, list your seven patients, 1-7, and tell which disorder(s) they have.

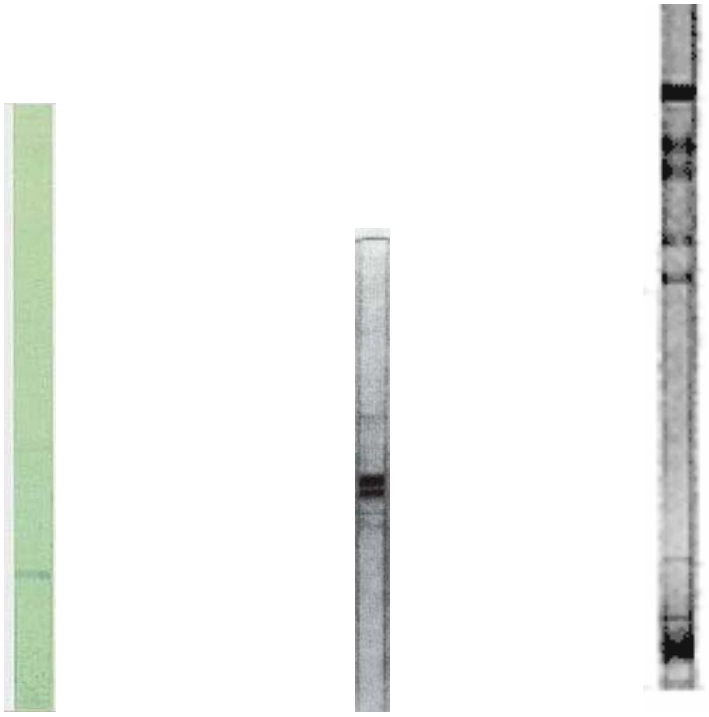
HIV

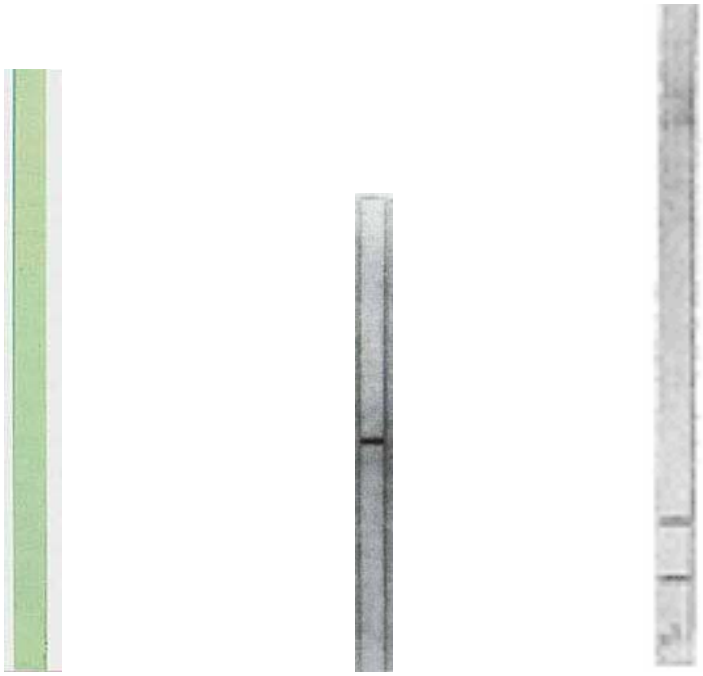
Lymes

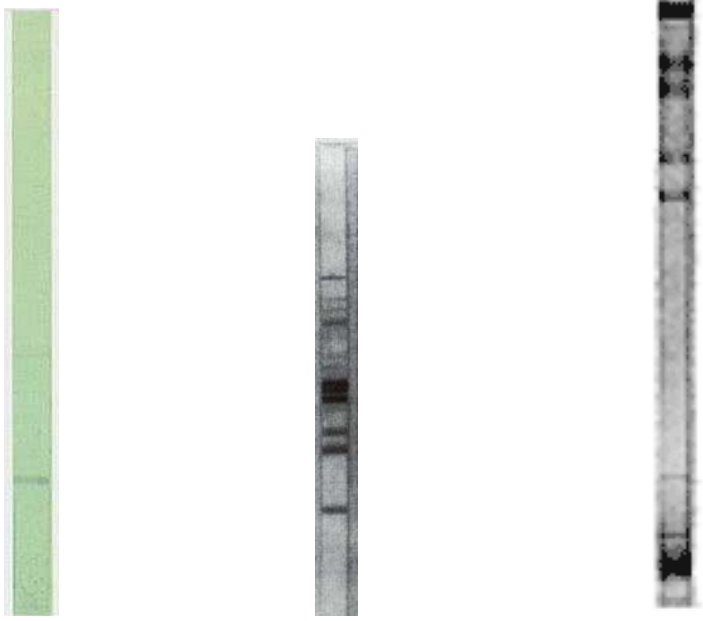
Ulcers

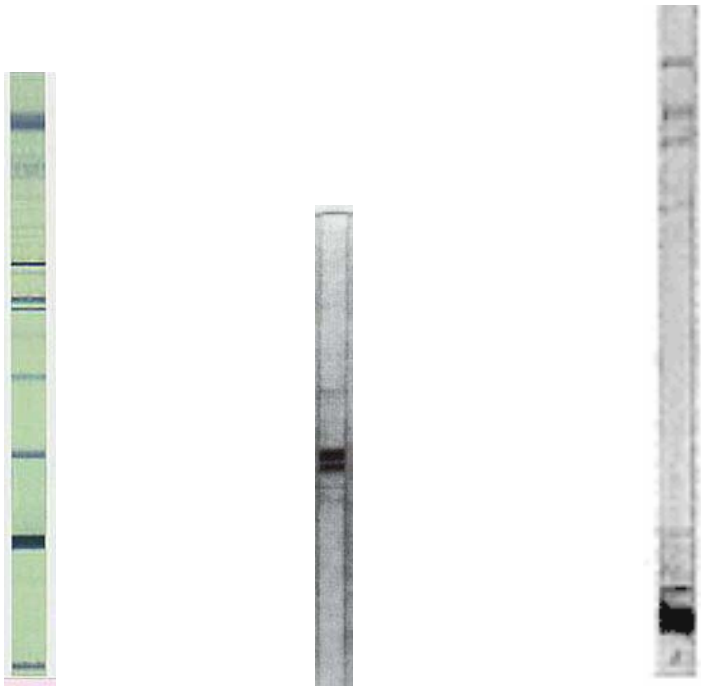
(Controls)

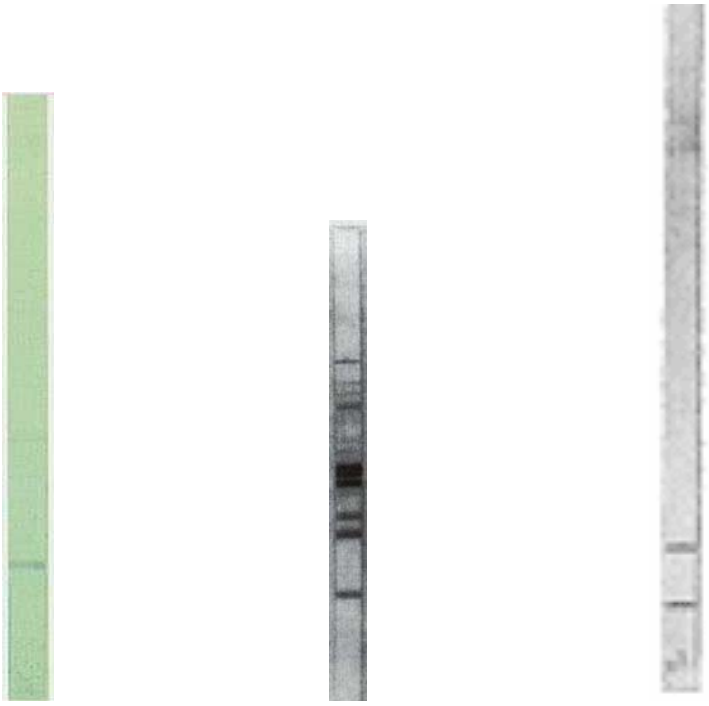
Western Blot Results

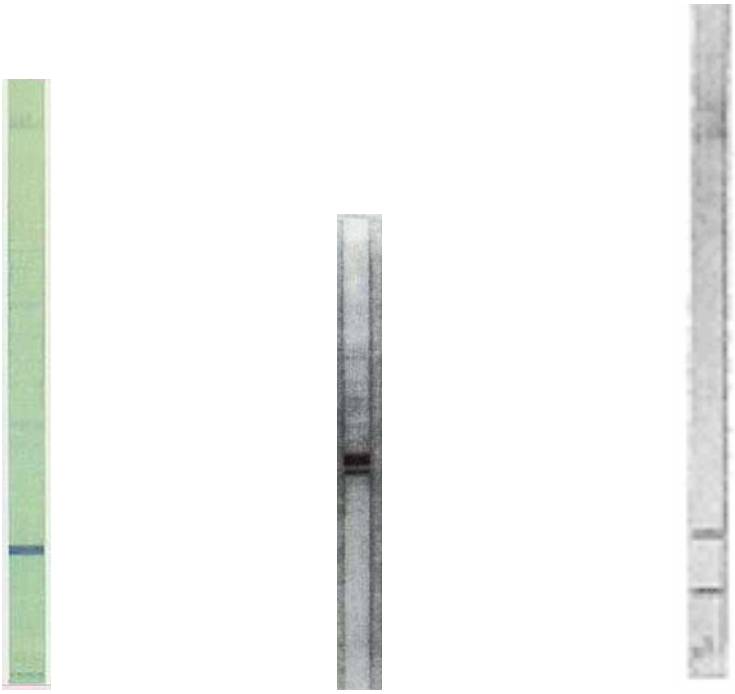
Patient 1: 

Patient 2: 

Patient 3: 

Patient 4: 

Patient 5: 

Patient 6: 

Patient 7: