SECTION ONE

General Information

Equipment List

Ordering Information

Solution Preparation

Aliquots for Students

ELISA LAB - General Information

Goals of Lab Exercise

ELISA is a standard test used in labs to confirm the existence of antibodies and to quantify the amount of antibody present. This lab fits easily into any unit that deals with disease, allergies and immunology in general. As a stand-alone exercise, it provides an opportunity for the student to learn several lab techniques and to learn about antibody/antigen relationships.

By using this ELISA exercise as part of your lab curriculum, students will:

1) Become familiar with an important lab test

2) Gain experience with the use of micropipettes to transfer microliters of liquids.

3) Learn to calculate serial dilutions

4) Learn the concept of complementarity

5) Learn the relationship between antigens and antibodies

6) Gain insight as to how ELISA might be used in asthma research and the detection of HIV in blood serum.

7) Learn the proper disposal of used materials

Science Background

In order to implement this lab, the teacher must be able to prepare some simple solutions. Also, familiarity with the use of micropipettes is required. Other than that, the lab protocols are self-explanatory. All the documents provided will help you to understand this lab and can be used as is, or can be modified for student use.

Section 1 contains the information you need to prepare for this lab.

Section 2 is a glossary of terms that are relevant to this lab. This should be provided to the students and be required reading.

Section 3 has some information on how ELISA is used.

Section 4 describes the concept of ELISA.

Section 5 covers the special skills needed to do this lab

Section 6 is the Lab Protocol.

Learning Objectives

1) The student should be able to demonstrate the proper use of a micropippette including when to change tips and the proper disposal of used tips

2) The student should be able to explain the value of the microliter unit in relation to milliliters and liters.

3) The student should be able to explain how a serial dilution is done; why it is done; and be able to calculate the relative concentrations produced by serial dilution.

4) The student should be able to explain why both a negative control and a positive control is needed as part of ELISA

5) The student should be able to explain complementarity and how it relates to antibody specificity

6) The student should be able to explain the parts of the "ELISA Sandwich" and the role of each part in the overall procedure.

7) The student should be able to explain the term "colorimetric" and how it relates to ELISA

8) The student should be able to give an example of when ELISA is used.

Time Requirements

A double length period is best for this lab but it can be divided into two parts and done on consecutive days. Some pre-lab time may be needed to familiarize students with the use of micropipettes. Class time can be used to describe and discuss some of the concepts related to ELISA. This can be partly done with homework readings and question sheets.

Prep Time

Making the solutions needed for this lab will take very little time. Aliquotting will take somewhat longer.

The amount of time it takes to prepare these aliquots depends, of course, on how many stations you will have in your lab. It is tedious work using a micropipette over and over again. For 15 stations, you will prepare a total of 135 tubes. This will probably take one to two hours. Also, the tubes must be labeled. It is easier to simply label the tubes by letter and allow the protocol to identify what each lettered tube holds.

The REAL Story

The standard ELISA protocol has four major steps. The protocols written here seem to have those four steps but we are "cheating" a little. In reality, we have omitted one step. This saves time and expense and tends to give a better result.

The four steps should be: a) add antigen b) add blood serum with target antibodies c) add antibody with enzyme d) add substrate.

The actual protocols in this lab omit the first step. The actual order of steps is add blood serum with target antibodies b) add antibody with enzyme c) add inert material d) add substrate.

The end result is the same.

Alternative to the Actual Lab-

Many of the included sheets can be useful even if you decide to use a pre-packaged ELISA lab kit. Doing this would save considerable preparation time but would not provide as "real" an experience as the actual protocols provided here.

Equipment

For each station-

7 microtubes

1 microtube holder

2- 50 ml centrifuge tubes with tops

1 50 ml centrifuge tube holder (a beaker can be used for this)

1 micropipette that will measure from 2 -20 microliters
1 micropipette that will measure from 20 to 200 microliters

NOTE- the actual volumes of these pipettes need not be as listed above. You will need micropipettes that can deliver the following volumes: 5µl, 45µl, 50µl, and 100 µl.

1 box of pipette tips for the smaller volume pipette
1 box pipette tips for the larger volume pipette

The micropipettes and tips can be shared between two groups.

1 96 well plate

Safety Glasses

Tip Disposal Container (a labeled coffee can will do)

Standard (as in cheap) paper table napkins

A box of Handiwrap or similar product

Storage of reagents may require several additional microtubes and centrifuge tube.

Ordering Information

Standard laboratory reagents have not been included here. Only those that would not normally be found in a high school stockroom.

3,3' 5,5' Tetramethyl-Benzidine (TMB) Liquid substrate system:

Company: Sigma
Catalog #: T-0440
Quantity: 100ml (this amount is sufficient for all your classes)
Price: $26.20

1M Tris, pH 8.0

Company: American Bioanalytical
Catalog #: AB14043-01000
Quantity: 1 liter (This amount is more than adequate for all your classes)
Price: $17.28

Rabbit Serum

Company: Sigma
Catalog #: S-2632
Quantity: 1 ml (this amount is adequate for all your classes)
Price: $12.70

This should be stored in the refrigerator when it arrives.

Goat anti-rabbit IgG with Peroxidase

Company: Sigma
Catalog #: A-6154
Quantity: 0.25 ml (this amount is adequate for all your classes)
Price: $21.40
This should be stored in a freezer when it arrives (packed in dry ice)

Microtubes

Company: Ward's
Catalog #: 18W 1361
Quantity: 500 microtubes
Price: $10.95

Microtube Storage Rack- Styrofoam (this can be cut into two separate racks)

Company: Carolina Supply
Catalog #: 21-5562
Quantity: 1-50 well rack
Price: $6.00

Microtube Storage Rack- Plastic

Company: Wards
Catalog #: 18W 4205
Quantity: 1-24 tube rack
Price: $9.95

96 Well U-Bottom non-sterile flexible PVC Plates

Company: VWR
Catalog #: 62406-220
Quantity: Case of 50
Price: $52.00

The ordering information below regarding micropipettes and tips gives you some choices as regards price. You should look through other catalogs to find what quality and price best suits your budget. As always, you only get what you pay for.

Micropipette: 5µl - 50µl

Company: Ward's
Catalog #: 15W 1726
Quantity: One micropipette
Price: $184.00

Micropipette: 50µl - 200µl

Company: Ward's
Catalog #: 15W 1727
Quantity: One micropippette
Price: $184.00

Micropipette Tips-0.5µl - 250µl (These fit both the above micropipettes)

Company: Ward's
Catalog #: 15W 2203
Quantity: 1000 (these come in a plastic bag without a rack to hold them)
Price: $39.95

Micropipette Tips- 0.5µl - 250µl (These fit both the above micropipettes)

Company: Ward's
Catalog #; 15W 2202
Quantity: 96 tips in a holding rack
Price: $7.50

Note- the first time you buy the tips, you should buy the ones that come in the rack to get enough holding racks. Later, the tips can be replaced in the rack using the ones that come 1000 to a bag.

Economy Micropipette: 5µl - 100µl

Company: Ward's
Catalog #: 15V 2072
Quantity: One micropipette
Price: $99.00

Micropipette Tips-0.5µl - 250µl (These fit the economy micropipettes)

Company: Ward's
Catalog #: 15W 2203
Quantity: 1000 (these come in a plastic bag without a rack to hold them)
Price: $39.95

Micropipette Tips- 0.5µl - 250µl (These fit the economy micropipettes)

Company: Ward's
Catalog #; 15W 2202
Quantity: 96 tips in a holding rack
Price: $7.50

Note again- the first time you buy the tips, you should buy the ones that come in the rack to get enough holding racks. Later, the tips can be replaced in the rack using the ones that come 1000 to a bag.

Gelatin-

Can be found in any catalog. You can buy this at the supermarket.

Handiwrap (or like product) and napkins- available at supermarket.

Solution Preparation

TBS (Tris Buffered Saline)

Add 4.83 g NaCl to a liter flask
Add 5ml 1M Tris, pH 8.0
Add 400 ml distilled water
Stir or gently swirl until dissolved
Add water to bring final volume to 500 ml
Pour into 500 ml bottle and put cap on loosely
Autoclave solution (not necessary but this can be stored indefinitely if sterile)

This amount is adequate for approximately 4 classes of 15 stations each

TBS-Gel

Mix 100 ml TBS with 2 g gelatin
Heat until the gelatin goes into solution
Allow to cool
Store in appropriate container

This amount is adequate for 15 stations

This can be stored in a refrigerator for about 1 month. When needed, place in warm water bath to liquefy the gelatin.

1% Rabbit Serum in TBS (This is your antigen)

Add 30 µl stock rabbit serum to 3 ml TBS

This is enough for 15 stations to use- increase the amounts as needed
This can be stored in the refrigerator for about 1 week.

0.04% Anti-Rabbit IgG with horseradish peroxidase

Add 3.0 µl stock goat anti-rabbit IgG-horseradish peroxidase to 7 ml TBS-Gel.
It may be necessary to warm the gel to the liquid state first.

These amounts are enough for 15 stations.

This should be stored in the refrigerator and is stable for about 1 week.

TMB- this can be used directly as ordered with no preparation.

Aliquots for Students
(Per Station)

Tube Label	Tube Contents
A) TBS:	550 μl TBS in a 1.5 ml microtube
B) Antigen:	175 μl 1% rabbit serum in a 1.5 ml microtube
C) TBS-Gel:	5 ml TBS-Gel in a 50 ml centrifuge tube
D) Patient 1 serum:	350 μl 0.04% anti-rabbit IgG in a 1.5 ml microtube
E) Patient 2 serum:	350 μl TBS-Gel in a 1.5 ml microtube
F) Positive Serum:	60 μl 0.04% anti-rabbit IgG in a 1.5 ml microtube
G) Anti-human antibody:	700 μl TBS-Gel in a 1.5 ml microtube
H) Distilled water:	3 ml distilled water in a 50 ml centrifuge tube
I) Substrate:	700 μl TMB in a 1.5 ml microtube

The amount of time it takes to prepare these aliquots depends, of course, on how many stations you will have in your lab. It is tedious work using a micropipette over and over again.

For 15 stations, you will prepare a total of 135 tubes. This will probably take between 1 and 2 hours. The tubes must be labeled. It is easier to label the tubes by letter and allow the protocol to identify what each lettered tube holds. The order of the aliquots above is the order in which they are used.

The amounts for the aliquots above are sufficient but it is a good idea to add a little more to each tube in case there are mistakes.

BE CAREFUL- the 1% rabbit serum and the 0.04% anti-rabbit IgG used above are **NOT** the stock reagents from the supply house. These are the solutions **YOU** made using the stock reagents.

Each station will require an ample supply of standard paper napkins.

If you plan to do the lab in two parts, Handiwrap is needed to cover the plates overnight.

SECTION TWO

Glossary of Terms

ELISA GLOSSARY

To better understand how the ELISA test works, it is necessary to be familiar with some terms. Below is a glossary of these terms.

Antibody- an antibody is a protein that is produced by B-lymphocytes, a type of white blood cell. Each antibody produced by the immune system is effective against only one antigen. Antibodies are specific for particular antigens (they show antigen specificity). Antibodies attach to the antigen and either neutralize it or label it for destruction by other parts of the immune system. Antibodies are also called immunoglobulins (Ig)

Antigen- an antigen is any substance that can stimulate the production of antibodies. The name antigen comes from "**anti**body **gen**erating". Antigens can be quite varied: certain molecules on the surface of viruses, bacteria, mold spores, cancer cells, pollen, house dust and transplanted organs are all included.
The term antigen is not synonymous with the term pathogen. A pathogen causes disease. An antigen stimulates the immune system. Antigens can be molecules that are part of pathogens. For example, a virus is a pathogen while a protein on its surface can be the antigen. The whole virus does not cause an immune response, just the protein on its surface. Some pathogens might have several molecules that are antigenic (i.e. they are antigens and cause an immune response)
Sometimes antigens are molecules that are not associated with any pathogen. Pollen, dust mites and animal dander are of this type.

Blood Serum - The part of the blood that contains antibodies is called blood serum. Blood serum is produced when whole blood is processed and the cellular components have been removed.

B-Lymphocytes - B-lymphocytes (B cells) are white blood cells that produce antibodies. When B-cells mature, they produce protein molecules that become part of their cell membranes. These molecules are called antigen receptors. Each antigen receptor can bind to a different antigen.
It has been estimated that we each produce at least 100 million and maybe even as many as 100 billion different antigen receptors - enough different kinds to bind with any antigen we might encounter. Any antigen that enters our bodies will eventually make contact with lymphocytes where one will have the matching antigen receptor that binds to that antigen. Antigen receptors and antigens bind as a result of having complementary shapes.
The antigen receptors in B cells are actually part of the antibody that the B cell can produce. When the antigen binds to the antigen receptor, this stimulates the production of those antibodies that combat that particular antigen.

Colorimetric Reaction - If a reaction produces a color change (from clear to blue, for example) and if the depth or intensity (saturation) of the color change can be measured, it is a colorimetric reaction. The amount of color saturation can be used to quantitate the extent of the reaction.

Complementary - In biology, when we say two things are complementary, we mean that they have configurations that allow them to "fit together". Sometimes, this can refer to two processes like photosynthesis and cellular respiration, each of which provides something the other needs.
At other times, complementary refers to the specific shapes of molecules that allow them to easily join together similar to how two pieces of a jigsaw puzzle join.
An antibody has a shape that is complementary to the antigen that it neutralizes. This complementary shape allows that antibody to attach (bind) to the antigen.

Enzyme - an enzyme is a protein that controls a chemical reaction within an organism. Enzymes are specific for the reactions that they control. Enzymes can cause reactions to occur under conditions where normally they would not. Enzymes are often called organic catalysts.

Immunoglobulins (**Igs**) - This is another term for antibodies. See entry for antibodies.

Mast Cell- A vertebrate body cell that produces histamines and other molecules that trigger the inflammatory response.

Microliters / Micropipettes - A basic unit of volume in the metric system (SI) is the liter. Another commonly used unit is the milliliter (ml). A milliliter is 1/1000 of a liter. A milliliter is not very much- about 5 ml is a teaspoon.

Even so, a ml is sometimes too much for certain procedures. In these cases, a smaller unit called the microliter is used. The symbol for microliter is μl.

A microliter is one millionth of a liter and is one thousandth of a millimeter. This is truly a small amount. In order to use microliters, special pipettes are needed. These are called micropipettes.
Note- a pipette is like a medicine dropper; it can draw up and dispense liquids. Pipettes, however, also measure the amount of liquid being used. The simplest pipettes are essentially medicine droppers with marked lines to measure volume. The more advanced pipettes can accurately work with very small and precise amounts of liquid.

96 Well Plates- A 96 well plate contains 96 semi-circular depressions in a rectangular plastic plate. Each depression is called a well. There are 8 rows of wells, each containing 12 wells. The rows are labeled A through H. The columns are labeled 1 through 12.

Each well provides a place where reactions can occur. A plate can therefore be used to run 96 separate (but usually related) reactions in a small area.

Although the plate contains 96 wells, only as many as are needed are actually used.
See diagram at the end of the glossary.

Serial Dilution- Solutions that are available for use in lab often contain solute concentrations (in this case, antibodies in sera) that are much too high to give meaningful results. This is also the case in bacteriology when the number of bacteria per milliliter of nutrient broth is too high to count by the standard colony count method. In both cases, when numbers exceed a certain upper limit per volume of sample, quantification accuracy sharply decreases.

To deal with this type of situation, the serial dilution technique is used. If one ml of a concentrated solution A (the sample) is mixed with 9 ml of water (or some other appropriate solvent), the concentration of the resulting solution B is 10% of the original solution A. If then, one ml of solution B is mixed with 9 ml of water, this solution C will be 10% of B. A little math will show that the concentration of solution C is 1% of solution A (10% of 10%). One more such dilution will produce solution D that is 0.1% of the original solution A.

If this is continued, the concentration of each subsequent solution will be 10% of the previous solution and the concentration of solute (or bacteria) will be decreasingly less by factors of 1/10 than that of the original sample A.

It is not necessary to dilute by 10% each time. Any dilution ratio can be used but the math is much simpler when using factors of ten.

It is also not necessary to do the dilutions using milliliters. Smaller amounts such as microliters are often preferred. Micropipettes are used to dispense these smaller volumes.

Specificity - Enzymes and antibodies exhibit specificity, that is, they attach and act on only one substrate or antigen respectively.

Spectrophotometer- A spectrophotometer is a device that can measure the color intensity of a liquid. Light of a specific wavelength is passed through the sample. The machine then measures how much of this light actually passes through the sample and how much is absorbed by the sample. The more intense the color, the more of this light will be absorbed. The readings from the machine can measure % transmittance, % absorbance and optical density.

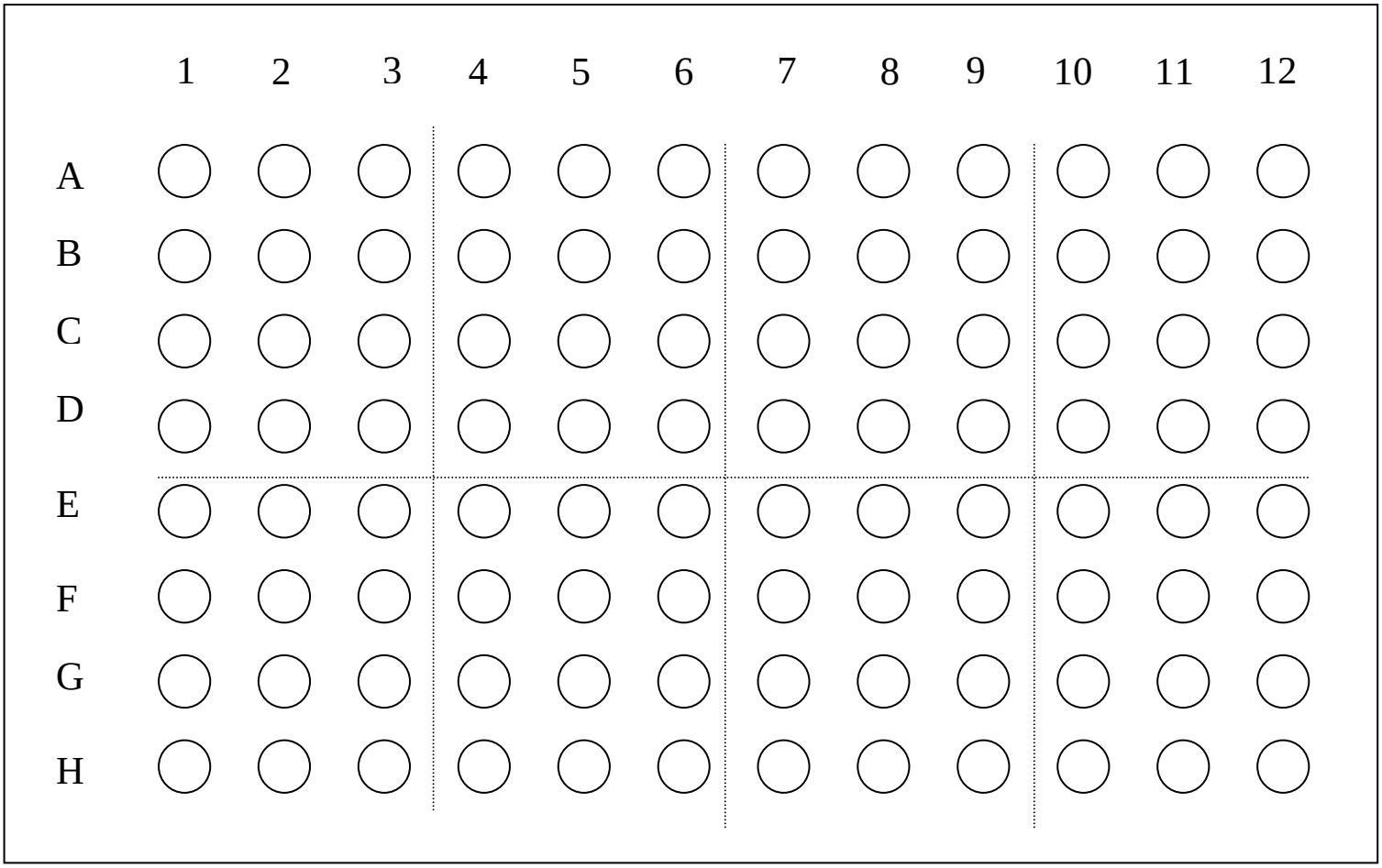
If the color of the sample is in the blue range, then the wavelength of light used is also in the blue range. Protocols or lab instructions generally indicate the wavelength that should be used for a specific sample.

Some spectrophotometers require that the sample be in a special type of test tube called a cuvette. This requires a minimum amount of sample for data collecting.

Other machines can read the color intensity of the liquid in the wells of a 96 well plate. These machines are called plate readers. The plate reader is connected to a computer with appropriate software and the results can be displayed in a number of different ways.

Substrate - A substrate is the molecule that an enzyme works on. If Enzyme X causes a reaction that breaks apart Molecule A, then Molecule A is the substrate that Enzyme X works on. . Enzyme specificity is illustrated by the Lock and Key Model of enzyme action (dear to the hearts of all biology teachers)

96 Well Plate



SECTION THREE

Antibody Information

Antibody Questions

Antibody Questions with Answers

Asthma Information

ELISA in Asthma Research

ELISA / Asthma Questions

ELISA / Asthma Questions with Answers

ELISA and HIV

ELISA and HIV Questions

ELISA and HIV Questions with Answers

ANTIBODIES

Classes-

Antibodies come in five classes. These are designated as IgG, IgM, IgA, IgE or IgD.
The Ig stands for immunoglobulin, another name for antibodies.

IgM - this class of antibody is produced when an antigen is encountered for the first time. This is part of what is called the primary response.

IgG - this class is the most prevalent in the body. IgGs are produced when the antigen is encountered for the second time. This is called the secondary response. IgGs can cross from the mother's blood into the fetus and thereby give protection to the baby until its own immune system is up and running (this is called passive immunity because the baby does not actively make its own antibodies).

IgA - these antibodies help protect from invasion along the body surfaces lined with mucous membranes- nose, eyes, lungs, digestive tract.

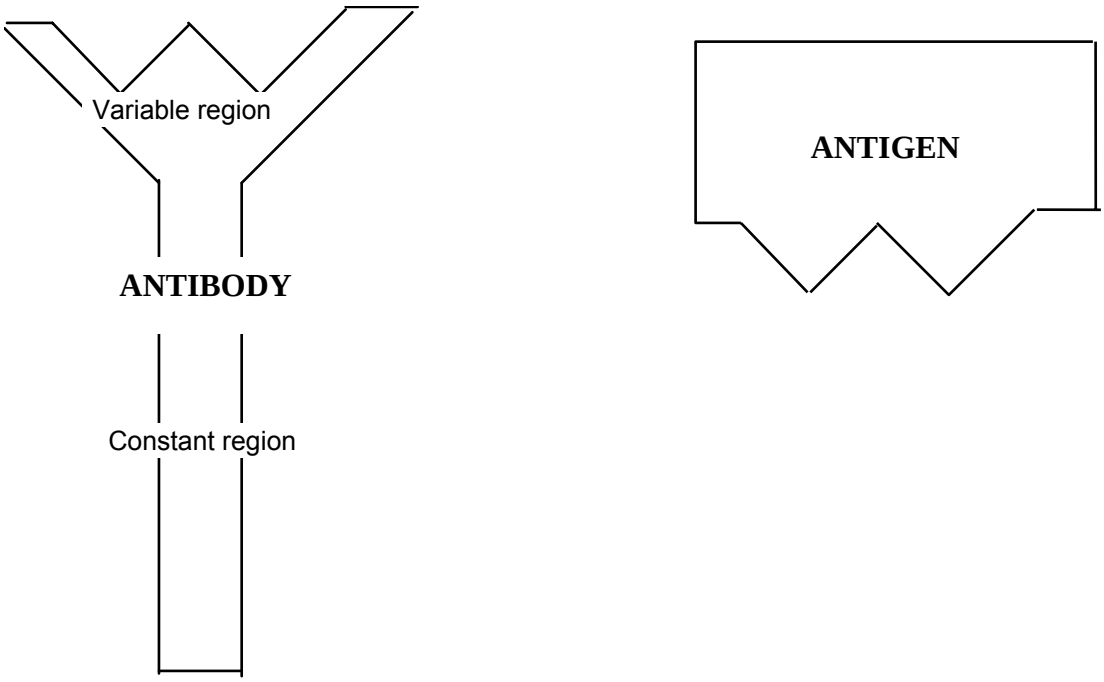
IgE - these antibodies trigger immediate allergic reactions, including the inflammatory response. These antibodies seem to do more harm than good although it is thought they help protect against certain parasitic worms common in Third World countries.

IgD - the function of these antibodies is not well understood.

Shape-

Molecular architecture refers to the shape of molecules. This is an extremely important characteristic of molecules since so many reactions occur based on one molecule's shape being complementary to another's.
The actual shape of molecules is quite complex and we often simplify things by using basic geometric shapes to represent these complicated configurations.
Antibodies have a Y shape. The forked part of the Y is the part that has a shape that allows it to bind to an antigen. This shape varies from one antibody to another and is specific for the particular antigen that the antibody targets. This part of the antibody is called the **variable region**. The bottom part of the Y is the **constant region**. It has one of five structures depending on which class the antibody is in. All antibodies in a particular class have the same constant region.

Figure 1.1 Model of antibody (left) and its complementary antigen (right).



The antibody shown in Fig. 1.1 to the left is specific for the antigen shown to the right. Figure 1.2 shows how they "fit together." In other words, they are complementary. Figure 1.3 shows another antibody and its complementary antige n. You can see how each antigen would require its own antibody in order for them to fit together.

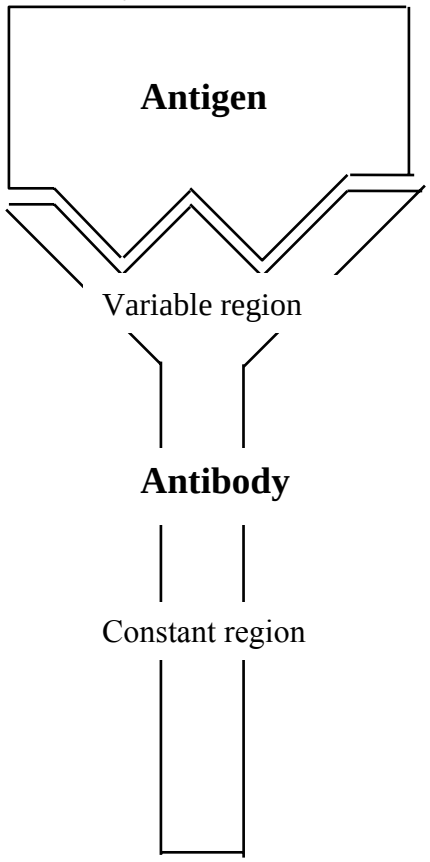


Figure 1.2

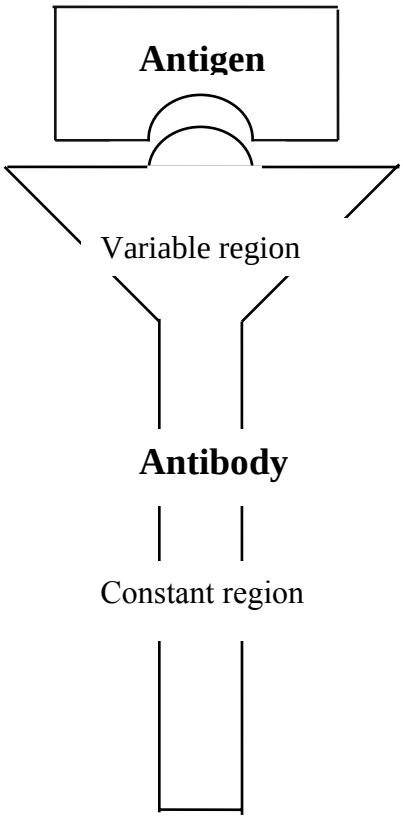


Figure 1.3

Antibody Questions

1) Draw two antigens different from those already seen. Draw the two antibodies that would be complementary to the antigens you drew.

What name is given to the part of an antibody that is the same in all antibodies?
_____

What name is given to the parts of an antibody that is complementary to the antigen?
_____

Explain what is meant by antibody specificity.

2) What is another name for antibodies? _____

3) Which type of antibody is involved in allergic reactions and the inflammatory response?
_____

These antibodies are thought to protect against _____ common in certain parts of the world.

4) Which type of antibody can be passed from mother to unborn child? _____
What type of immunity does this give the child? _____ Why is it called by this name? _____

Antibody Questions (with answers)

1) Draw two antigens different from those already seen. Draw the two antibodies that would be complementary to the antigens you drew.

Student answers to this will vary but the antibody shape should be a "Y" and the shape of the variable region of the "Y" should fit the antigen drawn.

What name is given to the part of an antibody that is the same in all antibodies?
Constant region

What name is given to the parts of an antibody that is complementary to the antigen?
Variable region

Explain what is meant by antibody specificity.

Because the variable region of the antibody has a shape that will fit only one antigen, the antibody is specific for that antigen.

2) What is another name for antibodies? **Immunoglobulins (Ig)**

3) Which type of antibody is involved in allergic reactions and the inflammatory response?
Immunoglobulin E (IgE)

These antibodies are thought to protect against **parasitic worms** common in certain parts of the world.

4) Which type of antibody can be passed from mother to unborn child?
Immunoglobulin G (IgG)

What type of immunity does this give the child? **Passive immunity**

Why is it called by this name? **The child does not actively make his own antibodies.**

Asthma

Approximately 20 million Americans suffer from asthma, a chronic, inflammatory lung disease characterized by breathing difficulties such as wheezing, coughing and shortness of breath. In some patients, exposure to allergens such as animal dander, dust and mold spores can trigger an allergic reaction that can cause asthma symptoms.

IgE and Asthma

For some people, allergen exposure can cause a reaction known as the allergic response. This occurs when allergens are inhaled into the respiratory tract (nose, throat and lungs) and attach to the mucous membranes. These allergens are recognized by the immune system as foreign invaders and a specific immune response is produced as the body prepares to fight them off. During the specific immune response, B-cells (B-lymphocytes) develop into plasma cells that produce IgE antibodies specific to the antigen.

These IgE antibodies are produced within a few weeks after antigen exposure and are released into the bloodstream. These IgE antibodies may attach to mast cells or remain free floating in the bloodstream. When an allergic individual is re-exposed to an allergen, these mast cells with attached IgE release chemicals such as histamines. Histamines cause protective inflammatory responses in the body such as dilation and increased permeability of blood vessels. However, an allergic response may occur when heightened inflammatory events such as bronchial constriction, coughing and wheezing accompany the specific immune response.

Current asthma therapies generally treat the symptoms of asthma by decreasing inflammation and reducing bronchial constriction. Further understanding of the role of IgE has led to new approaches in asthma management that intervene early in the allergic response.

Since IgE is a key component of the allergic response responsible for the symptoms of asthma, current research is focusing on the production and functioning of IgE, in order to better understand its role in asthma. It is thought that controlling IgE production and/or action, the disease might be reduced in severity or prevented altogether.

Research of this type often uses murine (mouse) models. Mice are injected with an allergen such as ovoalbumin (OVA). The mice will produce antibodies against this antigen. At a later time, they are exposed to OVA in aerosol form to stimulate an asthma-like allergic response. The amounts of IgE in the blood produced as a result of this exposure can be measured using ELISA. Additionally, histological studies are done to see the effects of the allergic response on certain cells.

Further experiments can test whether certain drugs will lower the amount of IgE produced or perhaps prevent its production altogether, and whether these IgE-lowering drugs alleviate the heightened allergic response of asthmatics.

ELISA IN USE

ELISA in Asthma Research-

One set of experiments done at the Channing Lab in Boston, MA, tests to see whether specific peptides can reverse the effects of OVA induced airway hyper-reactivity (AHR) in mice. AHR is a symptom of asthma and can be created in mice using OVA (ovalbumin- a protein obtained from chicken eggs)

Mice are first sensitized by injecting them with an OVA solution. Twenty-one days later, they will receive a booster shot of OVA. As a result of these injections, the immune system of these mice will have formed an immune response to OVA and the mice will react when exposed to it on a subsequent occasion.

Seven days later, OVA sensitized mice will be injected with the peptide being tested. Twelve hours after this injection, the mice will be "challenged."

The challenge is created by forcing the mice to breathe aerosolized OVA. This exposure to OVA in the lungs will cause IgE to be released, which will result in AHR. IgE is a type of antibody associated with allergies and inflammatory reactions as seen in asthma.

If the peptide being tested prevents the release of IgE, AHR will not occur. If the peptide reduces the amount of IgE released, the AHR will not be as severe. Positive results might indicate a possible treatment for the symptoms of asthma.

After being challenged, blood will be taken from a small cut on the mouse's tail and using ELISA, the amount of OVA specific IgE in the mouse blood serum will be measured.

The amounts of OVA specific IgE in the blood serum of these mice treated with the peptide will be compared to the amount of OVA specific IgE in:

a) mice sensitized to OVA, **not** given the peptide and challenged. This is the positive control. This is the amount of IgE released in a mouse that would have full-blown AHR. It is hoped that the OVA specific IgE levels in the test mice are much lower than in these mice.

b) mice **not** sensitized to ova, **not** given the peptide and **not** challenged. This is the negative control. This is how much OVA specific IgE is present in mice that have not been treated in any special way. This gives a "background reading" of OVA specific IgE in mice - how much is present under normal circumstances. It is assumed this will be zero since the mice have not been exposed to OVA in any way and therefore have not had the opportunity to form antibodies specific to it.

c) mice sensitized to OVA, **not** treated with the peptide and **not** challenged. This indicates how much OVA specific IgE is present as a result of only sensitization. Even if the peptide treatment were successful, the mice would be expected to have this amount of OVA specific IgE simply as a result of the sensitization. This is called the IgE control.

Further experiments with these mice would examine the blood for types of white blood cells present along with histological studies to evaluate any damage to lung tissue in the various groups. Blood serum would also be tested for other chemicals associated with AHR.

Since accuracy is crucial in these experiments, all steps in ELISA are done with greater care than would be done in a high school lab. Chemicals are left in the wells longer to assure maximum binding, washes are more thorough and the color changes are read by a spectrophotometer rather than evaluated by eye.

ELISA and Asthma Research Questions

1) What type of antibody is released that can cause the symptoms of asthma?

2) What do the letters AHR stand for?

3) What antigen can be used to create the symptoms of asthma in mice?

4) Why is research using mice called using a **murine** model? (look up the word murine)

5) What does it mean to "sensitize" the mice to OVA?

6) How are the mice "challenged?"

7) The positive control in these experiments would have a full-blown reaction to the challenge. Why?

8) What would it mean if the treated mice had the same concentrations of IgE as the positive controls?

9) Why would it be expected that the negative control have no OVA specific IgE?

10) Why is the IgE control necessary

ELISA and Asthma Research Questions (with answers)

1) What type of antibody is released that can cause the symptoms of asthma?
Immunoglobulin E (IgE)

2) What do the letters AHR stand for? **Airway Hyper-reactivity**

3) What antigen has been used to create the symptoms of asthma in mice?
Ovoalbumin (OVA)

4) Why is research using mice called using a **murine** model? (look up the word murine)
The word murine refers to rodents like rats and mice.

5) What does it mean to "sensitize" the mice to OVA?
Mice are sensitized by injecting them with OVA. This stimulates the immune system to form an immune response to OVA.

6) How are the mice "challenged?"
The challenge forces the mice to breathe OVA in aerosol form.

7) The positive control in these experiments would have a full-blown reaction to the challenge. Why?
They would have a strong reaction to the challenge because they have been sensitized to OVA but have not been treated to prevent the subsequent release of IgE.

8) What would it mean if the treated mice had the same concentrations of IgE as the positive controls**? It would mean that the treatment had no effect**.

9) Why would it be expected that the negative control have no OVA specific IgE?
These mice have not ever been exposed to OVA in any form.

10) Why is the IgE control necessary?
It is necessary to see how much IgE is present in mice that have been sensitized but not challenged. This is needed to figure out how much IgE was produced solely as a result of the challenge.

ELISA in USE - HIV Detection

When someone has been infected with HIV (human immuno-deficiency virus), the symptoms of AIDS do not appear for awhile, often not for several years. ELISA can be used to confirm a suspected HIV infection even when there are no outward signs of the infection.

Although the person is asymptomatic (without symptoms), the immune system is busily producing HIV antibodies in order to destroy the virus. ELISA is used to test for the presence of these HIV antibodies. If the antibodies are present, the person is HIV positive.

HIV antigen is put into the wells of a 96 well plate and it adheres to the plastic. Blood serum from the person is added. If the HIV antibodies are present they will bind to the HIV antigen.

Anti-human antibodies are then added and these will bind to the HIV antibodies. These anti-human antibodies have a conjugated (attached) enzyme.

A substrate is added and the enzyme causes a reaction with the substrate. The products of this reaction are colored.

The presence of a color change and its intensity can be used to confirm the presence and concentration of the HIV antibodies.

If there are no HIV antibodies in the blood serum, then there will be nothing to bind to the HIV antigen. As a result of this, there will be nothing for the anti-human antibodies to bind to and they (and the attached enzyme) will be removed by washing. In this case, when the substrate is added, there will be no enzyme to cause a reaction and no color change will occur.

When doing this type of test, a positive control and a negative control is used. A positive control is done in a well where HIV antibodies are added instead of the patient's blood serum. This assures that the anti-human antibodies with attached enzyme have something to bind to. This will produce a definite color change.

A negative control is done in a well where no blood serum is added and therefore, there will be no HIV antibodies to bind to the antigen. This means the anti-human antibodies with attached enzyme will have nothing to bind to and will be removed by washing. This should produce no color change.

The colors produced in the other wells can be compared to the positive and negative controls to better calibrate the results.

The following web site has an animation that shows ELISA being used to detect HIV antibodies: http://www.biology.arizona.edu/immunology/activities/elisa/main.html

HIV ELISA Questions

1) What does it mean to say a person is asymptomatic for a disease?

2) Even if an HIV infected person is asymptomatic for HIV, what will be present in the blood?

3) What is the difference between being HIV positive and having AIDS?(look this up)

4) In the HIV ELISA, what is added to the positive control well? What should happen in this well?

5) In the HIV ELISA , what is not added to the negative control well? What should (or should not) happen in this well?

6) What is the reason for having a positive and a negative control in the HIV ELISA?

HIV ELISA Questions (with answers)

1) What does it mean to say a person is asymptomatic for a disease?
They do not have any of the symptoms of that disease.

2) Even if an HIV infected person is asymptomatic for HIV, what will be present in the blood if they have been exposed to the virus?
A person exposed to HIV will have produced antibodies specific for that virus even if there are no outward symptoms of infection.

3) What is the difference between being HIV positive and having AIDS?(look this up)
Being HIV positive means that the human immuno-deficiency virus is present in your body. Having AIDS means the virus has affected your immune system enough that you can no longer properly defend yourself against infections. A variety of diseases can plague a person with AIDS but two diseases that are indicative of AIDS are Karposi's sarcoma and pneumocystis pneumonia. These are called opportunistic infections because they take advantage of the opportunity given to them by a compromised immune system.

4) In the HIV ELISA, what is added to the positive control well? **HIV antibodies**
What should happen in this well? **There should be a definite color change.**

5) In the HIV ELISA, what is not added to the negative control well? **Blood serum is not added to this well. What should (or should not) happen in this well? There should be no color change.**

6) What is the reason for having a positive and a negative control in the HIV ELISA?
These control wells are used for comparison to the test wells. They can also give indications that there was a problem with the test procedure if the expected color change (or lack of color change) does not occur

SECTION FOUR

ELISA: Four Basic Steps

ELISA: Another Look

ELISA Questions

ELISA Questions with Answers

THE ELISA

ELISA stands for **E**nzyme **L**inked **I**mmuno**S**orbent **A**ssay. It is a test that can detect the presence and concentration of specific antibodies. ELISA has been in use many years and is a basic tool of immunologists and other disease researchers.

If a person has been infected with a particular pathogen, the body will have made antibodies (immunoglobulins) to fight it. ELISA can detect the presence of these antibodies and thereby confirm the infection even if the person is asymptomatic and/or if the pathogen cannot be isolated for identification. The presence of antibodies can also confirm an earlier infection and can indicate that the person has immunity to successive infections by the same pathogen.

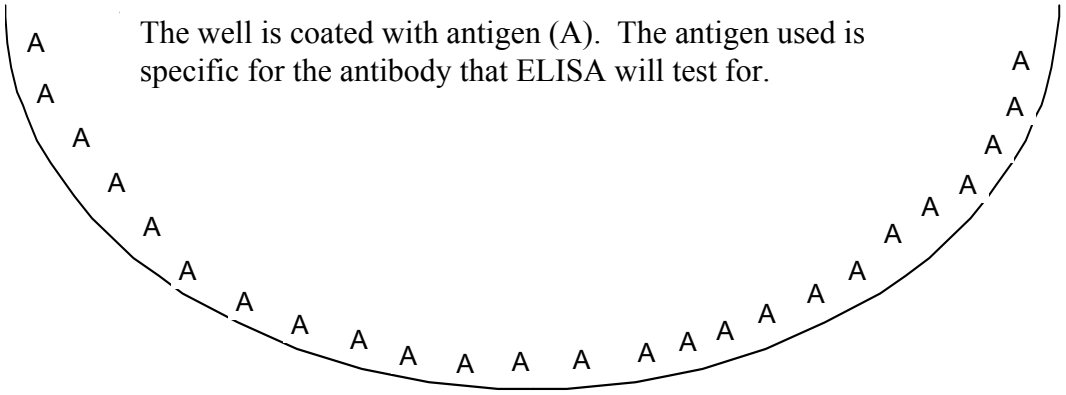
ELISA does not detect the antibody directly but does so by means of an enzyme that is linked to the antibody. This enzyme mediates a reaction that can be seen and measured colorimetrically. The depth of color intensity is dependent on the amount of enzyme present, which, in turn, indicates how much of the antibody is present.

There are several steps involved in the ELISA procedure and in between steps there is some waiting time. Adequate time must be allowed for linkages to occur between molecules. This is crucial for accurate results. In addition, excess material is washed away between steps. The test is usually done using 96 well plates although not all wells may be used.

OVERVIEW of the ELISA PROCEDURE: Four Basic Steps

1) Coat the wells in the test plate with the antigen that the antibody is specific for. If a medical technician were testing for HIV antibodies, the wells would be coated with HIV antigen. See Fig. 2.1

Fig. 2.1



2) Add the blood serum sample that is to be tested for the presence of these antibodies. If the antibodies are present, they will bind to the antigen. Any other antibodies present in the serum will not bind and will be removed by washing. See Fig. 2.2

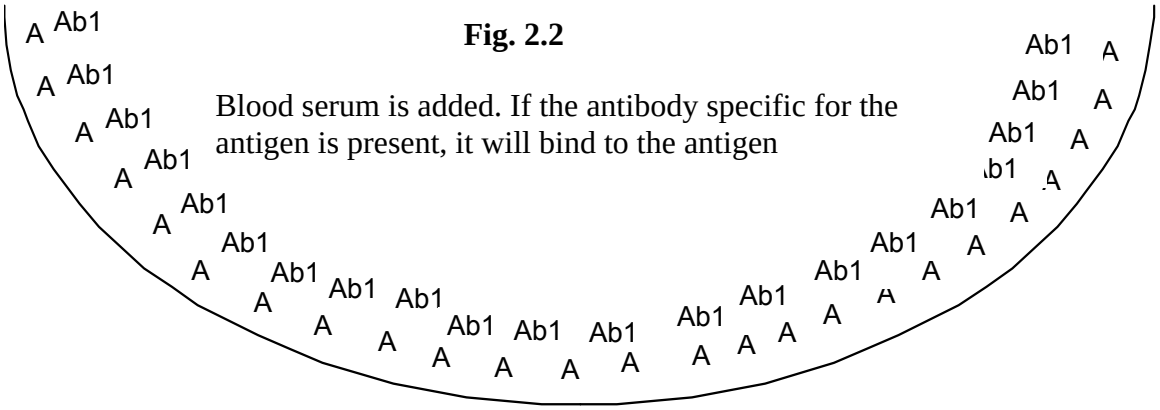


Fig. 2.2

Blood serum is added. If the antibody specific for the antigen is present, it will bind to the antigen

3) A second antibody is added that will bind to the first antibody. This second antibody has an enzyme attached to it. No binding will occur if there is none of the first antibody present. See Fig. 2.3

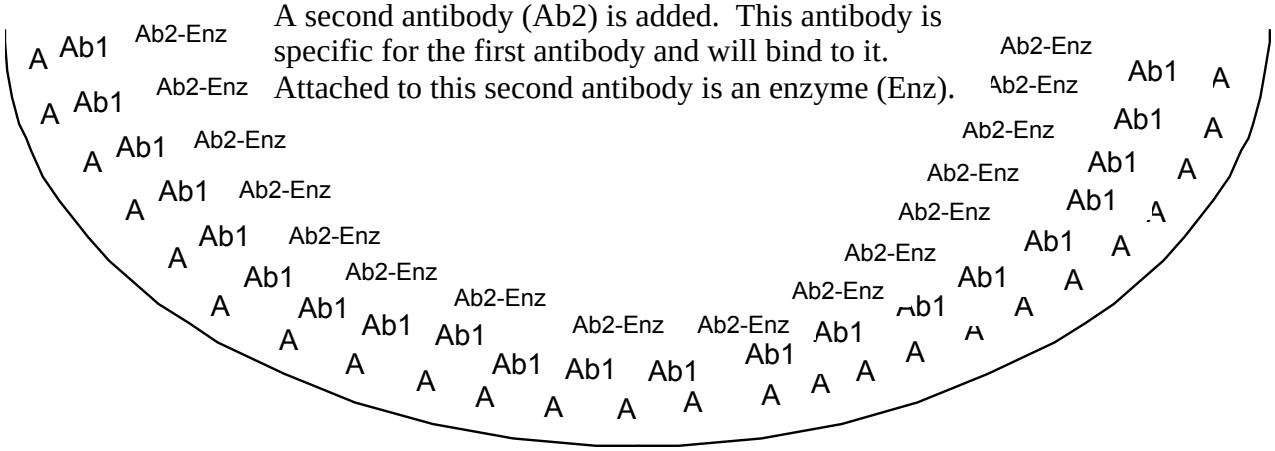


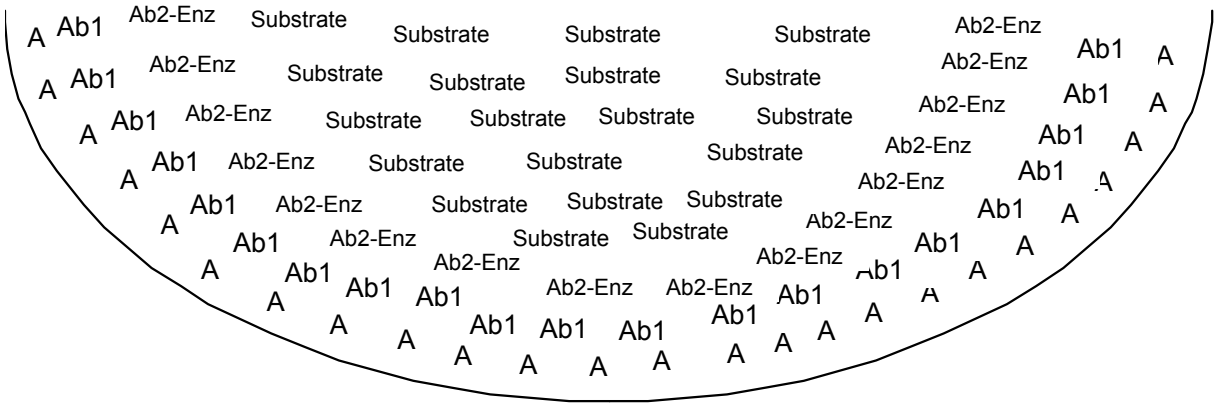
Fig. 2.3

A second antibody (Ab2) is added. This antibody is specific for the first antibody and will bind to it. Attached to this second antibody is an enzyme (Enz).

4) The substrate that the attached enzyme works on is added and a colorimetric reaction occurs. Each enzyme acts upon hundreds of substrate molecules in a very short time producing hundreds of colored product molecules. No reaction will occur if the original antibody is not present. See Fig. 2.4

Fig. 2.4

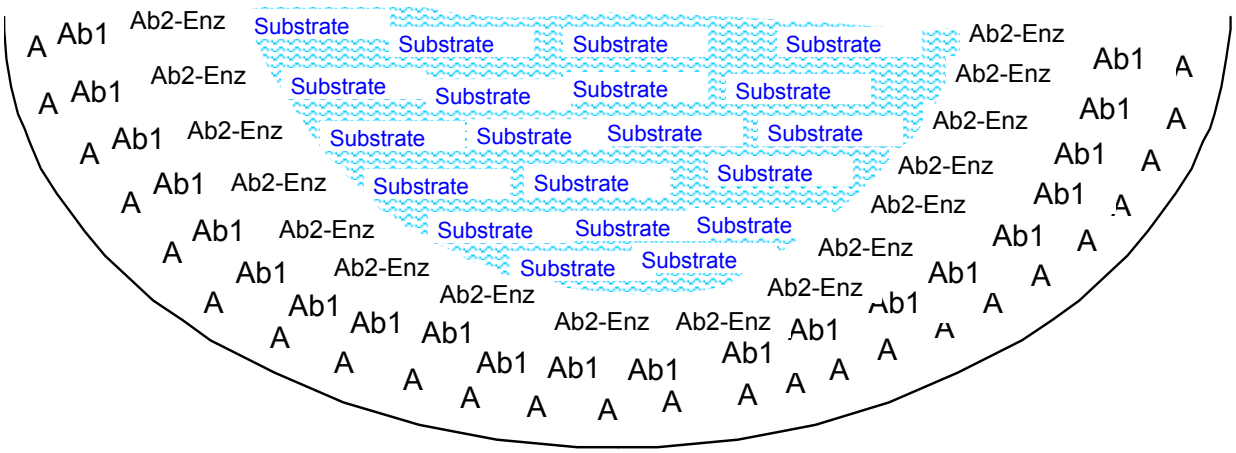
A substrate is added. The enzyme will cause a color change in the substrate.



The extent of the reaction is measured by a change in the intensity of the color. A spectrophotometer set to the particular wavelength of the particular color is used for accurate results. A large change in the depth of the color indicates a high concentration of the antibody. The absence of color (and a color depth change) indicates the absence of the antibody. See Fig. 2.5

Fig. 2.5

The intensity of the color is an indication of how much Ab2-Enz was present. This, in turn, indicates how much of Ab1 was present. ELISA is used to measure the amount of Ab1 in the blood serum of the patient. If there is no color change, there is no Ab1 present.



For teaching purposes, modifications to these steps can be made but the basic concept remains the same.

The following web site has an animation that shows the ELISA being used to detect HIV antibodies: http://www.biology.arizona.edu/immunology/activities/elisa/main.html

Some ELISA details

In general, proteins adhere well to plastic. There are 96 well plates available that have been coated with a substance that increases the binding affinity of the antigen to the plates.

After the antigen is added in the first step, a blocking buffer is used. A blocking buffer fills in any "empty" places where there was not enough antigen to bind. This prevents other molecules from binding to the plate during the other steps. The blocking buffer is an inert substance and will not interfere with the following steps of the experiment.

After each step, the plates are washed using washing buffer. Washing with the washing buffer removes any excess materials that did not bind to other molecules. An inadequate job of washing can give inaccurate results.

The second antibody that links to the first antibody is produced in organisms such as mice. A human antibody is injected into the mice. The mice then produce their own antibodies (Anti-human Ig) that will bind to the human antibody. These anti-human antibodies are then purified from the mouse blood serum and an enzyme is attached.

ELISA: Another Look

Step 1: The first layer applied to the wells is the antigen. The antigen will adhere to the plastic, as do all proteins. The antibody we are testing for (the target antibody) will bind to this antigen during the next step. See Fig. 3.1.

Fig. 3.1

****

2) Step 2: The next layer is the blood serum sample that may contain the target antibody we are testing for. If the target antibody is present, it will bind to the antigen. Remember that antibodies show antigen specificity. Other antibodies present in the serum will not bind to this antigen and will be removed later by washing. See Fig 3.2

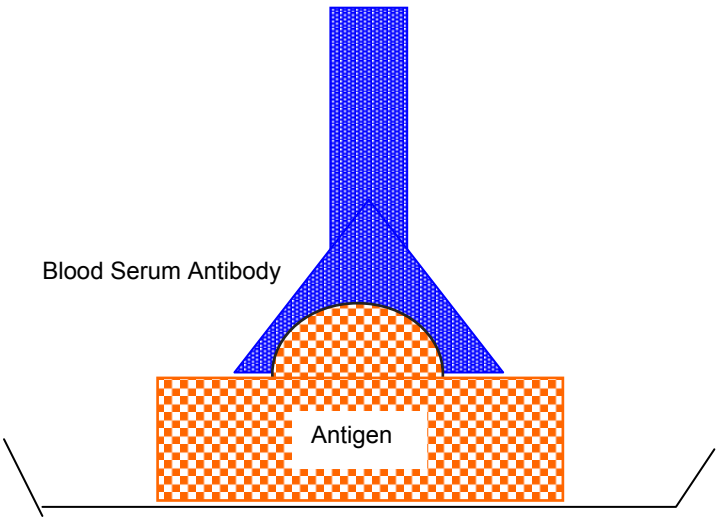


Fig.3.2

3) In Step 3, another antibody is added. This is called the secondary antibody. This antibody was produced in another organism (such as a mouse). The secondary antibody is complementary to the constant region of the human antibody and will bind to the human antibody if it is present in the sample. In other words, this secondary antibody is an anti-human antibody. The secondary antibody has an attached enzyme. See Fig.3.3.

4) In Step 4, a substrate reactant is added and the enzyme acts upon the substrate to produce a colored product.

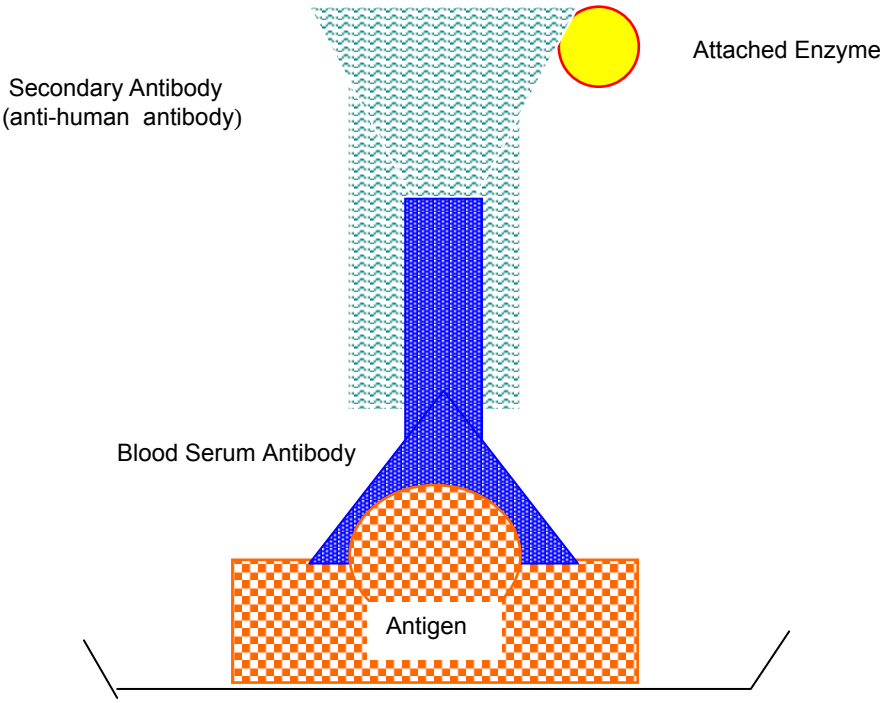


Fig. 3.3

****

In between the addition of each layer, the wells are washed to remove any free molecules (molecules not adsorbed onto the sandwich layer). If the process is done carefully, the number of enzyme molecules is proportional to the number of blood serum antibodies molecules present. In Fig.3.3, there is a one to one to one ratio of Antigen: Blood serum antibody: Anti-human with attached enzyme.

A substrate is added and the enzyme reaction on the substrate results in a colored product. In general, a large change in color intensity indicates a high concentration of enzyme activity and therefore, also a high concentration of the blood serum antibody. No color change indicates that the blood serum antibody is not present in the sample.

The color change is proportional to the amount of antibody present. A spectrophotometer is used to measure the amount of color change. A standard is used to calibrate the readings. The standard is a known quantity of blood serum antibody that is used as a control in the ELISA.

ELISA Questions

1) What do the letters in ELISA stand for?

2) ELISA is often used to test for the presence of _____ in a person's blood serum.

3) Why are the words "enzyme linked" used in the name ELISA?

4) What material coats the wells in the first step in ELISA?

5) What is then added to the wells in the second step?

6) ELISA tests this blood serum for the presence of what substances?

7) The antibody added in the third step will bind to the _____ that are attached to the antigen.

8) What is attached to the antibody that was added in the third step?

9) This attached enzyme will catalyze a chemical reaction when the proper _____ is added.

10) How do we know that a reaction has occurred?

11) What is the purpose of the blocking buffer?

12) Why is it necessary to wash the plates after certain steps?

13) The antibody added in the third step is usually an anti-human antibody (assuming we are testing humans). How is an anti-human antibody produced?

ELISA Questions (with answers)

1) What do the letters in ELISA stand for? **Enzyme Linked Immunosorbent Assay**

2) ELISA is often used to test for the presence of **antibodies** in a person's blood serum.

3) Why are the words "enzyme linked" used in the name ELISA? **An enzyme is attached to an antibody used in ELISA. This enzyme will catalyze a reaction which changes a clear substrate into a colored product. This indicates the presence of the target antibody.**

4) What material coats the wells in the first step in ELISA? **An antigen that is complementary to the target antibody being tested for.**

5) What is then added to the wells in the second step?
Blood serum that may (or may not) contain the target antibody

6) ELISA tests this blood serum for the presence of what substance? **The target antibody.**

7) The antibody added in the third step will bind to the **target antibodies** that are attached to the antigen.

8) What is attached to the antibody that was added in the third step? **An enzyme**

9) This attached enzyme will catalyze a chemical reaction when the proper **substrate** is added.

10) How do we know that a reaction has occurred? **The presence of color and the intensity of the color are indications of a reaction.**

11) What is the purpose of the blocking buffer? **After the antigen is added in the first step, we do not want any other materials to adhere to the plastic. The blocking buffer will adhere to any open places on the plastic and prevent other materials from doing so.**

12) Why is it necessary to wash the plates after certain steps? **Washing removes excess materials that did not bind to other molecules. If they do not bind, then they are not part of the process and must be removed so that they do not interfere with the results.**

13) The antibody added in the third step is usually an anti-human antibody (assuming we are testing humans). How is an anti-human antibody produced? **A human antibody of the correct class is injected into an animal (a rabbit, for example). This animal then makes antibodies that are complementary to the human antibodies and will attach to them.**

SECTION FIVE

Micropipette Information

Micropipette Practice

Serial Dilution Questions

Serial Dilution Questions with Answers

Small Volumes: Information and Questions

Small Volumes: Information and Questions with Answers

Micropipettes

Micropipettes are used to accurately withdraw and deliver very small amounts of liquid. Some micropipettes have fixed volumes; that is, they always withdraw the same amount. Others are variable. They can be set, within limits, to withdraw and deliver variable amounts.

The variable micropipettes are available for different amounts. Some measure from 0.5 μl to 10 μl; others may measure from 20 μl to 200 μl.; still others measure large volumes. There is great variation in the type of micropipettes you can buy.

The variable micropipettes are set to a specific volume usually by turning a knob on the top. The setting appears as numbers on the side. The numbers are read from top to bottom. The readings on the smaller volume pipettes often include the tenths place (which is usually a different color). The larger volume micropipettes generally measure only whole numbers.

Each time the pipette is used for a new solution, a new tip is attached. This is done by placing the end of the pipette into the tip and pushing down until the tip is firmly in place. When you are through with a tip, it is ejected into a disposal container. The ejector mechanism varies.

The size of the tip matches the volume amounts the pipette can handle.

The plunger on the top of the pipette can be pushed down until a stop point is reached. This is the withdrawal stop point. From there it can be pushed down a little further. This is the delivery stop point. The plunger will not go further down than this.

To withdraw liquid, push the plunger to the first stop point. Insert the tip of the pipette into the liquid and slowly allow the plunger to rise back up. The volume of liquid in the tip will be the amount the pipette was set for.

To deliver this liquid, the pipette tip is placed into the desired container and the plunger is pushed down past the first stop point to the delivery point from where it cannot go down any further. Remove the pipette from the container and allow the plunger to return to its normal position.

If you are going to be transferring the same solution to several different places, you may use the same tip. However, when using the same tip several times, you must not touch the tip to any liquid already in the microtube when making the transfer. If, however, you are going to transfer different solutions, the old tip is ejected into the disposal container and a new tip is attached.

Micropipette Practice

1) Get three microtubes and label them A, B and C.

2) Fill a 50 ml centrifuge tube with distilled water

3) Using the appropriate micropippette, place 90 µl of water from the centrifuge tube into tube A, 50 µl of water into tube B. Use the appropriate micropipette to put 10 µl of water into tube C.

4) Take 10 µl from tube B and put into tube C

5) Take 20 µl from tube A and put it into tube B

6) Take 20 µl from tube A and put it into tube C

7) Take 10 µl from tube B and put it into tube C.

Do the arithmetic to see how much water should be in each tube. What do your calculations show? _____

Compare by eye the amounts in all three tubes. Does this match what your calculations say should be the case? _____

Tiny volumes often stick to the side of the microtube. To prevent this, close the top firmly and snap the tube with a twist of your wrist. This should drive the droplet to the bottom of the microtube.

While you have the smaller volume micropipette out, take one µl of distilled water from the centrifuge tube. Is one µl a large amount? If you took a one liter soda bottle and divided the soda up equally to serve one million people, each person would get one µl.

Serial Dilution Questions

Method #1- If 1 ml of solution A is mixed with 9 ml of water, the original solution A has been diluted by 10%. This is calculated by taking the amount of solution A used and dividing it by the total amount of diluted solution produced (in this case, 1 divided by 10 ml [1 ml A and 9 ml water])

This new solution (call it B) is 0.1 (10%) of the concentration of the original solution A.

If 1 ml of B is mixed with 9 ml water, this will produce a solution that is 0.1 (10%) of the concentration of B. Call this new solution C.

How does the concentration of C compare to the concentration of A? (Hint-10% of 10%)

Note- Solution A was diluted to form Solution B which was then further diluted to form Solution C. Each solution is less concentrated than the one it was formed from. This procedure is called serial dilution.

Method #2-
If 1 ml of A were mixed with 99 ml of water. How would the concentration of this new solution compare with A?

If you do the math correctly, you will see that in both cases you get a solution that is (1%) of the original solution A.

How do the two methods differ from each other in the products produced?
(hint- a) volumes b) dilutions available)

What could you do using solution C to make a solution that is 0.001 (0.1%) of the concentration of solution A?

What would be another method you could use to make the same solution?

In our lab protocols, you mix 5 ml of antigen with 45 ml TBS. This dilutes the antigen to what percent of the original?_____ In these protocols, you do this 4 more times.
The final solution is what percent of the original?

Serial Dilution Questions (with answers)

Method #1- If 1 ml of solution A is mixed with 9 ml of water, the original solution A has been diluted by 10%. This is calculated by taking the amount of solution A used and dividing it by the total amount of diluted solution produced (in this case, 1 divided by 10 ml [1 ml A and 9 ml water])

This new solution (call it B) is 0.1 (10%) of the concentration of the original solution A.

If 1 ml of B is mixed with 9 ml water, this will produce a solution that is 0.1 (10%) of the concentration of B. Call this new solution C.

How does the concentration of C compare to the concentration of A? (Hint-10% of 10%)
Solution C is 1% (0.01) of solution A

Note- Solution A was diluted to form Solution B which was then further diluted to form Solution C. Each solution is less concentrated that the one it was formed from. This is called serial dilution.

Method #2
If 1 ml of A were mixed with 99 ml of water. How would the concentration of this new solution compare with A? **The concentration of the new solution would be 1% (0.01) of Solution A.**

If you do the math correctly, you will see that in both cases you get a solution that is (1%) of the original solution A.

How do the two methods differ from each other in the products produced?
(hint- a) volumes b) dilutions available)
One major difference is the amount of solution produced. In the first case, only 10 ml is produced. In the second case, 100 ml is produced. Also, using the first method, you will have some the original Solution A, some of Solution B and some of Solution C.

What could you do using solution C to make a solution that is 0.001 (0.1%) of the concentration of solution A? **Take one ml of Solution C and add it to 9 ml of water.**

What would be another method you could use to make the same solution?
Take 1 ml of Solution A and add it to 999 ml of water.

In our lab protocols, you mix 5 µl of antigen with 45 µl TBS. This dilutes the antigen to what percent of the original? **10%** In these protocols, you do this 4 more times.
The final solution is what percent of the original? **.001% (.00001)**

Small Volumes

A standard large soft drink bottle holds 2 liters of liquid. The smaller bottle holds one liter.

One liter equals 1000 milliliters (ml). How many ml are in the large bottle?

1) If you took the smaller bottle and divided the cola up equally to serve 100 people, how much would each person get? _____ml Is this a lot?_____ (it is actually about 2 teaspoons)

2) If the smaller bottle were divided equally among 1000 people, how much would each person get? _____ml Is a milliliter a small amount? _____

Certainly, a ml is a small amount compared to what a normal soft drink serving would be, but it is actually quite a large amount when doing certain experiments.

****

If a ml were divided into 1000 equal parts, each part is called a microliter (μl).

3) How many μl are in one ml? _____

4) How many μl are in one liter? _____
(hint - there are 1000 μl in a ml and 1000 ml in a liter)

5) What does the prefix "micro" mean? _____

To change μl to ml, divide μl by 1000
To change ml to μl, multiply ml by 1000

6) 100 μl is equal to how many ml? _____

7) 0.25 ml is equal to how many μl? _____

Small Volumes (with answers)

A standard large soft drink bottle holds 2 liters of liquid. The smaller bottle holds one liter.

One liter equals 1000 milliliters (ml). How many ml are in the large bottle? **2000 ml**

1) If you took the smaller bottle and divided the cola up equally to serve 100 people, how much would each person get? **10 ml** Is this a lot? **NO** (it is actually about 2 teaspoons)

2) If the smaller bottle were divided equally among 1000 people, how much would each person get? **One ml** Is a milliliter a small amount? **YES**

Certainly, a ml is a small amount compared to what a normal soft drink serving would be, but it is actually quite a large amount when doing certain experiments.

****

If a ml were divided into 1000 equal parts, each part is called a microliter (μl).

3) How many μl are in one ml? **1000 μl are in one ml.**

4) How many μl are in one liter? **One million μl are in one liter.**
(hint - there are 1000 μl in a ml and 1000 ml in a liter)

5) What does the prefix "micro" mean? **One millionth (.000001)**

To change μl to ml, divide μl by 1000
To change ml to μl, multiply ml by 1000

6) 100 μl is equal to how many ml? **0.1 ml (one tenth of a ml)**

7) 0.25 ml is equal to how many μl? **250 μl**

SECTION SIX

ELISA Protocol

(This protocol was adapted from one provided by CityLab,
a copyrighted program sponsored by Boston University School of Medicine)

ELISA Data Sheet and Questions

ELISA Data Sheet and Questions with Answers

Protocol Questions

Protocol Questions with Answers

Protocol Hints-

1) If you have a double length period, this lab can be done in one day.
If your students work efficiently, you might be able to finish in a regular length period but it will be tight.
If it seems that you must split the lab into 2 days, you can stop after Step 2 is completed. Cover the plate with a piece of Handiwrap (or similar product) and store in the refrigerator until the next day.
The plates should be taken out and allowed to reach room temperature before continuing with the lab. This should only take about 20 minutes or so.

2) There are several places where the plates are "tapped" on a napkin to remove the liquid in the wells. This is best done with some force. Holding the plate in your hand, bring it down to "hit" the stack of napkins. Do this two times. This will force the liquid onto the napkin. The softness of the stack will prevent the plate from being damaged and the napkin will absorb the liquid. You might want to take a plate, add some water to a few wells and practice this before showing your kids. The wells do not have to be "bone dry" after this procedure so don't be concerned if they still look wet.

3) The "story" behind the lab can vary. Most commonly it is that Patient One and Patient Two both may have been exposed to HIV. The ELISA is run using blood serum from both patients to detect the presence of HIV antibodies and thereby either confirm the infection or eliminate the possibility of infection.

4) You may wish to avoid the work of making all the solutions *etc*. and buy a pre-packaged kit for an ELISA lab. If so, simply ignore these protocols and use whichever of the other sheets that may seem appropriate.

STEP ONE- The antigen is added to the wells.

This antigen has been extracted from HIV. Since the concentration of the antigen may be too high, a serial dilution will be used to coat different wells with different concentrations of antigen. ELISA will test for the presence of antibodies specific for this antigen. Persons exposed to HIV will have these antibodies in their blood.

a) Use the larger micropipette to put 45 µl of TBS **(Tube A)** into each of wells B2 through B6 and D2 through D6. **Discard the micropipette tip and attach a new tip.**

b) Use the larger micropipette to put 50 µl of HIV antigen (Tube B) into wells B1, C1 and D1. **Discard the micropipette tip**

c) Use the smaller micropipette to take 5µl from B1 and add it to B2 then mix.
(this can be mixed by drawing the sample in B2 up and down in the pipette several times)
You may keep the same tip for steps d, e and f below.

d) Take 5µl from B2 and add it to B3 then mix as before.

e) Take 5µl from B3 and add it to B4 then mix as before.

f) Take 5µl from B4 and add it to B5 then mix as before.

DO NOT put any HIV antigen into Well B6 Well B6 is called a negative control. There should be no reaction at all in this well since there is no viral antigen added.

CHANGE THE MICROPIPETTE TIP

g) Use the smaller micropipette to take 5µl from D1 and add it to D2 then mix.
You may use the same tip for steps h, i and j below.

h) Take 5µl from D2 and add it to D3 then mix.

i) Take 5µl from D3 and add it to D4 then mix.

j) Take 5µl from D4 and add it to D5 then mix.

DO NOT put any HIV antigen into Well D6 Well D6 is also a negative control. There should be no reaction at all in this well since there is no viral antigen added.

Let the plate sit for 5 minutes. During this time, the viral antigen will stick to the plastic.

STEP TWO- Block and Wash the Plate

Blocking the plate will fill in any places where the HIV antigen did not stick thus preventing other molecules from adhering at a later step. This step will also remove any excess materials from the wells.

a) After the 5 minute waiting time, turn the plate upside down on the stack of napkins. Tap the plate against the towel to remove excess liquid.

b) Use the larger micropipette to add 100µl of TBS-Gel (Tube C) to each of wells B1 through B6, C1 and D1 through D6. **Discard the micropipette tip.**

c) Remove the TBS-Gel by tapping upside down on the paper napkins.

STEP THREE- Adding the patient serum

The patient serum contains antibodies. If any of these antibodies are specific for the HIV antigen used, they will bind to it. These are the target antibodies that ELISA tests for.

a) Use the larger micropipette to add 50µl of **Patient 1** serum **(Tube D)** to wells B1 to B6

CHANGE THE MICROPIPETTE TIP

b) Use the larger micropippette add 50µl of **Patient 2** serum **(Tube E)** to wells D1 through D6.

CHANGE THE MICROPIPETTE TIP

c)Add 50µl of Positive "+" serum (**Tube F**) to well C1. Positive serum definitely contains the target antibodies. Well C1 is a control well. This will show an undiluted positive result. **Discard the micropipette tip**

d) Let the plate sit for 5 minutes. During this time, the target antibodies (those specific for the antigen) will bind to it.

e) After 5 minutes, turn the plate upside down on the stack of paper napkins and tap the plate against the napkins to remove excess unbound antibodies.

f) Use the larger micropipette to add 100 µl of TBS-Gel **(Tube C)** to wells B1 through B6, D1 through D6 and C1. **Discard the micropipette tip.**

g) Turn the plate upside down on the napkins and tap to remove excess material.

STEP FOUR- Add the anti-human antibody with linked enzyme.

The antibody added in this step was produced in another animal (like a mouse) and will bind to the constant part of a human antibody. It has an enzyme attached to it.

a) Use the larger micropipette to add 50µl of anti-human antibody (**Tube G**) to each of wells B1 through B6, D1 through D6 and well C1. **Discard the micropipette tip**

Let the plate sit for 2 minutes.

b) Turn the plate upside down on the paper napkins and tap to remove excess material.

c) Use the larger micropipette to add 100 µl of distilled water (**Tube H**) to each of wells B1 through B6, C1 and D1 through D6. **Discard the micropipette tip**

d) Turn plate upside down on the paper napkins and tap to remove excess water.

STEP FIVE- Add the substrate

The enzyme that is attached to the anti-human antibody will react with this substrate to produce a color.

a) Use the larger micropipette to add 50µl of substrate (**Tube I**) to each of wells B1 through B6, C1 and D1 through D6. **Discard the micropipette tip**

b) Let the plate sit for 5-10 minutes while the color develops.

STEP SIX- Observations

On the data sheet, record your observations as to color intensity in each well.

When all observations have been recorded, discard the plate.

Answer the questions on the data sheet.

NOTE- If you have access to a spectrophotometer, the contents of each well can be pooled and placed into a cuvette with 2 ml distilled water and the optical density read using a wavelength of 450 nanometers. Doing this will require more time and it must be done soon after the color develops in the wells because the color will fade in a few hours. A special kind of spectrophotometer called a plate reader can read the color intensity while the liquid is still in the wells.

Protocol Questions

1) The first step adds _____ to the plates and then _____ it so the concentrations are not too high.

2) The second step does what two things? _____ and _____

3) What is the purpose of blocking the plate?

4) What is the purpose of the positive control in step three?

5) Where was the antibody added in step 4 produced? _____
What does it have attached to it? _____

6) How do we know if a reaction occurs between the enzyme and the substrate?

7) What machine can be used to measure the color intensity in each of the wells?
_____

8) Wells B6 and D6 are negative controls. Will there be a reaction in these wells? _____
Why not?

9) Why is well C1 called a positive control?

10) The positive and negative controls gives something to _____ the test results to.

Protocol Questions (with answers)

1) The first step adds **antigen** to the plates and then **dilutes** it so the concentrations are not too high.

2) The second step does what two things? **Blocks** and **washes**

3) What is the purpose of blocking the plate?
The blocking agent attaches to any places in the well where the antigen did not attach. This prevents other proteins from attaching later on and affecting the accuracy of the results

4) What is the purpose of the positive control in step three?
There will be a definite color change in the positive control well. This provides a comparison to the test wells.

5) Where was the antibody added in step 4 produced? **It was produced in some animal like a rabbit.**

What does it have attached to it? **It has an enzyme attached to it.**

6) How do we know if a reaction occurs between the enzyme and the substrate?
The reaction between the enzyme and the substrate produces a color change.

7) What machine can be used to measure the color intensity in each of the wells?
A spectrophotometer can be used to measure the color intensity in each well.
A specific kind of spectrophotometer called a plate reader can be used to measure the color intensity in each well without removing the liquid from the wells.

8) Wells B6 and D6 are negative controls. Will there be a reaction in these wells? **NO**
Why not? **There was no antigen added to these wells.**

9) Why is well C1 called a positive control? **Antibodies were added to this well in order to definitely produce a color change. This can be called a positive reaction.**

10) The positive and negative controls give something to **compare** the test results to.

ELISA Data Sheet

The squares below represent wells B1 through B6, C1 and D1 through D6. Examine the wells in your plate and pick a number from 0 to 5 that describes the intensity of the blue color in each plate. 0 for no color, 5 for the most intense. Put those numbers in the appropriate squares below.

	1	2	3	4	5	6
B						
C						
D						

Conclusions

1) Why does the color intensity vary from well B1 to well B6?

2) Why did we expect no color in well B6?

3) What does the color in wells B1 through B5 tells us about Patient One?

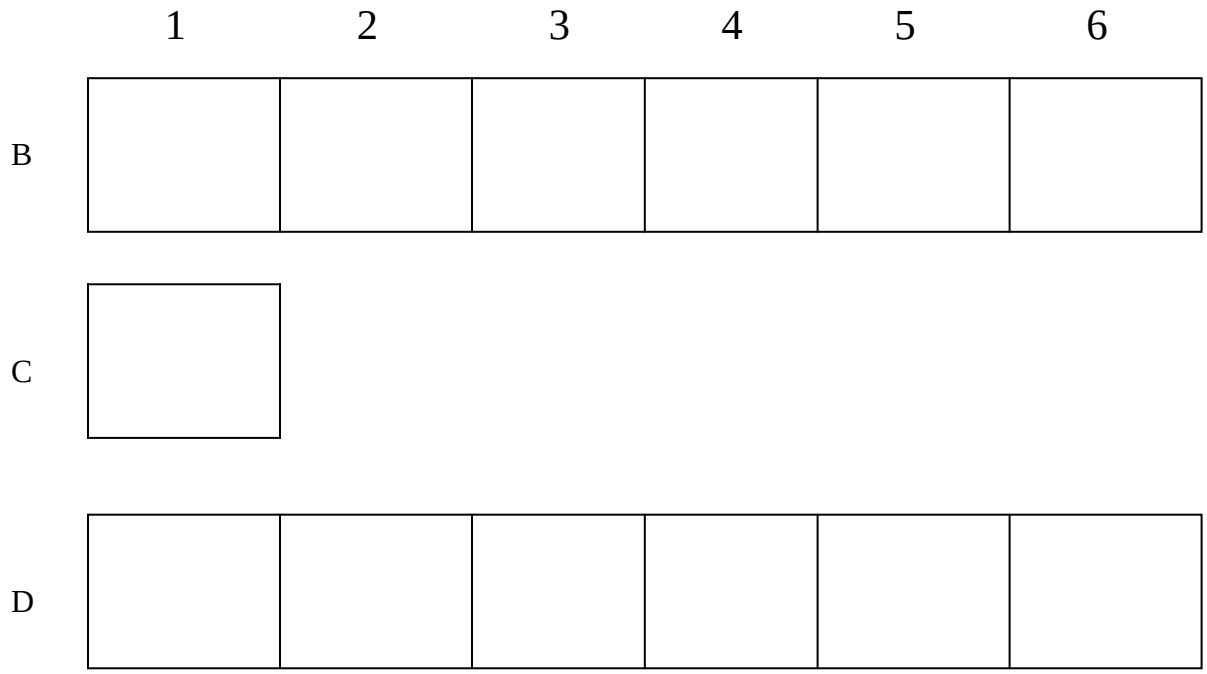
4) What does the lack of color in Wells D1 through D5 tell us about Patient Two?

5) Why did you know there would be a strong reaction in well C1?

6) How would you explain the results to Patient One?

ELISA Data Sheet
(with answers)

The squares below represent wells B1 through B6, C1 and D1 through D6. Examine the wells in your plate and pick a number from 0 to 5 that describes the intensity of the blue color in each plate. 0 for no color, 5 for the most intense. Put those numbers in the appropriate squares below.



Conclusions

1) Why does the color intensity vary from well B1 to well B6?
The antigen was diluted from highest concentration in well B1 to lowest in B6

2) Why did we expect no color in well B6?
No antigen was added to well B6

3) What does the color in wells B1 through B5 tells us about Patient One?
Patient One has been exposed to the virus. The patient has produced antibodies against the virus.

4) What does the lack of color in Wells D1 through D5 tell us about Patient Two?
Patient Two has no antibodies against the virus. The patient has not been exposed to it.

5) Why did you know there would be a strong reaction in well C1?
Full strength antigen was there and antibodies against the antigen were added.

6) How would you explain the results to Patient One? **Answers will vary**

Below are some websites of interest.

This is the Main Page for the Harvard University Outreach Program sponsored by the Department of Molecular and Cellular Biology and by the Howard Hughes Medical Institute.

http://outreach.mcb.harvard.edu/

This website has projects by participants in a summer program in immunology offered by Harvard University Outreach Program.

http://outreach.mcb.harvard.edu/teachermaterialsS04.shtml

This website lists other sites in immunology. It was put together by the Harvard University Outreach Program.

http://outreach.mcb.harvard.edu/summer04links.shtml

This website is "The Biology Project" produced by the University of Arizona. It covers several areas beyond immunology.

http://www.biology.arizona.edu/default.html

This website is for CityLab, a program sponsored by Boston University Medical School and the BU School of Education. It has several lab exercises of interest.

http://www.bumc.bu.edu/Dept/Home.aspx?DepartmentID=285

Acknowledgments

Richard G. Willets and Susan P. Mooney are very grateful to have been participants in the John H. Wallace Fellowship for High School Teachers and we wish to thank the following individuals, groups and supporters for their gracious accommodations, tutelage, and encouragement.

Dennis L. Kasper, M.D. Harvard Medical School William Ellery Channing Professor of Medicine; Brigham and Women's Hospital Senior Physician.

Arthur O. Tzianabos, Ph.D. Harvard Medical School Associate Professor of Medicine; Brigham and Women's Hospital Associate Microbiologist, Department of Medicine.

Gregory P. Priebe, M.D. Harvard Medical School Instructor in Pediatrics and Clinical Fellow in Anesthesia; Brigham and Women's Hospital Associate Physician.

Andrew B. Onderdonk, Ph.D. Harvard Medical School Professor of Pathology; Brigham and Women's Hospital Director of the Clinical Microbiology Laboratory.

Matthew A. Holsti, Ph.D. Harvard Medical School Research Fellow in Medicine; Brigham and Women's Hospital Research Fellow in Medicine.

Ronald J. Panzo, Harvard Medical School Channing Laboratory Research Lab Manager.

Emily S. Davie, Harvard Medical School Channing Laboratory, Researcher (Summer Intern)

Donald A. DeRosa, Ed.D., Director City Lab / Mobile Lab, Boston University School of Medicine; Boston University School of Education, Clinical Assistant Professor.

John H. Wallace Fellowship for High School Teachers.

American Association of Immunologists

Elizabeth Hiles and Jackie Miller, Ph.D., Curriculum Center for Science Education, Education Development Center, Inc., Newton, MA