SECTION ONE

General Information

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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.

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: 5 1, 45 1, 50 1, and 100 1.

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.

196 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

American Bioanalytical
AB14043-01000
1 liter (This amount is more than adequate for all your classes)
\$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:SigmaCatalog #:A-6154Quantity:0.25 ml (this amount is adequate for all your classes)Price:\$21.40This should be stored in a freezer when it arrives (packed in dry ice)

Microtubes

Ward's
18W 1361
500 microtubes
\$10.95

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

Carolina Supply
21-5562
1-50 well rack
\$6.00

Microtube Storage Rack- Plastic

0	
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 -2.58 -1.471 -1.1502 10.002 Tc

Economy Micropipette: 5 l - 100 l

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

Micropipette Tips-0.5 1 - 250 1 (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- 0.5	1-250 1 (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.

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)

Aliquots for Students (Per Station)

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

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 <u>organic catalysts</u>.

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. 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 <u>plate readers</u>. 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 <u>immunoglobulin</u>, 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

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.

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 th

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

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

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 negahappen in this

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

SECTION FOUR

ELISA: Four Basic Steps

ELISA: Another Look

ELISA Questions

ELISA Questions with Answers

THE ELISA

ELISA stands for Enzyme Linked ImmunoSorbent Assay. 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.

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

steps involved in the ELISA procedure and in between steps there is some uate time must be allowed for linkages to occur between molecules. This is sults. In addition, excess material is washed away between steps. The test well plates r[The well is coated with antigen 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



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



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



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

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 follo

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.



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: Antihuman 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	F	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

SECTION FIVE

Micropipette Information

Micropipette Practice

Serial Dilution Questions

Serial Dilution Questions with Answers

Small Volumes: Information and Questions

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 I to 10 I; others may measure from 20 I to 200 I.; 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 <u>slowly</u> 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 I of water from the centrifuge tube into tube A, 50 I of water into tube B. Use the appropriate micropipette to put 10 I of water into tube C.

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

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

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

7) Take 10 I 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 1 of distilled water from the centrifuge tube. Is one 1 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 1.

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.

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 ls 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 I).

3) How many lare in one ml?_____

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

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

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

7) 0.25 ml is equal to how many 1?_____

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 I).

3) How many lare in one ml? 1000 lare in one ml.

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

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

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

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

7) 0.25 ml is equal to how many 1?250 1

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.

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 100 I 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 50 | of Patient 1 serum (Tube D) to wells B1 to B6

CHANGE THE MICROPIPETTE TIP

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

CHANGE THE MICROPIPETTE TIP

c)Add 50 1 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, ththo md) L3ter th

a) Use the Ifrger micropipette to add 50P

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 50 | 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 | 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 50 l 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.

1	2	3	4	



ELISA Data Sheet (with answers)

Conclusions

1) Why does the color intensity

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

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