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EDVO-Kit

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AIDS Kit I: Simulation of HIV-1 Detection by ELISA

Store entire experiment in the refrigerator.

EXPERIMENT OBJECTIVE:

The objective of the experiment is for students to understand the molecular biology of the human immunodeficiency virus and the pathogenesis of acquired immune deficiency syndrome. The experimental concepts and methodology involved with enzyme linked immunosorbent (ELISA) assays will be introduced in the context of the clinical screening of serum samples for antibodies to the HIV virus.

Table of Contents

Safety Data Sheets can be found on our website:
www.edvotek.com/safety-data-sheets

This experiment is designed for 10 groups.

Store entire experiment in the refrigerator.

Experiment Components

A HIV Antigens (simulated)
B Positive Control (primary antibody)
C Donor 1 Serum (simulated)
D Donor 2 Serum (simulated)
E Anti-IgG-peroxidase conjugate (secondary antibody)
F Hydrogen peroxide, stabilized
G Aminosalicylic acid (peroxide co-substrate)
H Phosphate buffered saline concentrate

- Microtiter plates
- Transfer pipets
- Microtest tubes with attached caps
- 1 ml pipets
- Plastic tubes, 50 ml

This experiment does not contain HIV virus or its components. None of the components have been prepared from human sources.

Requirements

- Distilled or deionized water
- Beakers
- 37°C Incubation oven
- Disposable lab gloves
- Safety goggles
- Automatic micropipets (100 µl) and tips recommended

Make sure glassware is clean, dry and free of soap residue.
For convenience, additional disposable transfer pipets (Cat. # 632) can be purchased for liquid removal and washing steps.

Background Information

Acquired immune deficiency syndrome (AIDS) is a disease characterized by the progressive deterioration of an individual's immune system. The immunological impairment allows infectious agents such as viruses, bacteria, fungi and parasites to invade the body and propagate unchecked. In addition, the incidence of certain cancers dramatically increases in these patients because of their compromised immune system. AIDS is a serious threat to human health and is a global problem. Intensive research is being done to advance methods of detection, clinical treatment and prevention.

ABOUT HIV 1

The AIDS etiologic agent (HIV-1) is the human immunodeficiency virus type 1, which is a retrovirus. HIV-1 contains an RNA genome and the RNA dependent DNA polymerase also termed reverse transcriptase. Members of the retrovirus family are involved in the pathogenesis of certain types of leukemias and other sarcomas in humans and animals. The structure and replication mechanism of HIV is very similar to other retroviruses. HIV is unique in some of its properties since it specifically targets the immune system, is very immunoevasive, forms significant amounts of progeny virus *in vivo* during the later stages of the disease and can be transmitted during sexual activity.

The HIV viral particle is surrounded by a lipid bilayer derived from the host cell membrane during budding. The viral proteins are identified by the prefix gp (glycoprotein) or p (protein) followed by a number indicating the approximate molecular weight in kilodaltons. The lipid bilayer contains gp 160, gp 120 and gp 41. The gp 41 anchors gp 120 in the bilayer. Beneath the bilayer is a capsid consisting of p17 and p18. Within this shell is the viral core. The walls of the core consists of p24 and p25. Within the core are two identical RNA molecules 9000 nucleotides in length. Hydrogen bonded to each viral RNA is a cellular tRNA molecule. The core also contains approximately 50 molecules of reverse transcriptase.

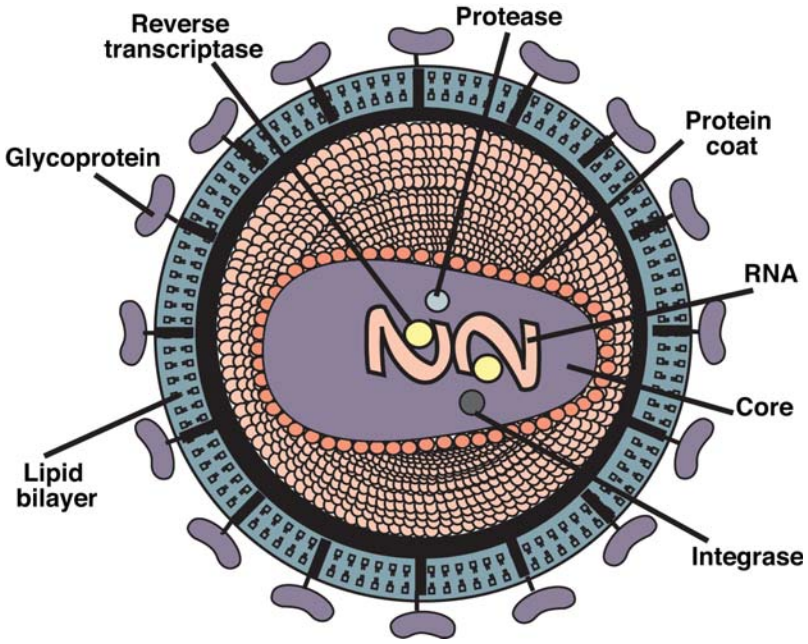


Figure 1: The HIV Virus

Large quantities of virus can be grown in tissue culture for diagnostic and research purposes. Several of the viral proteins have been cloned and expressed in relatively large quantities.

MECHANISM OF HIV INFECTION

An individual can be infected with HIV through an abrasion in a mucosal surface (e.g. genital and rectal walls), a blood transfusion or by intravenous

Background Information

injection with a contaminated needle. Virus or virally infected cells are found in bodily fluids such as semen and blood. During the early stages of infection in an immunocompetent person the HIV virus elicits humoral and cellular immunity responses that result in a variety of circulating IgG molecules directed at several viral epitopes. However, since the virus has a high mutation rate the variants survive and produce progeny having a similar capacity to escape immunosurveillance.

Unlike other cellular DNA polymerases, HIV DNA polymerase (reverse transcriptase) has a high error rate. These frequent mutations continually change the viral protein epitopes. This is believed to be the main mechanism of HIV immunoevasion. The most important target for the virus are hematopoietic cells such as bone marrow derived monocytes, myelocytes and immune system lymphocytes. Infection of immune system effector cells such as T cells and macrophages ultimately produce the most profound clinical consequences. gp 120 binds to the CD4 receptors on the surface of T helper (T_H) cells. These receptors are membrane bound glycoproteins involved in T cell maturation from precursor cells. T_H cells are required for the body's overall immunological responses. The viral lipid bilayer fuses with that of the cell's membranes and the viral protein capsid becomes internalized via receptor mediated endocytosis. Subsequently, the rest of the CD4 receptors are internalized and gp 120 appears on the T cell surface.

HIV REPLICATION AND TRANSCRIPTION

Through a complex mechanism involving several events, the reverse transcriptase synthesizes a double stranded DNA copy of the genomic RNA template. The tRNA molecule acts as the primer for the first strand synthesis. The reverse transcriptase, RNAse H activity, degrades the RNA strand of the RNA-DNA duplex and the polymerase activity synthesizes a complementary DNA strand. The DNA reverse transcripts (copy DNA) migrate into the cell nucleus where they become covalently integrated into the cellular genomic DNA. The integration is catalyzed by the HIV integrase. The copy DNA integrates via specific, self-complimentary sequences at both ends called long terminal repeats (LTRs). These sequences also have important functions in viral transcription. The integrated copy DNA is called proviral DNA or the provirus. The provirus enters a period of latency that can last for several years. The proviral DNA is replicated along with the cellular DNA and can be inherited through many generations. The HIV proviral DNA contains the major genes common to all non-transducing retroviruses. These genes are gag, pol and env. HIV also contains five or six other genes that are much smaller. Retroviral transcription is a complex process producing a variety of RNAs. Promotion of transcripts is controlled in the LTR and transcriptional termination signals are located in each major gene. Those RNA transcripts that remain unspliced become packaged in the new viral particles. The gag gene is translated into a polypeptide that is cleaved by a viral protease into four proteins that form the inner shells. Specific protease inhibitors are clinically being used to inhibit protein processing and control the further spread of the HIV virus in patients suffering from AIDS. The pol gene encodes the reverse transcriptase and the integrase which is responsible for the genomic incorporation of copy DNA. The env gene encodes the surface glycoproteins the viral particles acquire as they bud from the cells.

Background Information

IMMUNOLOGICAL RESPONSE

Macrophages are circulating monocytes and are involved in the non-specific engulfment of foreign material and normal cellular debris. These materials are degraded in the lysosomes of the cells. Peptides from foreign degraded proteins are transported to the macrophage surface where they remain bound by specialized receptors. Immunologically inactive T_H cells interact with these surface bound antigen-receptor complexes which enables them to become fully activated. HIV infects macrophages by binding to the cells' CD4 receptors. Fully activated T_H cells secrete several types of protein factors collectively known as lymphokines. Several of these factors are the interleukins which stimulate antibody secretions from B cells enable macrophage activation, stimulate general T cell growth and activate cytotoxic T cells. Cytotoxic T cells are involved in the actual destruction of foreign cells and body cells infected with different viruses. Inactive T_H cells that have been infected by HIV remain in a relatively quiescent state similar to uninfected cells. When a T_H cell containing provirus undergoes antigenic activation the integrated copy DNA becomes open to the transcription of viral RNA.

Viral replication causes the destruction of the T_H cells. Infected T_H cells also form syncytia, i.e. fused cells. Syncytia occur since the gp 120 on the infected T cell surface binds to CD4 receptors on other T_H cells. Cell to cell transmission of virus can occur in syncytia without the need for a free viral intermediate. Replication of virus also proceeds in activated macrophages which causes cell death and release of infectious viral particles. These and other events ultimately cause complete immune system collapse. The long latency period after HIV infection can be understood in terms of T_H cell activation. Only certain T_H cells are capable of responding to a particular antigen. HIV infected but asymptomatic individuals will experience the usual exposure to chemicals, viruses and bacteria. Each infection activates a subpopulation T_H cells containing the provirus which eventually leads to the death of these cells. After successive waves of infections the population of T_H and macrophage cells become depleted and clinical AIDS develops.

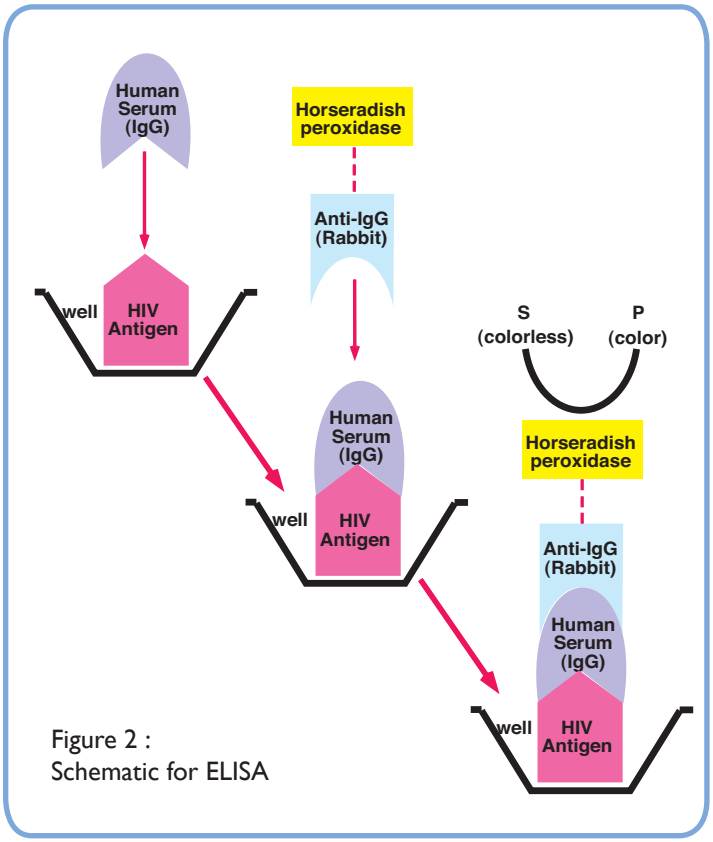


Figure 2 :
Schematic for ELISA

There are a very small number of individuals who have coexisted with HIV for over 15 years. Although the reasons for this coexistence with HIV is not understood, the immune system appears to remain intact. Such individuals tend to eat well, exercise and practice stress reduction techniques. Genetic analysis may determine whether or not subtle genetic differences in the immune system are significant factors.

Background Information

DESCRIPTION OF THE HIV SCREENING SIMULATION

Enzyme linked immunosorbent assay (ELISA) tests were originally developed for antibody measurement. These immunoassays have also been adapted to successfully detect samples that contain antigens. This HIV ELISA simulation experiment has been designed to detect a hypothetical patient's circulating IgG directed towards the viral (HIV) antigen. ELISAs are done in microtiter plates which are generally made of polystyrene or polyvinyl chloride. The plates are somewhat transparent and contain many small wells, in which liquid samples are deposited.

The following are the basic steps of the ELISA reaction.

Step 1
Antigens are added to the wells where some remain adsorbed to the wells by hydrophobic bonds. Antigens can be from whole HIV lysates, specific HIV proteins, or a mixture of the two. There is no specificity involved with the adsorption process to the wells, although some substances may exhibit differential binding. In certain cases, the antigens can be covalently cross-linked to the plastic using UV light.

Step 2
Wells are washed to remove unadsorbed antigens.

Step 3
Block the unoccupied sites on the walls of the plastic wells with proteins, typically gelatin or bovine serum albumin.

Step 4
Infection by HIV-1 causes the individual to mount an antibody response which eventually results in plasma IgG molecules that bind to different HIV proteins (or different areas or the same protein).

If these antibodies are present, as in the plasma sample of an HIV positive patient, they will bind to the adsorbed antigens in the well and remain there after washing.

If the antibody (from an HIV positive patient) remains bound to the antigen in the well, then the secondary antibody will bind to it and remain attached after washing. If the patient is negative for HIV, there will be no primary antibody to bind to the antigen and in turn, no secondary antibody binding.

Secondary antibodies are usually raised in rabbits and goats immunized with human IgG tractions. Secondary antibodies (anti - HIV - IgG) are purified and covalently cross linked to horseradish peroxidase. This modification does not usually affect the binding specificity and affinity of the antibody or the enzymatic activity of the peroxidase.

Step 5
Wells are washed to remove unbound secondary antibody.

Background Information

Step 6
After washing, a solution containing hydrogen peroxide and aminosalicylate is added to each well. Peroxidase possesses a high catalytic activity and can exceed turnover rates of 10^6 per second. Consequently, amplification of an HIV positive sample can occur over several orders of magnitude. Many hydrogen donor co-substrates can be used by peroxidase. These co-substrates include o-diansidine, aminoantipyrine, aminosalicylic acid and numerous phenolic compounds that develop color upon oxidation.

The substrate solution used for the ELISA reaction is nearly colorless. Peroxidase converts the peroxide to $H_2O + O_2$ using the salicylate as the hydrogen donor. The oxidized salicylate is brown and can be easily observed in wells containing anti - HIV - 1 IgG (positive plasma).

It should be noted that polyclonal antibody preparations to a given antigen can have variable binding affinities due to differences in the immunological responses between animals. Different immunizations with the same antigen in the same animal can also produce variable binding affinities. The use of monoclonal antibodies directed against a single epitope eliminates this variability. Western blot analysis of positive ELISA samples is used to confirm the presence of HIV in a patient.

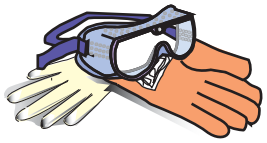
Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is to understand the molecular biology of the human immunodeficiency virus and the pathogenesis of acquired immune deficiency syndrome. The experimental concepts and methodology involved with enzyme linked immunosorbent (ELISA) assays will be introduced in the context of the clinical screening of serum samples for antibodies to the virus.

LABORATORY SAFETY

1. Exercise extreme caution when working with equipment which is used in conjunction with the heating and/or melting of reagents.
2. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
3. Gloves and goggles should be worn routinely as good laboratory practice.



Wear gloves and safety goggles

Student Experimental Procedures

Experiment Procedure

REMEMBER:

Equilibrate a 37°C incubation oven before starting the experiment.

GENERAL INSTRUCTIONS AND PROCEDURES

Labeling the Microtiter Plate

- Place the microtiter plate vertically as shown in Figure 1.
- Mark the plate with your initials or lab group number and number the rows 1-4 down the side.

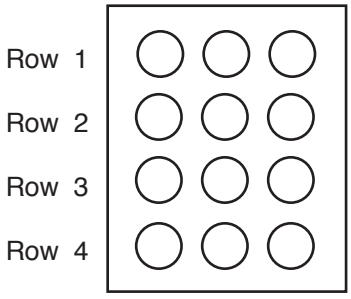


Figure 1

Labeling the Plastic Transfer Pipets

Label 5 transfer pipets as follows:

- (-) (negative)
- (+) (positive)
- DS 1 (Donor Serum 1)
- DS 2 (Donor Serum 2)
- PBS (Phosphate Buffered Saline)

Use the appropriately labeled plastic transfer pipet for liquid removals and washes.

INSTRUCTIONS FOR ADDING LIQUIDS AND WASHING WELLS

NOTE:

The general procedure for adding reagents to the wells will conserve the use of a large number of pipets in this simulated classroom ELISA.

If available, reagents should be dispensed with an automatic micropipet using disposable tips.

Adding Reagents to Wells

- For adding reagents to the wells, use the same 1 ml pipet.
- RINSE THE PIPET THOROUGHLY with distilled water before using the pipet for adding the next reagent.

Liquid Removal and Washes:

- When instructed in the experimental procedures, remove liquids with the appropriately labeled transfer pipet, and then wash the wells as follows:

  A. Use the transfer pipet labeled "PBS", to add PBS buffer to the wells in all rows. Add PBS buffer until each well is almost full.

  The capacity of each well is approximately 0.2 ml. Do not allow the liquids to spill over into adjacent wells.

  B. With the appropriately labeled transfer pipet, remove all the liquid (PBS buffer) from the wells in each row. Dispose the liquid in the beaker labeled "waste".

Student Experimental Procedures

Wear gloves and safety goggles

EXPERIMENTAL STEPS FOR THE ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

1. To all 12 wells, add 100 µl of "HIV" (viral antigens).
2. Incubate for 5 minutes at room temperature.
3. Remove all the liquid (viral antigens) with a transfer pipet.
4. Wash each well once with PBS buffer as described above ("Liquid Removal and Washes").

   In research labs, following this step, all sites on the microtiter plate are saturated with a blocking solution consisting of a protein mixture, such as BSA. We have designed this experiment to eliminate this step to save time.

5. Add reagents as outlined below:

   Remember to rinse the 1 ml pipet thoroughly with distilled water before adding a new reagent. If you are using automatic micropipets, use a clean micropipet tip for each reagent.

   - Add 100 µl of PBS Buffer to the three wells in Row 1. (This is the negative control.)
   - Add 100 µl of "+" (positive) to the three wells in Row 2. (This is the positive control.)
   - Add 100 µl of Donor Serum "DS1" to the 3 wells in Row 3.
   - Add 100 µl of Donor Serum "DS2" to the 3 wells in Row 4.

6. Incubate at 37°C for 15 minutes .
7. Remove all the liquid from each well with the appropriately labeled transfer pipet.
8. Wash each well once with PBS buffer (as described under "Liquid Removal and Washes").
9. Add 100 µl of the anti-IgG peroxidase conjugate (2°Ab) to all 12 wells.
10. Incubate at 37°C for 15 minutes.

    At this time you can obtain the substrate to be used in step 13. Since the substrate must be prepared just prior to use, your instructor will prepare it towards the end of the incubation in step 10.

11. Remove all the liquid from each well with the appropriately labeled transfer pipet.

Reminders:

ADDING REAGENTS:

Be sure to rinse the 1 ml pipet thoroughly before adding a new reagent. (Steps 1, 5, 9, & 13). Alternatively, if you are using automatic micropipets, use a fresh tip for each reagent.

LIQUID REMOVALS:

Use the appropriately labeled transfer pipet to remove all liquid from the wells in each row (steps 3, 7, & 11) and after washes (steps 4, 8 & 12)

Transfer pipet	(-)	Row 1
Transfer pipet	(+)	Row 2
Transfer pipet	DS 1	Row 3
Transfer pipet	DS 2	Row 4

Dispose the liquid in the beaker labeled "waste".

WASHES:

For all rows, use the transfer pipet labeled "PBS" to add PBS until each well is almost full. (Steps 4, 8, & 12)

Student Experimental Procedures

12. Wash each well once with PBS buffer (as described under "Liquid Removal and Washes").
13. Add 100 µl of the substrate to all 12 wells.
14. Incubate at 37°C for 5 minutes.
15. Remove the plate for analysis.
16. If color is not fully developed after 5 minutes, incubate at 37°C for a longer period of time.

Quick Reference:

The positive control, which contains IgG directed against HIV antigens, is the primary antibody. Positive serum samples will also contain anti-HIV IgG, while negative serum samples will not contain anti-HIV IgG.

Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. Describe the mechanism of ELISA. Why is ELISA so sensitive? Why is it necessary to block unoccupied binding sites in the microtiter wells? Why is it important to have a positive control?
2. Why can the onset of AIDS take several years?
3. Why is anti-HIV-1 IgG screened instead of the virus itself?
4. Why does the destruction of T_H cells compromise the entire immune system? How does HIV target T_H cells?
5. Why are there so many immunological variants of HIV?
6. The elimination of several steps in the ELISA could be accomplished if the primary antibody was made into an enzyme conjugate. Why is this generally not done? What can cause a false positive in an ELISA?

Instructor's Guide

If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK® web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

APPROXIMATE TIME REQUIREMENTS FOR PRELAB AND EXPERIMENTAL PROCEDURES

1. Pre-lab preparation of biologicals and reagents takes approximately one and one-half hours.

2. The student experimental activity requires approximately 60 minutes.

Safety Data Sheets can be found on our website:
www.edvotek.com/safety-data-sheets

Pre-Lab Preparations

PREPARATIONS BEFORE THE LAB

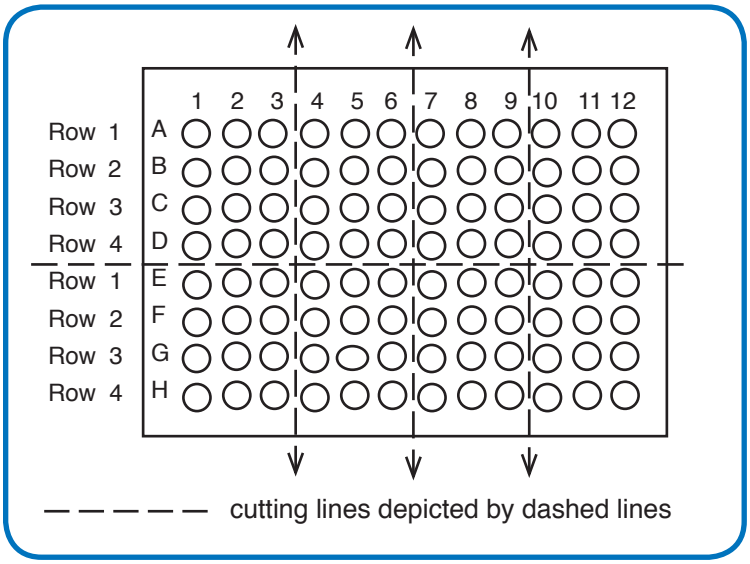
Microtiter Plates

1. As shown in the figure below, orient the microtiter plates so that the numbers 1-12 are at the top and the letters A-H are on your left.

2. Cut each plate on the dotted lines as shown in the figure. Each piece will be 3 wells on one axis and 4 wells on the other axis. Each lab group will receive one piece.

Dispensing Components A through D:

3. Use a FRESH 1 ml pipet (provided) for dispensing each of the components A-D directly from the component tubes provided in this experiment kit. Label microtest tubes and dispense volumes as outlined in the chart "Quick Reference: Preparation of experiment reagents".



Instructor's Guide

Pre-Lab Preparations

PREPARATIONS ON THE DAY OF THE LAB

Preparation of Phosphate Buffered Saline

1. Add all of the Phosphate Buffered Saline concentrate **(H)** to 270 ml of distilled water. Mix.
2. Label this diluted Phosphate Buffered saline as "PBS".
3. Dispense 25 ml into small beakers for each of the 10 lab groups.

Preparation of Anti-IgG Peroxidase Conjugate (Secondary Antibody)

4. Add 0.3 ml of diluted Phosphate Buffered Saline (PBS) to the concentrated Anti-IgG peroxidase conjugate **(E)**. Mix thoroughly by tapping and inverting the tube.
5. Transfer 15 ml of diluted Phosphate Buffered Saline (PBS) to a 50 ml plastic tube provided.
6. Transfer the entire contents of tube E prepared in step 4 to the 50 ml tube containing 15 ml of PBS. Label the tube "2°Ab" (Secondary Antibody). Mix.
7. Dispense 1.4 ml of the diluted Anti-IgG peroxidase conjugate for each group.

NOTE:

The sample volume of the secondary antibody is very small - the tube can be centrifuged to collect the sample at the bottom.

PREPARATION OF PEROXIDASE SUBSTRATE DURING THE LAB EXPERIMENT

Prepare 15 - 30 minutes before the last incubation:

1. Dispense 13.5 ml of diluted Phosphate buffered saline (PBS) to the second 50 ml tube provided.
2. Add Aminosalicylic acid **(G)** to the 13.5 ml of PBS. Cap and mix thoroughly by shaking and/or vortexing. There is usually undissolved material remaining.
3. Then add 1.5 ml of Hydrogen peroxide **(F)**. Cap and mix.
4. Dispense 1.4 ml of the peroxidase substrate for each group.

Quick Reference:

The substrate is prepared for the peroxidase enzyme, which is attached to the anti-IgG peroxidase conjugate (secondary antibody).

Prepare the substrate 15 - 30 minutes before students require it for plate development (last incubation).

Quick Reference Tables

Preparation of Experiment reagents

		Label	Dispense for each group
A*	HIV Antigens	HIV	1.4 ml
B*	Positive control	+	0.4 ml
C*	Donor Serum # 1	DS 1	0.4 ml
D*	Donor Serum # 2	DS 2	0.4 ml
E + PBS	Anti-IgG-peroxidase-conjugate	2°Ab	1.4 ml
PBS + F + G	Peroxidase-enzyme substrate	Substrate	1.4 ml
H + water	Phosphate Buffered Saline	PBS	25.0 ml

* **Components A - D** can be dispensed before the actual day of the lab and stored in the refrigerator. If these components are dispensed on the day of the lab, leave at room temperature.

STUDENT MATERIALS
Each Lab Group Should Receive:

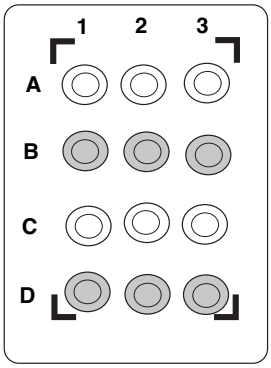
1	Piece of microtiter plate
1	Tube labeled "HIV"
1	Tube labeled "+"
1	Tube labeled "DS 1"
1	Tube labeled "DS 2"
1	Tube labeled "2°Ab"
1	1 ml pipet (or 1 automatic micropipet with tips)
1	Pipet pump
5	Transfer pipets
1	Beaker containing 16 ml of PBS
1	Beaker containing approximately 100 ml of distilled water
1	Empty beaker labeled "waste"
1	Tube labeled "Substrate" (just before the last incubation)

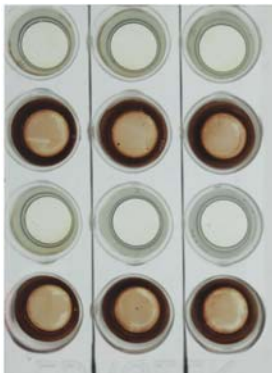
Avoiding Common Pitfalls

1. Students should be advised to be very careful when transferring solutions into and out of the microliter plate wells.
2. Use only clean or appropriately labeled pipets and avoid contaminating adjacent wells.
3. Do not attempt to empty the microliter wells by shaking it out. This will not work - it will result in contaminating adjacent wells.
4. Wash the wells gently and slowly, without force.
5. As an optional activity, the plates can be read in a microplate reader at 405 nm.

Expected Results

Donor 2 should show positive for HIV. The color should look similar to the positive control.





Please refer to the kit insert for the Answers to Study Questions