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Edvo-Kit #

271

Edvo-Kit #271

AIDS Kit I: Simulation of HIV Detection by ELISA

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.

See page 3 for storage instructions.

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

Experiment Components

COMPONENTS Store components A-G in the refrigerator.		Check (✓)
A	10X ELISA Wash Buffer	❑
B	ELISA Dilution Buffer	❑
C	Antigen (lyophilized)	❑
D	Primary Antibody (lyophilized)	❑
E	Secondary Antibody (lyophilized)	❑
F	ABTS (lyophilized)	❑
G	ABTS Reaction Buffer	❑

Experiment #271 is designed for 10 lab groups.

REAGENTS & SUPPLIES Store all components below at room temperature.	Check (✓)
• Microtiter plates	❑
• Transfer pipets	❑
• Snap-top microcentrifuge tubes	❑
• 15 mL conical tubes	❑

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Requirements *(not included with this kit)*

- Distilled or deionized water
- Beakers
- Disposable lab gloves
- Safety goggles
- Recommended: Automatic micropipettes (50 µL) and tips

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

None of the components have been prepared from human sources.

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 termed reverse transcriptase. The structure and replication mechanism of HIV is very similar to other retroviruses. Members of the retrovirus family are involved in the pathogenesis of certain types of leukemia and other sarcomas in humans and animals.

HIV is unique in some of its properties since it specifically targets the immune system, is very immune-evasive, forms significant amounts of progeny virus *in vivo* during the later stages of the disease, and can be transmitted during sexual activity.

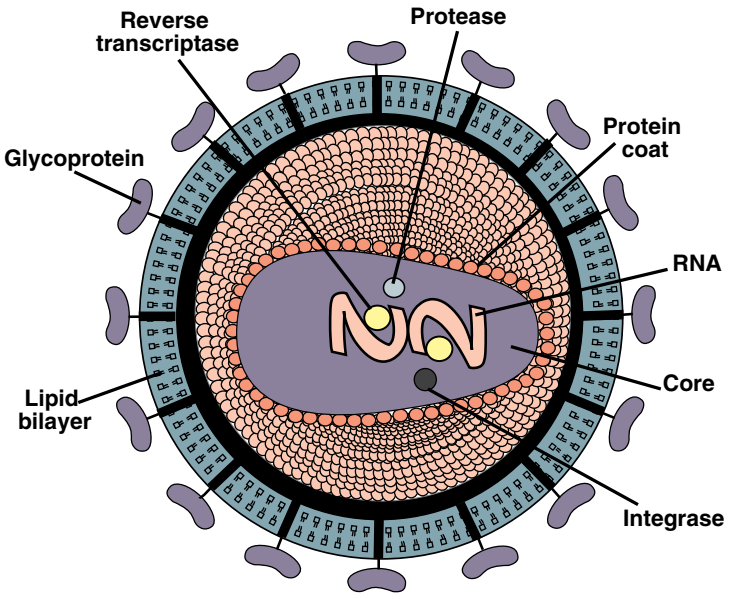


Figure 1: The HIV Virus

The HIV viral particle is surrounded by a lipid bilayer, derived from the host cell membrane during budding, and a protein capsid (Figure 1). 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. The viral proteins are identified by the prefix "gp" (glycoprotein) or "p" (protein) followed by a number indicating the approximate molecular weight in kilodaltons (Table 1). In total, the HIV genome encodes for 19 proteins necessary for the virus's structure, integration, replication, and disruption of the host cell.

Table 1: Examples of HIV Proteins

HIV Protein Name	Category	Protein Description
gp41	Envelope Antigens	Transmembrane protein
gp120	Envelope Antigens	CD4 binding protein
p17	Core Antigens	Matrix protein
p24	Core Antigens	Capsid protein
p31	Enzymes	Integrase
p51	Enzymes	Reverse transcriptase

Detecting HIV Infection in Patient Samples

An individual can be infected with HIV through unprotected sex, a blood transfusion, or by intravenous 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 immune responses. However, the viral reverse transcriptase has a high error rate, leading to an extremely high rate of mutations. This allows some of the viral variants to survive and produce progeny having a similar capacity to escape immunosurveillance.

Due to the highly infectious nature of HIV it is essential that patients are identified as quickly as possible following infection. This allows for patients to understand their potential risks, minimize transmission of the virus, and immediately begin treatment if necessary. Almost all modern HIV detection tests collect serum or saliva samples from the patient to identify the presence of antibodies against HIV proteins. These antibodies are produced within a few weeks of HIV infection and are incredibly specific, making them ideal for HIV screening.

ANTIBODIES

Antibodies (also called immunoglobulins, or Igs) are specialized proteins that allow the immune system to distinguish between "self" and "non-self" proteins or polysaccharides. These Y-shaped molecules comprise four linked polypeptide chains: two identical "heavy chains" and two identical "light chains" (Figure 2). The antigen binding sites are located at the ends of the short arms of the Y. The amino acid sequence in this region is variable, allowing for each antibody to recognize a unique epitope (a particular location within an antigen).

Because of their specificity, researchers can use antibodies to detect the presence of specific biomolecules (i.e. peptides, proteins, antigens and hormones) in a complex sample. One technique, called western blotting, identifies a particular protein in a mixed sample. Immunohistochemistry uses antibodies to label specific antigens within a cell or tissue. To quantitatively measure the amount of an antigen within a sample, scientists use the Enzyme Linked ImmunoSorbent Assay (ELISA). The assay produces a signal that is easy to detect and to quantify, even when starting with complex mixtures like cellular lysates.

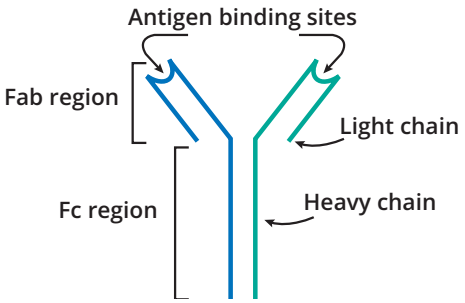


Figure 2:
General Structure of an antibody.

THE ELISA

ELISAs were originally developed to measure the quantity of antibodies in a solution, but have since been adapted to detect many different types of antigens. Traditional ELISAs require two antibodies. The first antibody, called the "primary antibody", recognizes the antigen of interest. For example, an ELISA that detects the HIV virus might be designed to use an antibody that recognizes one of the virion's coat proteins. In a clinical HIV ELISA, the assay will determine if HIV antibodies are present in the patient samples.

The "secondary antibody" recognizes the primary antibody – since our primary antibody is produced by human immune cells we would use a secondary antibody that specifically recognizes human antibodies. The secondary antibody is covalently linked to an enzyme called Horseradish Peroxidase (HRP) that lets us detect the presence of the antibody-antigen complex (Figure 3A). HRP has a high catalytic activity – its substrate turnover rates exceed 10^6 per second – allowing us to quickly detect even the smallest amount of antigen.

To perform an ELISA, the samples are added to the wells and the antigens are allowed to adsorb to the surface through hydrophobic associations (Figure 3B). In an HIV ELISA, the antigens will be viral proteins prepared ahead of

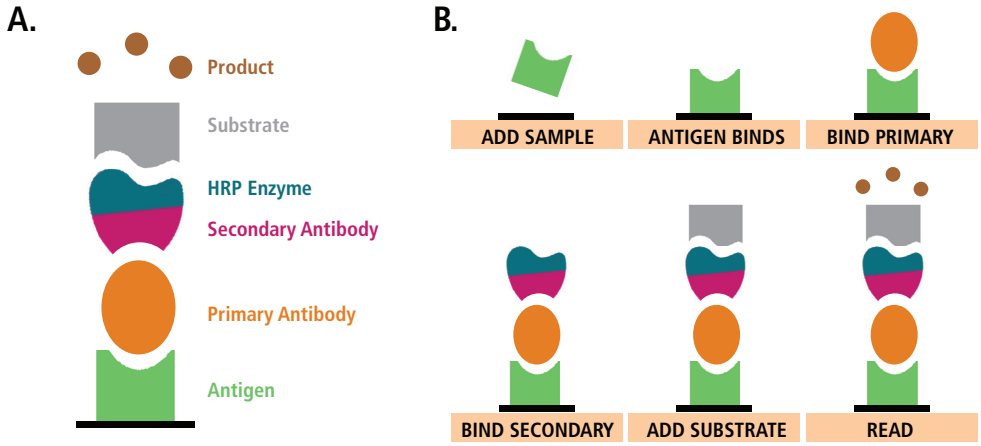


Figure 3: Optimized ELISA workflow

time. ELISAs are often performed in transparent microtiter plates made of polystyrene or polyvinyl chloride plastics. Scientists add antigens to the wells and allow them to non-specifically stick to the plastic through hydrophobic and electrostatic interactions. After washing away any excess fluid, the wells are "blocked" with a protein-containing buffer, which prevents non-specific interactions between the antibody and the plastic wells.

Next, a patient sample, or a control sample, is added to the wells and the mixture is allowed to incubate for a short time. If anti-HIV antibodies are present in the patient sample, they will recognize and bind to the HIV antigens. Following the incubation period, the wells are washed to remove any primary antibody that did not bind with the antigen.

After the wash, an enzyme-linked secondary antibody is added to the wells where it recognizes and binds to the primary antibody (if present). Importantly, if there was no primary antibody in the patient sample there will be nothing for the secondary antibody to bind. As before, the excess antibody is removed from the wells by washing with buffer. If the secondary antibody has bound to the primary antibody, it will stay in the well.

Finally, a clear, colorless solution of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and hydrogen peroxide is added to each well. The HRP enzyme on the secondary antibody oxidizes ABTS in wells where the antigen-antibody complex is present, turning the clear substrate solution blue-green. Since each enzyme breaks down many substrate molecules, the ELISA can detect even the smallest amount of antigen. While the color change from clear to blue-green is detectable by eye, measuring the sample's absorbance at 405 nm provides a quantitative result.

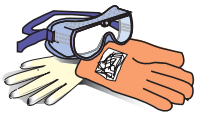
This experiment replicates a clinical screen to detect HIV antibodies in a simulated patient blood sample. Students will incubate prepared antigens in a microtiter plate, wash to remove unadsorbed protein, and then incubate with control and patient samples. If a patient is positive for HIV, their blood will contain anti-HIV antibodies that can bind to the proteins in the well. An HRP-linked secondary is then added to detect primary antibodies, if present. Finally, substrate is added to each well and monitored to determine the status of the assay. At the conclusion of the experiment students will provide an HIV diagnosis for each 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



Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
3. Always wash hands thoroughly with soap and water after handling contaminated materials.

LABORATORY NOTEBOOKS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

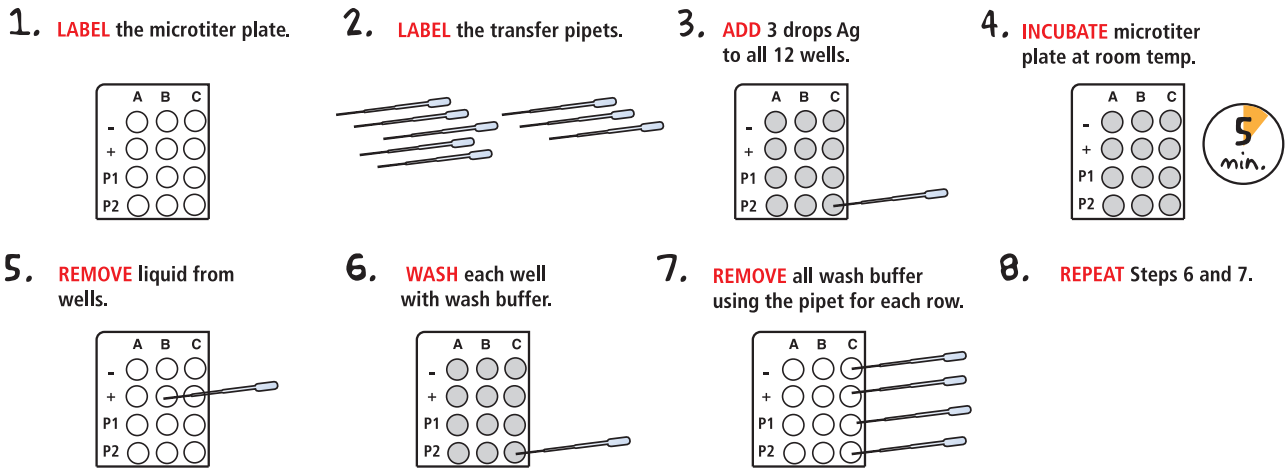
During the Experiment:

- Record (draw) your observations, or photograph the results.

After the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

Student Experimental Procedures



PERFORMING THE ELISA

Wear gloves and safety goggles

1. **LABEL** the wells of the microtiter plate as shown.

2. **LABEL** the transfer pipets as outlined in the box below. These 8 pipets will be used to add and remove liquid from the wells.

(Wash)	1x PBST Wash Buffer	(P1)	Patient 1 Sample
(Ag)	HIV Antigen	(P2)	Patient 2 Sample
(-)	Negative Control	(2°AB)	Secondary Antibody
(+)	Positive Control	(ABTS)	ABTS Substrate

3. Using the "Ag" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Antigen (Ag) to all 12 wells.

4. **INCUBATE** the plate at room temperature for 5 minutes.

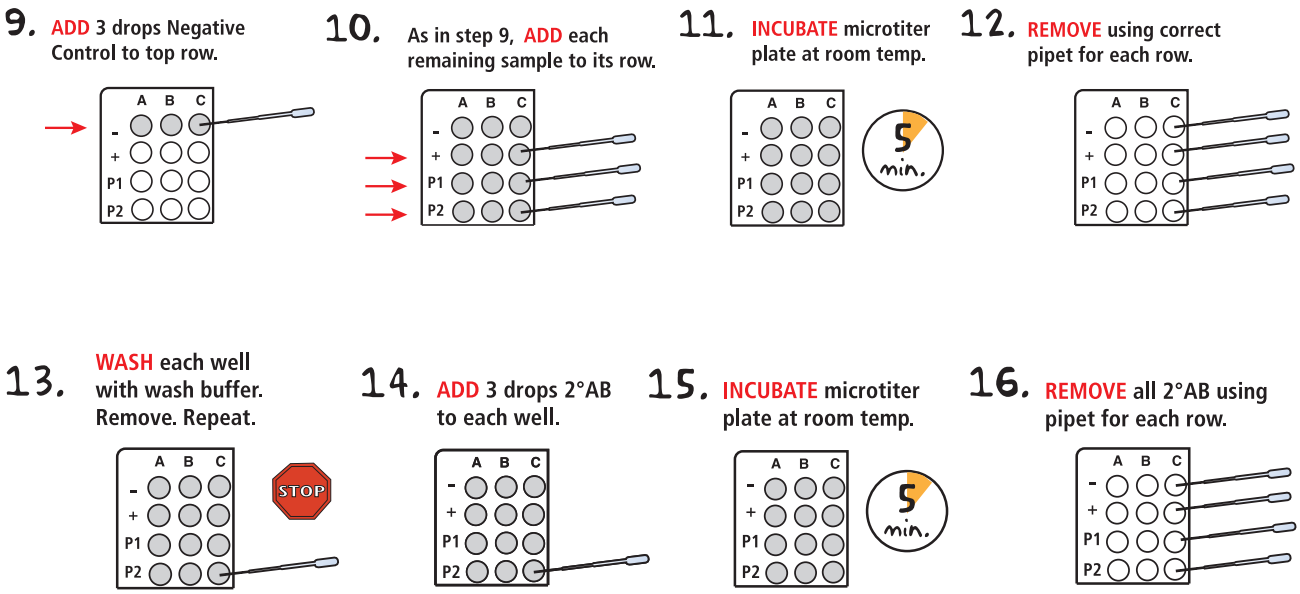
5. Using the "Ag" pipet **REMOVE** all of the liquid from the wells.

6. Using the "Wash" transfer pipet **WASH** each well by adding wash buffer until the wells are almost full (~200 µL). Do not allow the buffer to spill over into adjacent wells.

7. **REMOVE** all of the wash buffer using the transfer pipet designated for each row.

8. **REPEAT** steps 6 and 7 to wash the wells once more.

Student Experimental Procedures, continued



9. Using the "-" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of the negative control to all three wells in the top row.

10. As in step 9, **ADD** the "+", "P1", and "P2" samples to all three wells in the appropriate rows, taking care to use the correct pipets or changing tips between each sample.

11. **INCUBATE** the plate at room temperature for 5 minutes.

12. Using the correct transfer pipet for each row, **REMOVE** all of the primary antibody from each well.

13. **WASH** each well twice with fresh wash buffer. Between washes **REMOVE** all of the wash buffer using the transfer pipet designated for each row.

OPTIONAL STOPPING POINT: For overnight storage, **ADD** 200 µL of wash buffer to each well. Carefully cover the samples and place the plate in the refrigerator. The experiment should be resumed during the next lab period. Remove the wash buffer and continue with Step 14.

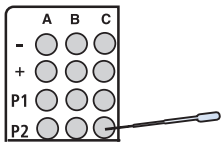
14. Using the "2°AB" labeled transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of the secondary antibody to each well.

15. **INCUBATE** the plate at room temperature for 5 minutes.

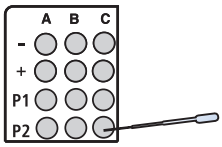
16. Using the labeled transfer pipet for each row, **REMOVE** all of the secondary antibody from each well.

Student Experimental Procedures, continued

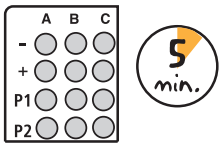
17. WASH each well with wash buffer. Remove. Repeat.



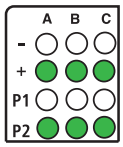
18. ADD 3 drops "ABTS" to all wells.



19. INCUBATE microtiter plate at room temp.



20. ANALYZE plate for color changes.



17. **WASH** each well twice with fresh wash buffer. Between washes **REMOVE** all of the wash buffer using the transfer piper designated for each row.

18. Using the "ABTS" labeled transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of ABTS substrate to all wells.

19. **INCUBATE** the plate at room temperature for 5 minutes.

20. Immediately **ANALYZE** the plate for color changes in the substrate. If the color is not fully developed it can be left for a longer period of time.

Study Questions

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 is anti-HIV-1 antibody screened instead of the virus itself?

3. Why are there so many immunological variants of HIV?

4. 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

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATIONS:

Some of the components can be prepared ahead of time, aliquoted, and stored in the refrigerator (4° C) until needed. See the table below for information on advanced preparation of reagents.

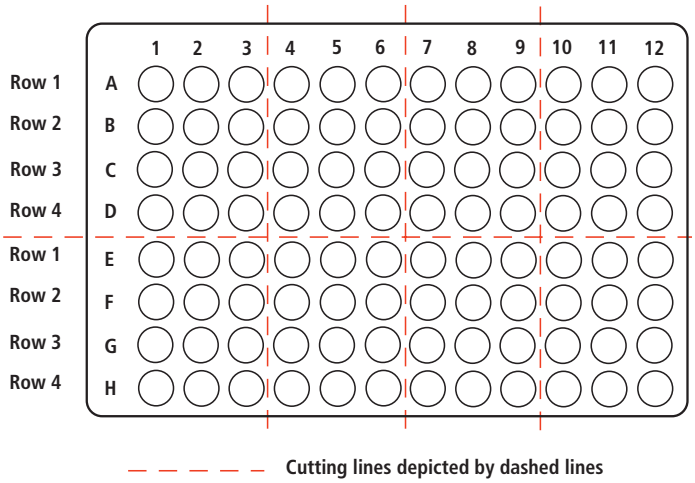
Component:	What to do:	When:
10X ELISA Wash Buffer (A)	Dilute to 1X solution and aliquot	Anytime before the experiment. Cover and store in the refrigerator.
ELISA Dilution Buffer (B)	Aliquot for negative control and patient samples	Anytime before the experiment. Store tubes in the refrigerator.
Whey Antigen (C)	Rehydrate and aliquot	Up to one week before performing the experiment.
Anti-Whey Primary Antibody (D)	Rehydrate and aliquot	Up to one week before performing the experiment.
Secondary Antibody (E)	Rehydrate and aliquot	Up to one day before performing the experiment.
ABTS Substrate (F)	Rehydrate and aliquot	Up to one week before performing the experiment.

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

Pre-Lab Preparations

Preparation of the Microtiter Plates

1. As shown in the figure (right), orient the microtiter plate so that the numbers 1-12 are at the top and the letters A-H are on the left.
2. Cut each plate on the dotted lines as shown in the figure. Each piece will contain 3 wells on one axis and 4 wells on the other axis. Each lab group will receive one piece.



Preparation of the Wash Buffer

1. Add all of the 10x ELISA Wash Buffer (A) to 180 mL of distilled water and mix well. Label as “Wash Buffer”.
2. Dispense 18 mL into small beakers for each lab group.

Preparation of the Antigen

1. Transfer 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label the tube “Antigen”.
2. Carefully remove the stopper from the vial of lyophilized Antigen (C) and transfer approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. Close the stopper and gently shake the vial to mix.
3. Transfer the entire contents of reconstituted Antigen back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "Ag" and dispense 650 µL into each tube.

Preparation of the Controls and Patient Samples

1. Label 10 microcentrifuge tubes "-CTRL" and 10 tubes as "P1". Dispense 200 µL ELISA Dilution Buffer (B) into each tube.
2. Transfer 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label the tube “1°AB”.
3. Carefully remove the stopper from the vial of lyophilized Primary Antibody (D) and transfer approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. Close the stopper and gently shake the vial to mix.
4. Transfer the entire contents of reconstituted Primary Antibody back to the 15 mL tube from step 1. Mix well.
5. Label 10 microcentrifuge tubes "+ CTRL" and 10 tubes as "P2". Dispense 200 µL of the primary antibody into each tube.

Pre-Lab Preparations

Preparation of Secondary Antibody

(NOTE: Prepare on same day as needed for the experiment.)

1. Transfer 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label the tube "2°AB".
2. Carefully remove the stopper from the vial of lyophilized Secondary Antibody (E) and transfer approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. Close the stopper and gently shake the vial to mix.
3. Transfer the entire contents of reconstituted Secondary Antibody back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "2°AB". Dispense 650 µL per tube.

Each Lab Student Group Should Receive:

1	Microtiter plate (3 x 4 well)
1	Microcentrifuge tube containing 650 µL Antigen
1	Microcentrifuge tube containing 650 µL Secondary Antibody
1	Microcentrifuge tube containing 650 µL ABTS
1	Microcentrifuge tube containing 200 µL - Control
1	Microcentrifuge tube containing 200 µL + Control
1	Microcentrifuge tube containing 200 µL P1
1	Microcentrifuge tube containing 200 µL P2
8	Transfer pipets
1	Beaker containing 18 mL Wash Buffer
1	Empty beaker for waste

Preparation of ABTS Substrate

1. Transfer 10 ml ABTS Reaction Buffer (G) into a 15 mL conical tube. Label the tube "ABTS".
2. Carefully remove the stopper from the vial of lyophilized ABTS (F) and transfer approximately 0.5 mL of the ABTS from the tube in step 1. Close the stopper and gently shake the vial to mix.
3. Transfer the entire contents of re-hydrated ABTS to the 15 mL conical tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "ABTS". Dispense 650 µL per tube.

Avoiding Common Pitfalls

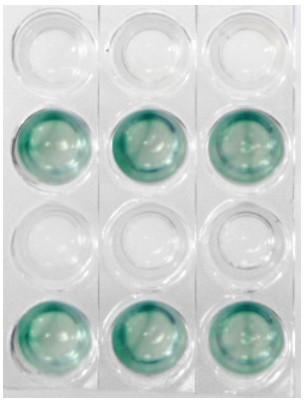
1. Students should be advised to be very careful when transferring solutions into and out of the microtiter plate wells.
2. Use only clean or appropriately labeled pipets.
3. Do not attempt to empty the microtiter wells by shaking it out. This will often result in contaminating adjacent wells.
4. Wash the wells gently and slowly, without force.

Experiment Results and Analysis

The ABTS substrate will change color to dark green in wells containing a positive result on the ELISA.

Students should first confirm that the results from the control samples are correct. The wells in the first row (-CTRL) should have no color change, while wells in the second row (+CTRL) should be dark green.

The patient samples should identify Patient 2 as positive for HIV, with all 3 wells showing a dark green color similar to the positive control samples.



Answers to Study Questions

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?**

   Adsorbed antigen is bound by an antibody. The bound antibody is itself bound by an antibody from another species (this is the secondary antibody). The secondary antibody is conjugated to a marker such as HRP which can produce colored products from certain substrates. If sites not occupied by antigens are not blocked, the primary and secondary antibodies will be non-specifically adsorbed as well, producing false positives. A positive control assures that the reagents and plates are working optimally. The sensitivity of the ELISA is due to the high catalytic turnover rates of the enzyme linked conjugate. One conjugate can generate millions of product molecules in a few minutes.

2. **Why is anti-HIV-1 antibody screened instead of the virus itself?**

   The HIV has a proviral phase. During the early stages of the disease very little circulating virus is present. In addition, the viral mutation rate is too high to dependably monitor.

3. **Why are there so many immunological variants of HIV?**

   The high error rate of reverse transcriptase leads to rapid mutations in the virus.

4. **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?**

   For every antigen the corresponding IgG would have to be purified and conjugated. This is too labor intensive and not cost effective. False positives can be caused by the cross reactivity of an antibody. Occasionally, two unrelated antigens will be recognized by the same antibody. Inadequate blocking of the microtiter well will also give a false positive.