Biotechnology Explorer™ ELISA Immuno Explorer™ Kit

Instruction Manual

Catalog #166-2400EDU

BIO-RAD

To the Instructor

Immunology is the study of the immune system and how the body protects itself against foreign, potentially disease-causing microorganisms and molecules. The immune system has three fundamental functions. They are:

1. To recognize intruders
2. To respond appropriately to intruders in a way that protects the body
3. To respond the next time the intruders are encountered

Mammalian immune systems produce molecules called antibodies that recognize intruder molecules with incredible specificity. Like magic bullets, antibodies locate and attach themselves to their targets. By attaching to the invading foreign entities, antibodies make the invaders recognizable to other cells of the immune system so that they can be destroyed. Antibodies have become vital high-tech tools, used in biotechnology research and disease diagnosis and treatment.

This kit facilitates teaching about the immune system, particularly about antigen-antibody interactions and the unique properties of antibodies that have revolutionized modern medicine, biotechnology, and research.

What Is ELISA?

ELISA stands for enzyme-linked immunosorbent assay. This powerful antibody-based test is used to diagnose diseases such as HIV/AIDS and SARS and to track pathogenic agents in water, food, and the air, whether these emerge naturally or through acts of aggression. ELISA is also used to identify genetically modified organisms (GMOs) and to trace food allergens and molecular markers of pregnancy and drug use.

Three approaches to ELISA may be taken with this kit (see page 2). Individual curriculum guides and instructions for each approach are included in the kit, complete with instructor's guides and student manuals. The instructor or the students may select the test protocol most relevant to the concurrent coursework.

Teaching Strategy: Guided, Inquiry-Based Investigation

The intent of this curriculum is to guide students through the thought processes of a laboratory-based scientific investigation. Students who engage in Biotechnology Explorer activities develop a positive sense of their ability to apply real research tools to relevant questions. Thought-provoking questions embedded in the student manuals are designed to maximize students' engagement in the laboratory. Student involvement in this process results in an increased understanding of the value of approaching a scientific challenge in an organized and logical fashion.

You can download this complete instruction manual on the Internet. Visit us on the Web at explorer.bio-rad.com or call us in the US at 1-800-4BIORAD (1-800-424-6723).

We strive continually to improve our curricula and products and welcome your stories, ideas, and suggestions!

Respectfully,

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Table of Contents

Kit Inventory Checklist

This section lists the components provided in this ELISA kit. It also lists required and optional accessories. Each kit contains sufficient materials to outfit 12 student workstations of up to four students per workstation. Use this checklist to inventory your supplies before beginning your advanced preparation.

Kit Components	Quantity	(✔)
Antigen, chicken gamma globulin, lyophilized	1 vial	❑
Primary antibody, rabbit anti-chicken polyclonal antibody, lyophilized	1 vial	❑
Secondary antibody, goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP), lyophilized	1 vial	❑
HRP enzyme substrate 3,3',5,5'-tetramethyl benzidine (TMB)	1 bottle	❑
10x phosphate buffered saline (PBS)	1 bottle	❑
10% Tween 20	1 bottle	❑
Disposable plastic transfer pipets	80	❑
Microplates with 12-well strips	3 plates of 8 strips x 12 wells	❑
Yellow microcentrifuge tubes, 2.0 ml	1 bag	❑
Colored microcentrifuge tubes, 2.0 ml (15 each of green, blue, orange, violet, and brown)	1 bag	❑
Required Accessories		
Micropipets (catalog #166-0515EDU), 50 µl fixed-volume or 20–200 µl adjustable volume (catalog #166-0507EDU)	12	❑
Pipet tips (catalog #223-9035EDU)	150	❑
Paper towels	4 rolls or packs	❑
Beakers, 100–200 ml	12	❑
Reagent bottles or tubes, 50 ml	3	❑
Marking pens, black	12	❑
Graduated cylinder, 100 ml	1	❑
Graduated cylinder, 1 L	1	❑
Distilled water	1 L	❑
Optional Accessories		
Microplate reader (catalog #168-1002EDU)	1	❑
Microcentrifuge tube racks (catalog #166-0481EDU)	12	❑

Refills Available Separately

Catalog #	Description
166-2401EDU	**ELISA kit reagent refill package**, includes antigen, primary antibody, secondary antibody, 10x phosphate buffered saline, 10% Tween 20, and HRP enzyme substrate
166-2402EDU	**HRP enzyme substrate**
166-2403EDU	**Phosphate buffered saline, 10x**, 100 ml
161-0780EDU	**Phosphate buffered saline, 10x**, 1 L
166-2404EDU	**Tween 20, 10%**, 5 ml
161-0781EDU	**Tween 20, 10%**, 1 L
166-2405EDU	**Microplates** with 12-well strips, 3 plates of 8 strips x 12 wells
166-2406EDU	**Antigen**, chicken gamma globulin, lyophilized
166-2407EDU	**Primary antibody**, rabbit anti-chicken polyclonal antibody, lyophilized
166-2408EDU	**Secondary antibody**, goat anti-rabbit antibody conjugated to HRP, lyophilized

166-0474EDU	**Disposable plastic transfer pipets,** sterile, 500
166-0480EDU	**Disposable plastic transfer pipets,** nonsterile, 500
166-0473EDU	**Colored 1.5 ml microcentrifuge tubes,** 6 colors, 600
223-9480EDU	**EZ Micro™ test tubes, 1.5 ml,** natural, 500
223-9430EDU	**EZ Micro test tubes, 2.0 ml,** natural, 500
166-0481EDU	**Green racks**, set of 5 racks

How This Kit Works

This kit gives you the flexibility to perform three different ELISA-based protocols with your students. The ELISA tests for the presence of antigen (protocols I and II) or antibody (protocol III) in unknown samples. The positive control is either antigen or antibody, depending on the type of test performed.

Type of ELISA	Real-World Application	Positive Control
Protocol I		
ELISA for Tracking Disease Outbreaks Step 1: Classroom exchange of simulated bodily fluids Step 2: Antigen detection ELISA protocol Step 3: Track disease transmission	HIV, SARS and West Nile viruses, common cold, cholera, smallpox, anthrax, influenza, and STDs	Antigen
Protocol II		
Antigen Detection ELISA ELISA for detecting specific antigens in a sample	Pregnancy, drug, GMO, and allergen tests Air, food, and water testing HIV, smallpox, West Nile and SARS viruses	Antigen
Protocol III		
ELISA Antibody Test ELISA for diagnosing exposure to a disease by testing for the presence of antibodies to the disease in a simulated blood sample	HIV, Lyme disease, trichinosis, West Nile virus, and SARS	Antibody

Protocol I: ELISA for Tracking Disease Outbreaks

Fully engage your students before performing the antigen detection ELISA or ELISA antibody test. With this interactive approach, students track the spread of a simulated disease agent through their classroom, exemplifying the spread of a contagious disease through a population. Beginning with one or two randomly "infected" individuals, students exchange simulated "body fluids" in vitro. Then they perform an ELISA on the resulting shared samples and use their results to track the progress of the disease through the class. This activity is designed to generate additional discussion about health issues and epidemiology. Topical diseases that work well with the tracking protocol include SARS, West Nile virus, HIV/AIDS, colds, smallpox, anthrax, influenza, and sexually transmitted diseases (STDs). As the instructor, you can determine the context most appropriate for your lesson and your students.

Protocol II: Antigen Detection ELISA

Test for the presence of an antigen in a simulated sample. This protocol can be used to discuss how an ELISA can detect disease agents in samples such as body fluids (before the body has had a chance to mount a detectable immune response). Smallpox is one great real-world application of this protocol. If detected and treated with vaccine within 2–3 days of exposure, infected patients do not develop smallpox. Alternatively, the protocol can be used to discuss how ELISA detects for pregnancy, drugs, air or water contamination, or the presence of pathogens or genetically modified organisms (GMOs) in food.

Protocol III: ELISA Antibody Test

Test for the presence of antibodies to specific disease antigens in a sample of simulated patient serum. This type of ELISA is used to detect and diagnose infection when the antigen is undetectable or after the body has mounted an immune response and antibodies are present in the blood serum. AIDS/HIV testing is a classic real world application. Until recently, testing for exposure to the HIV virus was limited to ELISA detection of antibodies to the virus, rather than direct detection of the virus itself. The body produces antibodies at a high enough level to detect by ELISA, far earlier than the HIV virus replicates to detectable levels.

How Do You Choose a Protocol?

We provide a separate manual for each protocol, with a complete instructor's guide and student manual for each. The instructor or the students may select a diagnostic test of most relevance to the coursework at hand. The three activities reflect increasing depth of knowledge of the subject matter from Protocol I, which gives a broad introductory approach to immunology and health issues, to Protocol III, which gives an opportunity to delve deeply into the biological mechanisms of the immune system. Please note that in order to provide separate manuals for each protocol, some information is repeated throughout the manuals.

All three protocols are variations on the same theme, but each approach places real-world applications of ELISA in a different context. To help create relevant and meaningful classroom contexts for these activities, the introductory pages to this manual (pages 4–9) as well as appendices A and B provide background vocabulary and factual and conceptual lecture points. Appendix C (pages 96–109) contains useful information about specific diseases and classroom presentation scenarios.

Once you have chosen, read pages 4–9 and then go to the relevant manual for the protocol you have chosen:

Important Note for Instructor's Advance Laboratory Preparation

Before beginning advance preparation for the activity, select the protocol to be used and a scenario. Please ensure that the correct advance preparation is followed for the protocol you wish to perform. The Instructor's Advance Laboratory Preparation varies subtly for each of the three protocols.

Safety Issues

Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Wearing protective eyewear and gloves is strongly recommended. Students should wash their hands with soap before and after this exercise. If any of the solutions gets into a student's eyes, flush with water for 15 minutes.

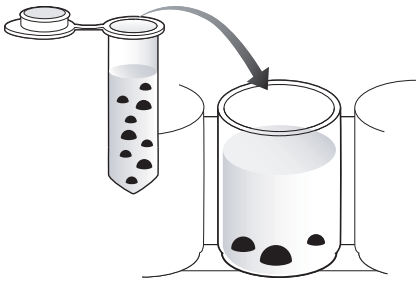
Storage Temperatures

Open the kit immediately upon receipt and store components at 4°C or room temperature as indicated.

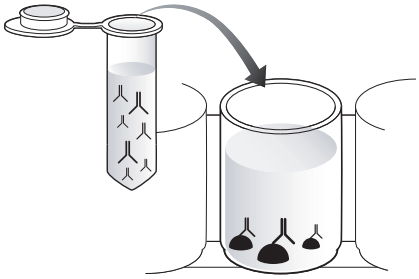
Step-by-Step Description of ELISA

The protocols in this kit rely on indirect antibody capture ELISA. The steps in this assay are:

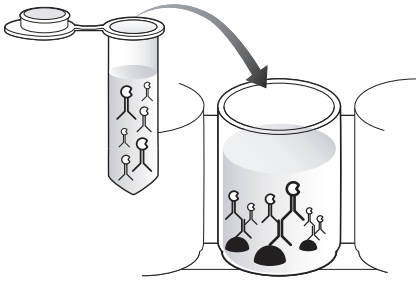
Step 1: Antigen is added to the wells of the microplatestrip and incubated to allow binding, after which unbound antigen is washed from the wells with detergent. The detergent also serves as a blocking agent, binding to all unused protein binding sites in the wells and preventing nonspecific binding of antibody.



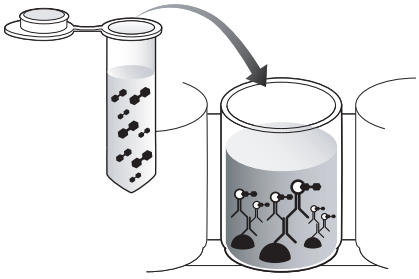
Step 2: Primary antibody solution is added to the wells and incubated to allow the antibody to bind to the antigen. Then unbound primary antibody is washed from the wells.

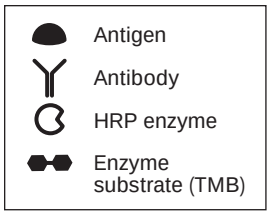


Step 3: Enzyme-labeled secondary antibody solution is added to the wells and incubated to allow the secondary antibody to bind to the primary antibody. Then unbound secondary antibody is washed from the wells.



Step 4: Chromogenic (color-producing) enzyme substrate is added to the wells and incubated to allow color to develop. Results of the assay are evaluated. Wells that remain colorless are negative and wells that turn blue are positive.



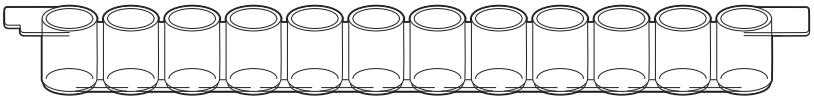


General Introduction to This ELISA Kit

To create a relevant and meaningful classroom context for this activity, the in-depth information in Appendices A and B provides background vocabulary and factual and conceptual lecture points. In addition, useful reading and web sites are included in Appendix E. Of course, there is no substitute for a good textbook and the knowledge and expertise of the instructor.

The following section briefly describes the technical and conceptual points that are directly related to the laboratory activities in this curriculum. Student understanding of these points is extremely important to a successful outcome.

Microplate strips: Microplates are made of polystyrene which adsorbs (binds) proteins by hydrophobic interaction. The plates provided in this kit have 96 wells, arranged in 8 removeable rows of 12-well strips. Two students share one strip. Each well holds approximately 250 microliters (µl).



Antigen: In this kit, the antigen is chicken gamma-globulin (purified from egg yolks) which serves as a generic representative of any hypothetical antigen, protein or otherwise.

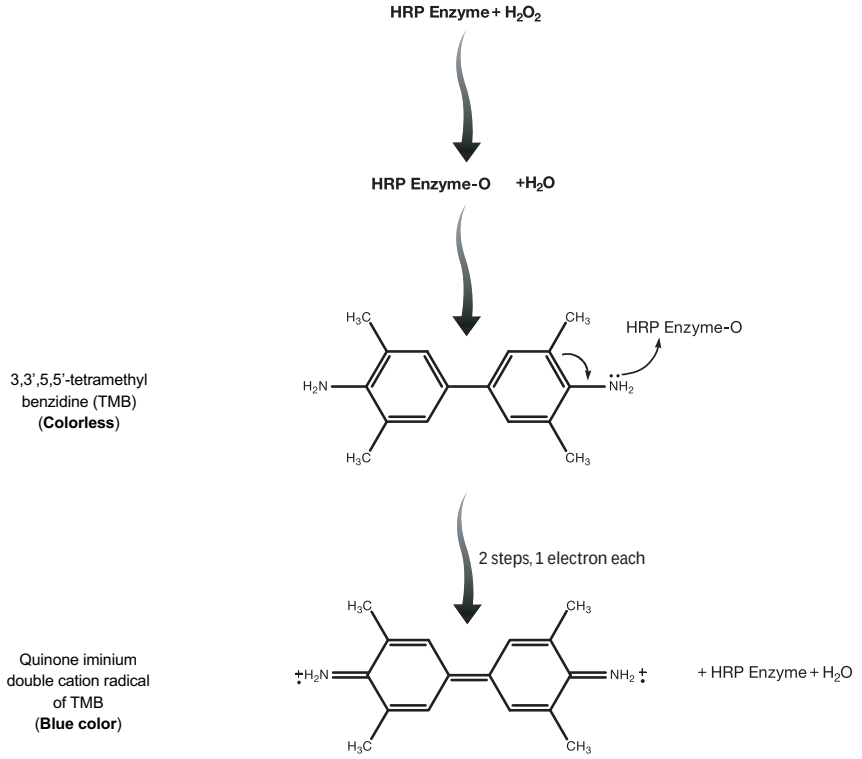
Incubation times: The rate of binding depends on the incubation temperature and the concentrations of the reagents. This kit has been optimized so that each incubation can be performed for 5 minutes at room temperature. Exceeding this time or temperature will cause an increase in color intensity and possibly some background color in the negative controls.

Blocking: Blocking agents are added after antigen adsorption to prevent nonspecific binding of antibodies to the plastic, which would produce false positive results. The blocking agent may be a protein or a detergent (or both). Common blocking agents include Tween 20 (a nonionic detergent that is used in this kit), nonfat dry milk, gelatin, and bovine serum albumin (BSA). Although Tween 20 is a sufficient block for this protocol, you may wish to add the following blocking step for teaching purposes: have the students add 50 µl of 1% gelatin in wash buffer to their wells for 15 min after the addition of the antigen and then perform a wash step.

Primary (1°) antibodies: The antibodies that recognize and bind to the antigen in an immunoassay are primary antibodies. In this kit, the primary antibody is a polyclonal rabbit antibody raised against chicken gamma-globulin. In the ELISA antibody test starting on page 55, this primary antibody simulates human antibodies in a sample of human serum.

Secondary (2°) antibodies: Secondary antibodies recognize and bind to primary antibodies. They are made in animals of a different species than that used to make the primary antibody. For this kit, goats were immunized with rabbit IgG to make the secondary antibodies.

Colorimetric detection: Secondary antibodies for ELISA are linked to enzymes. Detection of secondary antibodies that are bound to primary antibodies occurs by an enzyme-substrate reaction. In this kit, the secondary antibody is linked to horseradish peroxidase (HRP). In the presence of hydrogen peroxide (H_2O_2), HRP catalyzes the oxidation of the chromogenic substrate 3,3',5,5'-tetramethylbenzidene (TMB). This oxidation of TMB by HRP forms a blue product. Note: TMB is light sensitive, and the assay results should be determined 5–10 minutes after the substrate is added to the wells. If the microplate strips sit longer, nonspecific color may develop. Color that develops after the 5-minute incubation should not be considered in the assay results. After 20–30 minutes, the blue color may begin to fade as TMB precipitates out of solution.



Colorimetric Detection: Oxidation of TMB by HRP

Controls: Controls are always run side by side with actual samples to make sure that the procedure is working correctly. Controls can resolve ambiguous results that occur due to human error or contaminated reagents; controls must be included in any valid ELISA. For the negative control, the antigen or primary antibody is either omitted (as in this kit) or the antigen is replaced by a factor that will not bind specifically to the antibody. The positive control always contains the target antigen or antibody. A negative sample that gives a positive assay result is called a **false positive**. A positive sample that gives a negative assay result is called a **false negative**.

Many diagnostic assays give a percentage of false positive or false negative results, so confirmation of diagnosis by a second type of assay is important. For example, immunoassays for antibodies to human immunodeficiency virus (HIV) can give either false positive or false negative results. False positives can result from recent vaccinations, and false negatives can result from immunosuppression (e.g., from drugs given after transplants) or from administering the test too soon after infection with HIV. (Antibodies against HIV do not appear until some weeks after HIV infection; the appearance of specific antibodies is called seroconversion.)

Because of this, positive HIV ELISA results are always confirmed by western blot (see page 91).

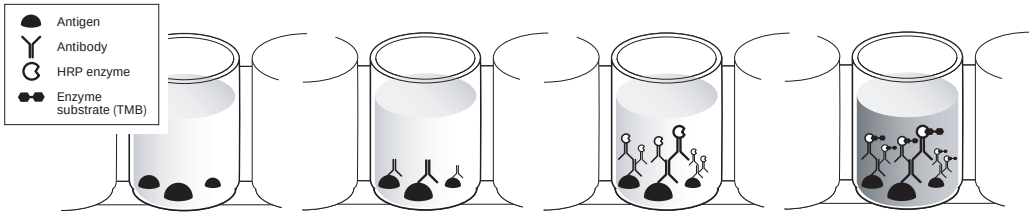
In an ELISA like those in Protocols I and II (in which antigen concentration is the experimental variable), an appropriate negative control would be wells with antigen omitted. Any color product in those wells would be the result of either 1) nonspecific binding of the antibodies, or 2) experimental error. An appropriate positive control would be a sample known to contain the antigen. In an ELISA antibody test like that in Protocol III (in which primary antibody concentration is the experimental variable), an appropriate negative control would be wells with primary antibody omitted. Any color product in those wells would be the result of either 1) nonspecific binding of the secondary antibody, or 2) experimental error. An appropriate positive control would be a sample known to contain primary antibody. For many clinical ELISAs, control solutions are provided with the commercial kits.

Analysis of Results: An ELISA can give qualitative (yes or no) or quantitative (how much?) information. Qualitative results can be determined visually without the use of complicated instrumentation. Quantitative results can be estimated visually and scored symbolically, e.g., (++) for strong signal, (+) for weak signal, (+/–) for an ambiguous signal, and (–) for no detectable signal. For accurate and precise determination of concentrations, a microplate reader is required. Microplate readers quantitate the absorbance of light by the colored substrate in each well of a microplate. They use the negative control wells to set a baseline and then read the absorbance of each well at a specified wavelength. For example, the peak absorbance for TMB is at 655 nm. Quantitative ELISA controls include a dilution series of known concentrations that is used to create a standard curve. This standard curve allows the concentration of antigen in a sample to be quantitated, which in turn may help a researcher, clinician, or physician determine the infection level of a particular disease. A lesson extension to perform a quantitative ELISA is included in Appendix D.

ELISAs are performed so routinely in both clinical and research laboratories that assays for many antigens are available in kit form. Kits normally include all components and controls needed for a given test except for the experimental samples. For example, Bio-Rad's Clinical Diagnostics Group produces over 100 kits that are used to detect autoimmune diseases, blood viruses, genetic disorders, microorganisms, toxins, and bovine spongiform encephalopathy (BSE or mad cow disease).

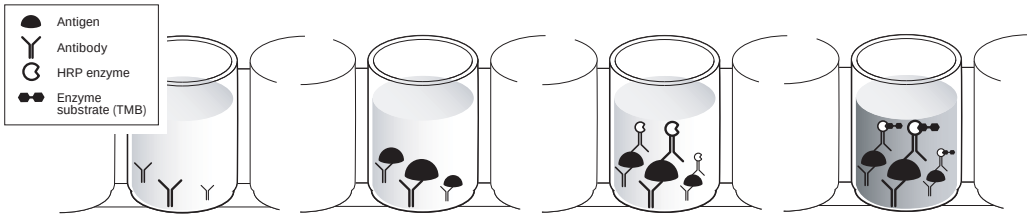
A commercial ELISA kit to test for antibodies to HIV-2, from Bio-Rad's Clinical Diagnostics Group.

The Bio-Rad ELISA Immuno Explorer kit demonstrates a method to detect the presence of specific antigens or antibodies in a variety of samples. A number of different ELISA methods have been developed that differ primarily in the sequence in which antigens and antibodies are added to the wells. In an **antibody capture** assay (as used in this kit), antigen is bound in the plastic wells and the primary antibody binds to (or is captured by) the immobilized antigen. A secondary antibody is linked to the enzyme horseradish peroxidase (HRP), which oxidizes its substrate (TMB), turning the assay solution blue.



Antibody capture ELISA.

In an **antigen capture** assay, primary antibody is bound in the plastic wells, antigen is captured by the immobilized primary antibody, and the captured antigen is detected by a secondary antibody, also linked to HRP, that turns the assay solution blue upon reaction with TMB.



Antigen capture ELISA.

Real-World Applications of ELISA

Although ELISA is a powerful diagnostic tool in human medicine, the technique is used in a variety of other fields, including veterinary medicine, food testing, and agriculture. Some examples include:

Field	Use
Human and veterinary medicine	• Diagnose a variety of diseases, such as West Nile virus (in people or animals), HIV, SARS, Lyme disease, trichinosis, tuberculosis, and many more by detecting serum antibodies
Veterinary	• Detect viruses such as feline leukemia virus (FLV) and feline immunodeficiency virus (FIV) in cats • Detect parasites such as heartworms in dogs • Diagnose thyroid problems in dogs and cats by measuring serum thyroxine (t4) concentrations • Diagnose equine encephalitis in horses by detecting arboviruses
Agriculture: crops	• Detect viruses such as potato leaf roll virus and cucumber mosaic virus in food crops • Detect mycotoxins in crops, such as aflatoxin in cereal grains and corn • Detect viruses in decorative plants, such as bean yellow mosaic virus in gladiolus • Track adulteration of non-genetically modified (non-GMO) crops with GMO products
Environmental	• Test indoor air quality, such as detecting mold toxins in buildings
Food safety and quality	• Prevent transmission of bovine spongiform encephalitis (mad cow disease, BSE) by screening for central nervous system tissues in raw meat, in processed and cooked meats, and on surfaces • Determine if food labeling is correct, e.g., by checking for cow milk proteins in goat milk products or for non-durum wheat in durum wheat products • Prevent allergic reactions by detecting ingredients that aren't listed on food content labels, e.g., detecting peanuts in products in which peanuts are not listed as an ingredient
Other	• Detect restricted or illegal drug use, e.g., performance-enhancing drugs, marijuana, methamphetamine, cocaine, etc. • Confirm pregnancy by detecting human chorionic gonadotropin (hCG) in urine

Chemical Weapons, Biological Warfare, and ELISA

We feel it is important to include a short treatment of biological warfare since many educators are finding it necessary to address this subject due to recent concerns over this phenomenon.

Biological warfare and bioterrorism have been much in the news in recent years; however, the use of biological agents to cause harm to an enemy is not a recent phenomenon. In the 6th century BC, the Assyrians poisoned the wells of their enemies with rye ergot, and Athenians poisoned the water supply of their enemies with skunk cabbage (a purgative). In the 18th century, there were several cases in which Native Americans were given gifts intentionally contaminated with smallpox. More recently, a Bulgarian defector was killed in London with ricin (a toxin from castor beans); the toxin was injected into his leg using an umbrella tip as he waited for a bus. In 2001 in the US, weaponized anthrax spores were sent through the mail to the news media and government offices.

During a biological attack, detection, diagnosis, and identification of the biological agent and its related disease(s) are vital to disease containment. Diagnostic tests are needed to identify the agent and to determine who has been infected so that those exposed can undergo treatment and/or be quarantined. For example, if smallpox infection is detected within 2–3 days of exposure, post-exposure vaccination protects against the disease. Vaccination within 4–5 days of exposure may prevent a fatal outcome. However, the smallpox vaccine itself is associated with risks, so the question arises as to whether only infected individuals should be treated.

The CDC prioritizes biological agents based on their danger, primarily their ease of dissemination/transmission:

- The highest priority agents (category A) are those that are easily transmitted, have high mortality rates, and may cause public panic. Examples of high priority agents include anthrax (*Bacillus anthracis*), botulism (*Clostridium botulinum* toxin), plague (*Yersinia pestis*), smallpox (*Variola major*), tularemia (*Francisella tularensis*), and viral hemorrhagic fevers [filoviruses (e.g., Ebola and Marburg) and arenaviruses (e.g., Lassa and Machupo)].
- The second priority agents (category B) are those that are somewhat easy to transmit and have lower mortality rates, such as brucellosis (*Brucella* species), epsilon toxin of *Clostridium perfringens*, food safety threats (e.g., *Salmonella* species, *Escherichia coli* O157:H7, *Shigella*), glanders (*Burkholderia mallei*), melioidosis (*Burkholderia pseudomallei*), psittacosis (*Chlamydia psittaci*), Q fever (*Coxiella burnetii*), ricin toxin from castor beans (*Ricinus communis*), staphylococcal enterotoxin B, typhus fever (*Rickettsia prowazekii*), viral encephalitis [alphaviruses (e.g., Venezuelan equine encephalitis, eastern equine encephalitis, and western equine encephalitis)], and water safety threats (e.g., *Vibrio cholerae* and *Cryptosporidium parvum*).
- Lower priority agents (category C) are emerging pathogens that may become a threat in the future, such as Nipah virus and hantavirus.

The diagnosis and identification of biological agents are important parts of the response to biological attack. Also important are plans for prevention of such attacks and preparedness in case they occur, including using intelligence sources to prevent bioterrorism attacks, formulating emergency plans, establishing surveillance methods to detect attacks, training medical and law enforcement professionals, preparing vaccines and treatments, inoculating populations as needed, and educating the public.

Protocol I: ELISA for Tracking Disease Outbreaks

Protocol I: ELISA for Tracking Disease Outbreaks

Instructor's Guide

This interactive procedure provides a context for introducing a real-world, topical application of ELISA. Students first model the spread of disease in a population by the sharing of simulated "body fluids". Each student is given a sample to share, one or two of which are positive for the "disease agent". After students share their "body fluids", they assay their shared samples using ELISA.

Students' ELISA results reveal that a large portion of the class now tests positive for the disease! This leads to a guided, inquiry-based activity about how the disease has spread through the population. Because students have a personal connection to the results, this activity tends to captivate students' imaginations and is particularly relevant in light of the recently emerged contagious disease SARS.

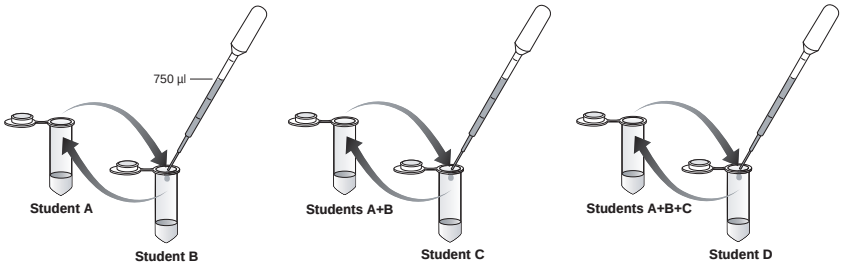
Many other diseases also work with this protocol, including West Nile virus, HIV, colds, influenza, and STDs, to name a few. A review of Appendix C will provide useful information about appropriate diseases and scenarios for implementation in the classroom. A simple approach may be to leave the actual disease unspecified for a generic lesson.

Implementation Timeline

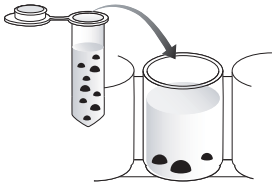
Lesson 1	Set the stage	Lecture and discussion
Lesson 2	Sharing of simulated body fluids	ELISA lab
Lesson 3	Analyze ELISA results	Tracking exercise

Instructor's Laboratory Overview

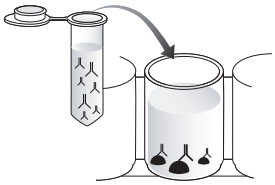
Step 1: Students share "body fluids" by mixing their sample with those of other students. Within each pair of students who share, each student takes back half of the combined sample. Each student repeats the sharing process with a different student either 1 or 2 more times (depending on class size) and records sharing partners. Note: To ensure dissemination of the "disease", the sharing must be performed in two or three separate rounds.



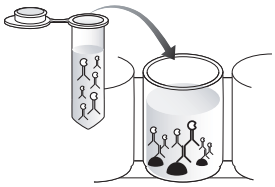
Step 2: Using a pipet, 50 µl of each student's sample (unknowns) along with positive and negative controls are added to the wells of the microplate strip and incubated for 5 minutes, allowing proteins in the sample to bind to the wells. The wells are rinsed with wash buffer (PBST: phosphate buffered saline containing 0.05% Tween 20) that also blocks the unoccupied protein binding sites in the wells.



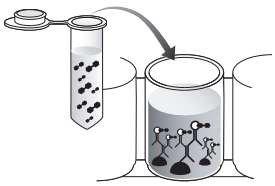
Step 3: Primary antibody (50 µl) is added to each well of the microplate strip and incubated for 5 minutes at room temperature. The primary antibody is an antibody that recognizes and binds to the "disease agent"/antigen. The wells are rinsed with wash buffer to remove unbound antibody.

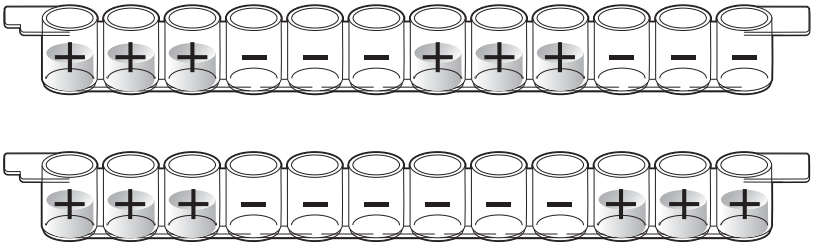


Step 4: Horseradish peroxidase (HRP)-labeled secondary antibody (50 µl) is added to each well and incubated for 5 minutes at room temperature. The secondary antibody is antibody that recognizes and binds to the primary antibody. HRP is an enzyme that will oxidize a color-producing substrate. Wells are rinsed with wash buffer to remove unbound secondary antibody.



Step 5: The enzyme substrate (50 µl) is added to each well and students watch color development. If HRP is present (meaning that the antigen was present in the sample), the solution in the wells will turn blue within 5 minutes. If the antigen was not present in the sample, the wells will remain colorless.





Typical ELISA results.

Using Students' ELISA Results to Track the "Disease"

The number of positive tests in the class results will depend on how many positive samples you released at the beginning. You can now track the progress of the disease through your class.

You may want the students to work out a method to track the disease to its source by themselves. For a more guided inquiry approach, we recommend using the class results table on page 32 in the student manual. Make a transparency from the page with the students' names listed and place it on an overhead projector. Ask each student to come up and write a plus (+) or a minus (-) in the second column to indicate if their ELISA tested positive or negative. Then, depending on whether their ELISA tested positive or negative, they should also write a plus (+) or a minus (-) respectively next to the names of the students with whom they shared their sample.

For example, if Kiko tested positive and shared with Alexander, Florence, and Mustafa, she would write a "+" by Alexander, Florence's, and Mustafa's names.

The students with all pluses against their names will be revealed as early sources of the infection.

Question: Why will the class not be able to track the infection to a single student?

Answer: When a single student who is the primary source of infection first shares his or her sample with a second student, the second student will also have all pluses. This is representative of the kind of problem that epidemiologists face in the real world. You may turn this occurrence to your advantage by discussing why epidemiologists investigate many factors when tracking diseases, such as patients' locations, histories, and behaviors, in addition to testing for the infection. You may also have your students perform a more detailed analysis involving tracking the order in which the samples were shared and deducing if some of the students can be eliminated from the pool of students suspected as being the original source.

Epidemiologists rarely have patient samples prior to the outbreak of infection, and rarely are they able to track an outbreak to a single source. However, you have the advantage of keeping a record of which students received the infected samples, which for the sake of this exercise may prove helpful. Alternatively, for a more anonymous approach, you may sequentially number all the student samples and record the numbers of tubes that are "infected". The source can be revealed at the end of the activity to see if it matches your students' data analysis.

Instructor's Advance Laboratory Preparation

This section is designed to help you prepare for the laboratory efficiently. We recommend that you read this section of the manual (Protocol I: Tracking Disease Outbreaks) in its entirety before beginning your preparation. In addition, if you are choosing to perform a scenario-based activity (for example, HIV testing), we recommend using the information given in Appendix C to help plan your lesson.

The most important thing for the students to do is to put the correct components in the assay wells in the correct order, so having the tubes clearly labeled and properly color-coded is crucial to a successful outcome.

Objectives

Step 1. Prepare buffers

Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody to make 50x stocks

Step 3. Dilute 50x stock solutions

Step 4. Dispense reagents for student workstations

Step 5. Set out student workstations

Time Required 1–3 hours

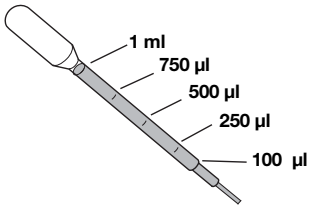
Preparation Timeframe

We recommend rehydrating and diluting the antigen and primary antibody no more than 3 days before the lesson, and the secondary antibody less than 24 hours before the lesson. We also suggest using sterile distilled water to prepare the 1x PBS to avoid contaminating rehydrated reagents. These reagents must be kept on ice or in the refrigerator if prepared more than 4 hours before the lesson.

Note: If you are planning to use this kit for multiple lab sessions over a 1- or 2-week period, we strongly suggest using sterile water to prepare the PBS buffer in order to avoid contaminating reagents. (Water can be sterilized by boiling it in a microwave oven for 5 minutes in a loosely capped bottle; after you remove the bottle from the microwave oven, let it cool, then secure the cap.) Dilute only as much concentrated antibody and antigen as required for each lab session. The rehydrated antibodies are 50x concentrates. Store the remaining concentrated antigen and antibodies in the refrigerator at 4°C. We do not recommend storing the concentrated antibody and antigen for more than 2 weeks, even at 4°C. Do not freeze the solutions.

Volume Measurements

This kit contains graduated disposable plastic transfer pipets (DPTPs) to use for preparing some of the reagents where volumes between 250 microliters (µl) and 5 milliliters (ml) are required. In addition, adjustable- or fixed-volume micropipets are needed to measure 50 µl volumes. The illustration shows the marks on the DPTP corresponding to the volumes to be measured. Volumes over 1 ml will require multiple additions. For each step of the laboratory preparation, use a fresh DPTP or a fresh pipet tip.



Measuring liquids that contain detergents that foam (e.g., the wash buffer) requires that you read the volume at the interface of the liquid and the bubbles.

PROTOCOL I: Step-by-Step Instructor's Advance Preparation Guide

These instructions are for the setup of 12 student workstations of 4 students each.

Supplied Reagents	Quantity
Antigen, chicken gamma globulin, freeze-dried	1 vial
Primary antibody, rabbit anti-chicken polyclonal antibody, freeze-dried	1 vial
Secondary antibody, goat anti-rabbit antibody conjugated to (HRP), freeze-dried	1 vial
HRP enzyme substrate (TMB)	1 bottle
10x phosphate buffered saline (PBS)	1 bottle
10% Tween 20	1 bottle

Required Reagent	
Distilled water, sterile is recommended, see note on page 15	1 L

Step 1. Prepare buffers.

We recommend you use a 100 ml and a 1 liter (L) graduated cylinder for preparing the buffer solutions. You will also need 1 L of distilled water.

Buffer	Volume	Reagent	Used for
1x PBS, 100 ml	90 ml	Distilled water	• Rehydrating antigen, Primary and secondary antibodies to make 50x reagent stock solutions • Diluting 50x antigen to make positive control and "infected" student samples • Negative control • Negative student samples
	10 ml	10x PBS	
Wash Buffer, 900 ml	805 ml	Distilled water	• Dilution of 50x antibody stocks • Plate washing
	90 ml	10x PBS	
	4.5 ml	10% Tween 20	

Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody.

Carefully remove the stoppers from the three freeze-dried reagents and use a fresh DPTP to add 0.5 ml 1x PBS to each. Close the stoppers and shake to mix. These solutions are 50x concentrates, or stock solutions. **NOTE: You must <u>not</u> use wash buffer in this step**.

Freeze-Dried Reagent	Protocol for 50x Stock Solution	Used for
Antigen	Add 0.5 ml of 1x PBS to vial	• Positive control • "Infected" student samples
Primary antibody	Add 0.5 ml of 1x PBS to vial	• Primary antibody
Secondary antibody	Add 0.5 ml of 1x PBS to vial	• Secondary antibody

Step 3. Dilute 50x stock reagents.

Label one 50 ml bottle or tube for each of the diluted solutions below. Use a fresh DPTP to add the contents of the appropriate 50x concentrated stock to the corresponding 50 ml bottle or tube.

Diluted solution	Volume	Reagent	Used for
Positive control (1x antigen), label one 50 ml bottle or tube	7.5 ml	1x PBS	Positive control
	150 µl	50x antigen stock	
1x primary antibody, label one 50 ml bottle or tube	**NOTE: you must not add any buffer containing Tween 20 to the antigen, or the experiment will not work.**		
	24.5 ml	Wash buffer	Primary antibody
	0.5 ml	50x primary antibody stock	
	• Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used.		
1x secondary antibody, label one 50 ml bottle or tube	• Close the cap and shake to mix.		
	24.5 ml	Wash buffer	Secondary antibody
	0.5 ml	50x secondary antibody stock	
	• Dilute the secondary antibody less than 24 hours before the start of the lesson. Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		

Step 4. Dispense reagents for student workstations.

Tubes	Description	Label	Contents (Each Tube)
Violet tubes, 12	Positive control	"+"	0.5 ml positive control solution (1x antigen)
Blue tubes, 12	Negative control	"–"	0.5 ml 1x PBS
Green tubes, 12	Primary antibody	"PA"	1.5 ml 1x primary antibody solution
Orange tubes, 12	Secondary antibody	"SA"	1.5 ml 1x secondary antibody solution
Brown tubes, 12	Enzyme substrate	"SUB"	1.5 ml HRP enzyme substrate (TMB)
	Note: TMB is light sensitive, so it is important to use the dark tubes to store this reagent.		
Yellow tubes, # depends on # of students (1–3 tubes)	"Infected" student sample(s) (6.6x antigen)	Determined by instructor	100 µl 50x antigen stock solution 650 µl 1x PBS
	The number of students in the class will determine the number of "infected" samples you mix with the blanks. For a result where about half the students become infected, we recommend making one infected sample per 16 students. (Note: if your class is less than 10 students, use a single sample and perform just 2 rounds of sharing.) **Note: You must not add any buffer containing Tween 20 to the antigen, or the experiment will not work**. You may want to keep the infected samples separate from the blank samples until the lesson to keep track of who receives them.		

Tubes	Description	Label	Contents (Each Tube)
Yellow tubes, # depends on # of students	Blank (uninfected) student samples	Determined by instructor*	750 µl 1x PBS
	Make enough blank yellow tubes for your student number minus the "infected" samples. **Note: You must use 1x PBS and not wash buffer for the blank students samples, or the experiment will not work.**		
* (Optional) For your own information, you may wish to number each tube of student sample (infected and blank) from 1 to 48 and record which numbered tubes contain the infected samples.			

Step 5. Set out student workstations.

Student Workstation Checklist

One workstation serves 4 students.

Item (Label)	Contents	Number	(✔)
Yellow tubes	Student test samples (0.75 ml)	4 (1 per student)	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipets		5	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

Note on sharing protocol: Make sure that the students share “body fluids” with students from other parts of the room, not just with their near neighbors. The best way to do this is to have orderly sharing: Tell the students to share with one other person, then return to their lab station. After all students are finished with the first sharing and are back in place, then tell them to share with a different person. The degree of sharing will depend on your class size. We recommend one "infected" sample per 16 students. If your class size is less than 10, use a single "infected" sample and perform just two rounds of sharing.

Stopping points: Although this procedure is designed to fit into a single lesson period, you may stop the laboratory activity after sharing the “body fluids” and place all the reagents in the refrigerator at 4°C overnight. Alternatively, if you wish to stop during the ELISA you may add wash buffer to the microplate wells at any stage after the addition of antigen and prior to the addition of enzyme substrate. Place the microplate strips and all the reagents in the refrigerator at 4°C overnight.

Setting Up The Activity to Test for a Specific Disease (e.g., HIV)

Appendix C provides information on a variety of diseases that can be diagnosed using ELISA. In addition, for each disease, we provide a table describing what the reagents for the activity represent in a real-world diagnostic ELISA. Below is an example of a diagnostic test to detect HIV viral proteins in a patient's blood sample.

Detecting p24 HIV-1 Capsid Protein.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Student samples (unknowns)	Yellow	1x antigen or	Sample derived from patient's blood 1x PBS
Primary antibody	Green	1x primary antibody	Anti-p24 capsid protein antibody from mouse
Secondary antibody	Orange	1x secondary antibody	Anti-mouse immunoglobulin antibody conjugated to HRP
Positive control	Violet	1x antigen	Heat-inactivated viral antigen (p24 protein)
Negative control	Blue	1x PBS	HIV negative human serum

Instructor's Answer Key and Discussion Points

Pre-Lab Focus Questions

1. **How does the immune system protect us from disease?**

   The immune system includes physical barriers, such as the skin and mucous membranes that prevent pathogens from entering the body, and cellular responses, such as circulating macrophages that respond to foreign invaders. Our acquired immune system mounts a specific antibody response when the body is exposed to a foreign invader, and our immune cells attack the invader.

2. **How do doctors use the immune response to protect you from disease?**

   Doctors use the immune response when we are vaccinated against diseases. Our immune system remembers the pathogens to which we have been exposed, and the next time we are exposed to the pathogens our immune system attacks them more quickly and efficiently. Doctors take advantage of this priming effect by exposing us to inactivated pathogens (killed or weakened organisms that cannot make us sick) so that if we are later exposed to the live pathogen, our body will mount a strong and immediate antibody response, reducing or eliminating the chance that it will make us sick.

3. **What are some ways that diseases spread?**

Diseases Can Spread Through:	Examples:
Exchange of bodily fluids	• HIV, SARS, Epstein-Barr virus (cause of mononucleosis), STDs
Ingestion of contaminated food or water	• *E. coli* O157:H7, prions that cause Creutzfeldt-Jakob and mad cow diseases, protozoa that cause giardiasis, nematodes that cause trichinosis
Inhalation	• Viruses that cause the flu, bacteria that cause tuberculosis
Vector transfer	• Mosquito-borne diseases (malaria, West Nile virus, dengue fever, yellow fever), tick-borne diseases (Lyme disease, Rocky Mountain spotted fever)

4. **What is an example of a disease that attacks the human immune system?**

   Diseases that attack the immune system include autoimmune diseases (e.g., rheumatoid arthritis, lupus, asthma, eczema, SCID) and AIDS. An extensive list can be found in Appendix A.

5. **What problems can prevent the immune system from working properly?**

   Problems with the immune system fall into three categories: hypersensitivity, immunodeficiency, and autoimmune diseases. Hypersensitivity occurs when the immune system overreacts to an antigen; hypersensitivity reactions include anaphylactic reactions, allergies, and contact sensitivity (e.g., reaction to poison ivy). Immunodeficiency means that an individual cannot mount an effective immune response. Immunodeficiency may be genetic (e.g., SCID or "bubble boy" disease) or induced by a disease (e.g., immunodeficiency from HIV infection) or by immunosuppressive drugs (e.g., drugs given after organ transplant to prevent rejection). Autoimmune disease results from the immune system inappropriately mounting an immune response to

itself, for example, diseases like lupus, rheumatoid arthritis, multiple sclerosis (MS), insulin-dependent diabetes, and celiac disease.

6. **Why are immunosuppressant drugs necessary when someone has an organ transplant?**

   Immunosuppressive drugs (like prednisolone and cyclosporine) prevent the body from treating the transplanted organ as a foreign invader; availability of immunosuppressive drugs is largely responsible for the success of transplantation as a treatment for organ failure. Organs are rejected when the body mounts a strong immune response to the transplant. On the negative side, the action of immunosuppressive drugs is not specific and they suppress all immunological reactions. As a result, transplant recipients are very vulnerable to infections.

7. **Why is rapid detection of disease exposure important?**

   Rapid detection of disease exposure is important for several reasons. For many diseases, detecting the infection and beginning treatment early may reduce the severity of the symptoms or even prevent the disease completely. Rapid detection of disease exposure is also important to prevent further spread of the disease.

8. **What does ELISA stand for?**

   Enzyme-linked immunosorbent assay.

9. **Why are enzymes used in this immunoassay?**

   Enzymes provide a way to see whether the primary antibody has attached to its target (antigen) in the microplate well. Primary and secondary antibodies are invisible, so a detection method is necessary. The enzyme HRP is linked to the secondary antibody. HRP reacts with a colorless substrate in a chemical reaction that turns blue. If the secondary antibody is present in the well, the color change indicates a positive result.

10. **Why do you need to assay positive and negative control samples as well as your experimental samples?**

    Controls are needed to make sure that the experiment worked. If there are no positive controls and the sample is negative, we can't know if the sample was truly negative or if the assay didn't work. Conversely, without a negative control, there is no way of knowing if all samples (positive or not) would have given a positive result.

Post-Lab Focus Questions

1. **The samples that you added to the microplate strip contain many proteins and may or may not contain the disease antigen. What happened to the proteins in the plastic well if the sample contained the antigen? What if it did not contain the antigen?**

   In either case, all the proteins present in the sample bind to the plastic wells.

2. **Why did you need to wash the wells after each step?**

   Washing removes any proteins that have not bound to the plastic wells and any antibodies that have not bound to their targets, thus preventing unbound proteins (either antigen or antibodies) from giving false positive results.

3. **When you added primary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?**

   If the sample contained the antigen, the primary antibody bound the antigen. If it did not contain the antigen, the primary antibody did not bind and was flushed out in the wash step.

4. **When you added secondary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?**

   If the sample contained antigen, the secondary antibody bound to the primary antibodies already bound to antigen in the wells. If the test sample did not contain antigen, primary antibody did not bind in the wells, so the secondary antibody had nothing to bind and was flushed out in the wash step.

5. **If the sample gave a negative result for the disease-causing agent, does this mean that you do not have the disease? What reasons could there be for a negative result when you actually do have the disease?**

   A negative result does not necessarily mean that you do not have the disease. It could be a false negative. The ELISA may not be sensitive enough to detect very low levels of disease agent, such as the levels that might be present soon after infection. Another cause of false negatives is experimental error, such as putting a negative control into a well where you thought you were putting an experimental sample.

6. **Why did you assay your samples in triplicate?**

   Assaying the samples in triplicate is another control. If you do not get the same result in all triplicate wells, you have a problem with your experimental technique or you have made a pipetting error. In a clinical laboratory, the experiment would have to be repeated. If this error occurs in this activity, take the result of the two matching wells since this is probably correct.

7. **What antibody-based tests can you buy at your local pharmacy?**

   Test kits that are based on the same principles as the ELISA include home pregnancy and ovulation tests, and tests for the presence of illegal drugs such as marijuana and cocaine.

8. **If you tested positive for disease exposure, did you have direct contact with one of the original infected students? If not, what conclusions can you reach about transmissibility of disease in a population?**

   Having intimate contact with another person means that you are exposed to any germs that a person may have contracted from any previous intimate contacts.

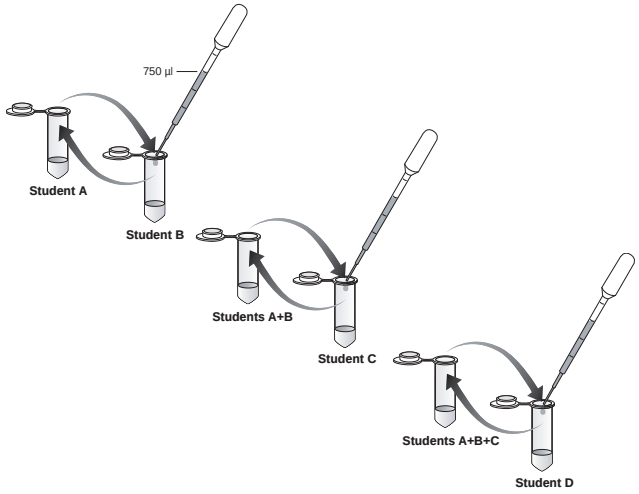
Laboratory Quick Guide

Student Workstation Checklist

One workstation serves 4 students.

Item (Label)	Contents	Number	(✔)
Yellow tubes	Student test samples (0.75 ml)	4 (1 per student)	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipets		5	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

ELISA for Tracking Disease Outbreaks



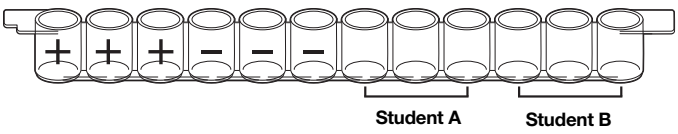
1. Label a yellow tube and a plastic transfer pipet with your initials.
2. Use the pipet to transfer all your "bodily fluid" sample into the tube of another student. Gently mix the samples, then take back half of the shared sample (750 µl) to your own tube. Write down the name of the student next to "Sharing Partner #1".
3. When instructed to do so, repeat the sharing protocol two more times. Discard this transfer pipet after this step.

   Optional stopping point: Samples may be stored at 4°C overnight.
4. Label your 12-well strip. On each strip label the first 3 wells with a "+" for the positive controls and the next 3 wells with a "–" for the negative controls. Label the remaining wells with your and your lab partner's initials (3 wells each).

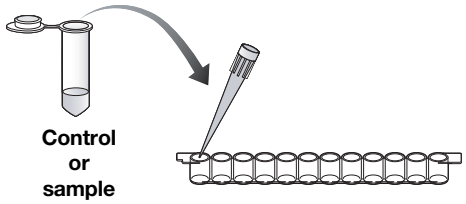
Sharing Partner #1 _____

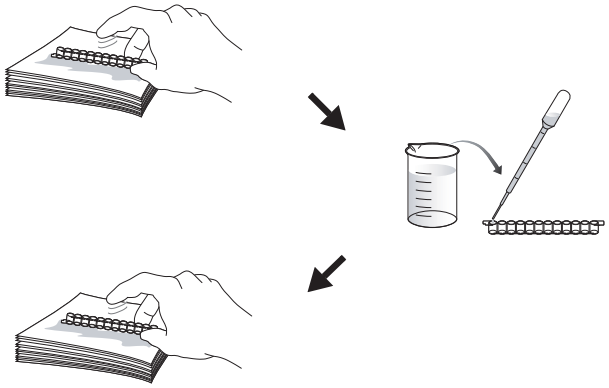
Sharing Partner #2 _____

Sharing Partner #3 _____

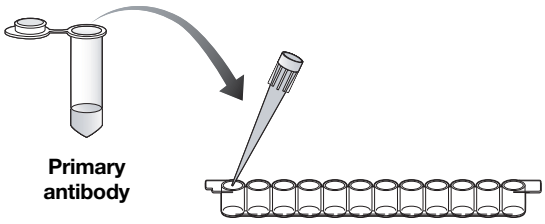


5. Use a fresh pipet tip to transfer 50 µl of the positive control (+) into the three "+" wells.
6. Use a fresh pipet tip to transfer 50 µl of the negative control (–) into the three "–" wells.
7. Transfer 50 µl of each of your team's samples from step 3 into the appropriately initialed three wells, using a fresh pipet tip for each sample.
8. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
9. WASH:
   a. Tip the microplate strip upside down onto the paper towels, and gently tap the strip a few times upside down. Make sure to avoid samples splashing back into wells.
   b. Discard the top paper towel.
   c. Use a fresh transfer pipet to fill each well with wash buffer, taking care not to spill over into wells. Note: the same transfer pipet is used for all washing steps.
   d. Tip the microplate strip upside down onto the paper towels and tap.
   e. Discard the top 2–3 paper towels.
10. Repeat wash step 9.
11. Use a fresh pipet tip to transfer 50 µl of primary antibody (PA) into all 12 wells of the microplate strip.
12. Wait 5 minutes for the antibodies to bind to their targets.
13. Wash the unbound primary antibody out of the wells by repeating all of wash step 9 **two** times.



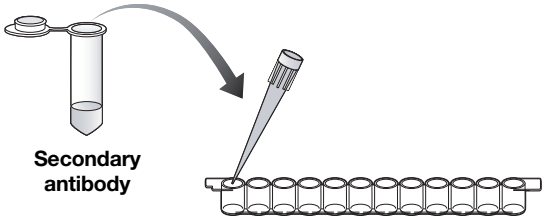


WASH



WASH 2x

14. Use a <u>fresh</u> pipet tip to transfer 50 µl of secondary antibody (SA) into all 12 wells of the microplate strip.

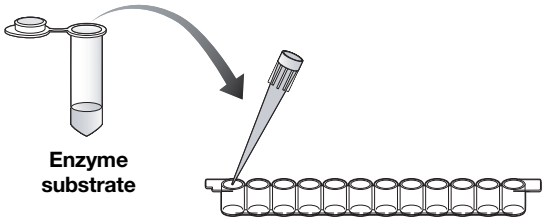


15. Wait 5 minutes for the antibodies to bind to their targets.

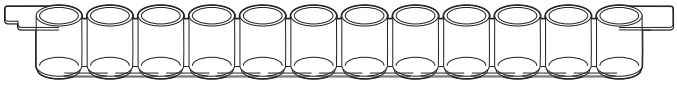
16. Wash the unbound secondary antibody out of the wells by repeating wash step 9 **three** times.

WASH 3x

17. Use a <u>fresh</u> pipet tip to transfer 50 µl of enzyme substrate (SUB) into all 12 wells of the microplate strip.



18. Wait 5 minutes. Observe and record the results.



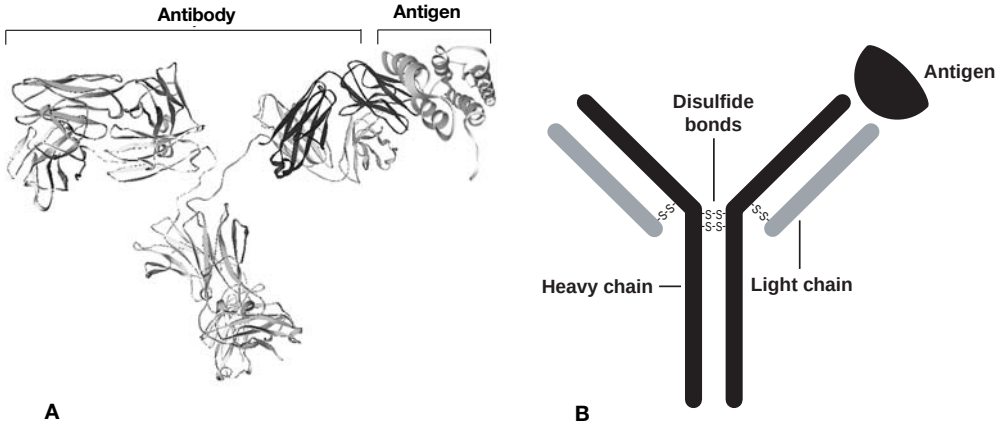
Student Manual

Introduction

You are about to perform an experiment in which you will share simulated "body fluids" with your classmates. After sharing, you will perform an enzyme-linked immunosorbent assay or ELISA to determine if you have been exposed to a contagious "disease". The ELISA uses antibodies to detect the presence of a disease agent, (for example, viruses, bacteria, or parasites) in your blood or other body fluid. You will then track the disease back to its source.

When you are exposed to a disease agent, your body mounts an immune response. Molecules that cause your body to mount an immune response are called antigens, and may include components of infectious agents like bacteria, viruses, and fungi. Within days, millions of antibodies — proteins that recognize the antigen and bind very tightly to it — are circulating in your bloodstream. Like magic bullets, antibodies seek out and attach themselves to their target antigens, flagging the invaders for destruction by other cells of the immune system.

Over 100 years ago, biologists found that animals' immune systems respond to invasion by "foreign entities", or antigens. Today, antibodies have become vital scientific tools, used in biotechnology research and to diagnose and treat disease. The number of different antibodies circulating in the blood has been estimated to be between 10^6 and 10^{11}, so there is usually an antibody ready to deal with any antigen. In fact, antibodies make up to 15% of your total blood serum protein. Antibodies are very specific; each antibody recognizes only a single antigen.



A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, (Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

How Are Antibodies Made?

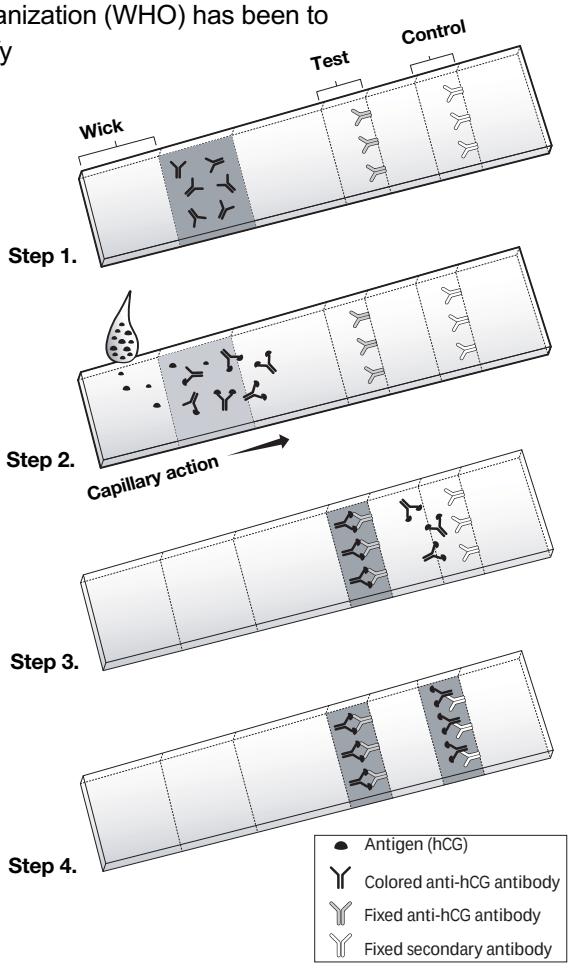
Scientists have learned to use the immune response of animals to make antibodies that can be used as tools to detect and diagnose diseases. The study of the immune system is called "immunology". Animals such as chickens, goats, rabbits, and sheep can be injected with an antigen and, after a period of time, their serum will contain antibodies that specifically recognize that antigen. If the antigen was a disease agent, the antibodies can be used to develop diagnostic tests for the disease. In an immunoassay, the antibodies used to recognize antigens like disease agents are called primary antibodies; primary antibodies confer specificity to the assay.

Other kinds of antibody tools, called secondary antibodies, are made in the same way. In an immunoassay, secondary antibodies recognize and bind to the primary antibodies, which are antibodies from another species. Secondary antibodies are prepared by injecting antibodies made in one species into another species. It turns out that antibodies from different species are different enough from each other that they will be recognized as foreign proteins and provoke an immune response. For example, to make a secondary antibody that will recognize a human primary antibody, human antibodies can be injected into an animal like a rabbit. After the rabbit mounts an immune response, the rabbit serum will contain antibodies that recognize and bind to human antibodies. The secondary antibodies used in this experiment are conjugated to the enzyme horseradish peroxidase (HRP) which produces a blue color in the presence of its substrate, TMB. These antibody and enzyme tools are the basis for the ELISA.

Where Is ELISA Used in the Real World?

With its rapid test results, the ELISA has had a major impact on many aspects of medicine and agriculture. ELISA is used for such diverse purposes as pregnancy tests, disease detection in people, animals, and plants, detecting illegal drug use, testing indoor air quality, and determining if food is labeled accurately. For new and emerging diseases like severe acute respiratory syndrome (SARS), one of the highest priorities of the US Centers for Disease Control (CDC) and the World Health Organization (WHO) has been to develop an ELISA that can quickly and easily verify whether patients have been exposed to the virus.

Some tests give positive or negative results in a matter of minutes. For example, home pregnancy dipstick tests are based on very similar principles to ELISA. They detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization. The wick area of the dipstick is coated with anti-hCG antibody labeled with a pink compound (step 1). When the strip is dipped in urine, if hCG is present it will bind to the pink antibody, and the pink hCG-antibody complex will migrate up the strip via capillary action (step 2). When the pink complex reaches the first test zone, a narrow strip containing an unlabeled fixed anti-hCG antibody, the complex will bind and concentrate there, making a pink stripe (step 3). The dipsticks have a built-in control zone containing an unlabeled fixed secondary antibody that binds unbound pink complex (present in both positive and negative results) in the second stripe (step 4). Thus, every valid test will give a second pink stripe, but only a positive pregnancy test will give two pink stripes.



Why Do We Need Controls?

Positive and negative controls are critical to any diagnostic test. Control samples are necessary to be sure your ELISA is working correctly. A positive control is a sample known to be positive for the disease agent, and a negative control is a sample that does not contain the disease agent.

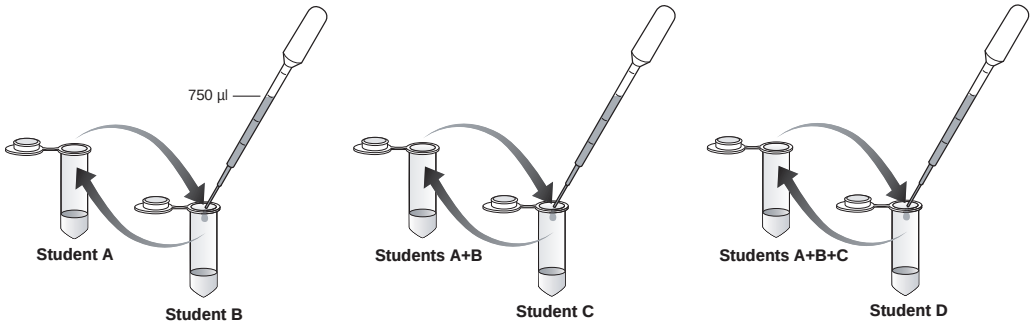
Your Task Today

You will be provided the tools and an experimental protocol to perform an ELISA. You will be given a simulated “body fluid” sample that you will share with your classmates. One or two of the samples in the class have been “infected”. You will also be provided with positive and negative control samples. Then you and your fellow students will assay your samples for the presence of the “disease agent” to track the spread of the disease through your class population.

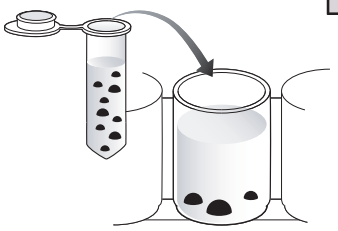
Now let’s put this all together.

Your task will be to:

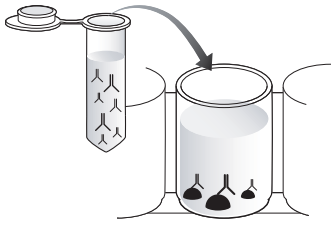
1. Share your (simulated) body fluids randomly with your classmates.



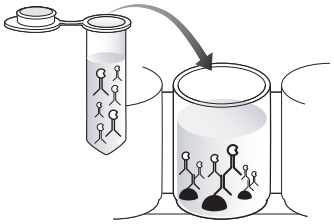
2. Add your shared sample plus control samples to the wells in a microplate strip. Your samples contain many proteins and may or may not contain the disease agent (antigen). Incubate for 5 minutes to allow all the proteins in the samples to bind to the plastic wells via hydrophobic interaction. This is an immunosorbent assay because proteins adsorb (bind) to the plastic wells.



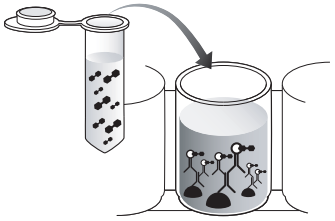
3. Add anti-disease antibody (primary antibody) to the wells and incubate. The primary antibody will seek out the antigen from the many proteins bound to the well. If your sample was "infected", the antibodies will bind tightly to the disease agent (antigen) in the wells.



4. Detect the bound antibodies with HRP-labeled secondary antibody. If the primary antibodies have bound to the antigen, the secondary antibodies will bind tightly to the primary antibodies.



5. Add enzyme substrate to the wells, wait 5 minutes, and evaluate the assay results. If the disease antigen was present in your sample, the wells will turn blue. This is a positive diagnosis. If the wells remain colorless, the disease antigen was not present in your sample and the diagnosis is negative.



Pre-Lab Focus Questions

1. How does the immune system protect us from disease?

2. How do doctors use the immune response to protect you from disease?

3. What are some ways that diseases spread?

4. What is an example of a disease that attacks the human immune system?

5. What problems can prevent the immune system from working properly?

6. Why are immunosuppressant drugs necessary when someone has an organ transplant?

7. Why is rapid detection of disease exposure important?

8. What does ELISA stand for?

9. Why are enzymes used in this immunoassay?

10. Why do you need to assay positive and negative control samples as well as your experimental samples?

Laboratory Guide

Student Workstation Checklist

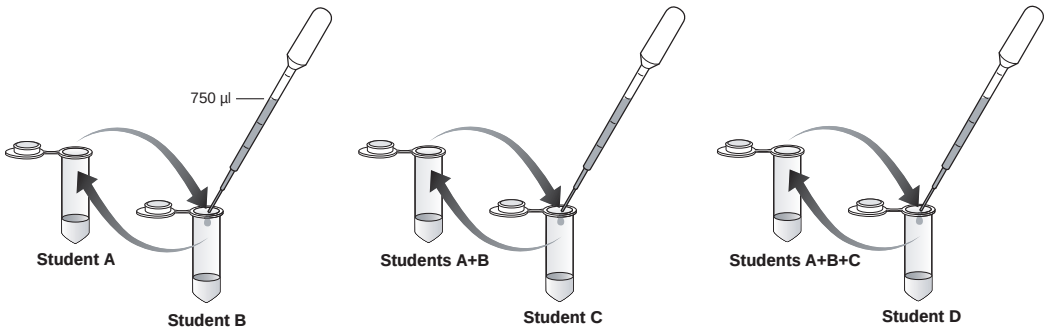
One workstation serves 4 students

Items	Contents	Number	(✔)
Yellow tubes	Student samples (0.75 ml)	4 (1 per student)	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipets		5	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

Laboratory Procedure

Share Body Fluids

1. Label each yellow tube with your initials. These are your "body fluid" samples that will be shared randomly with your classmates.
2. Label a plastic transfer pipet with your initials; you will use this to mix your sample with your fellow students.
3. When you are told to do so, find another student and use a pipet to transfer all 750 µl of your sample into the tube of the other student. (It doesn't matter whose tube is used to mix both samples.) Gently mix the samples by pipetting the mixture up and down. Then take back half of the shared sample (about 750 µl) to your own tube. Write down the name of that student next to "Sharing Partner #1" below.



Sharing Partner #1 _____

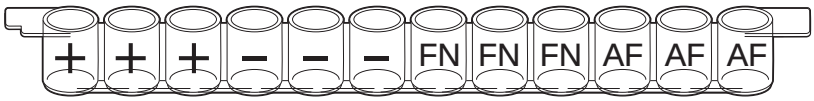
Sharing Partner #2 _____

Sharing Partner #3 _____

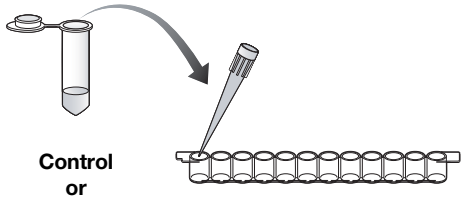
4. When told to do so, repeat the sharing protocol two more times with 2 other students so that you have shared your sample with 3 other students total. Make sure that you record their names in the order in which you shared. Discard your transfer pipets after this step. You may proceed directly to the next step or store your samples overnight at 4°C.

Perform ELISA

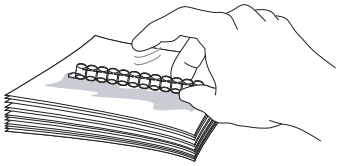
5. Label the outside wall of each well of your 12-well strip. Two students may share a strip of 12 wells. On each strip, label the first three wells with a "+" for the positive controls and the next three wells with a "–" for the negative controls. On the remaining wells write your and your partner's initials. For example, Florence Nightingale and Alexander Fleming would label their shared strip like this:



6. Bind the antigen to the wells:
    a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
    b. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
    c. Use a fresh pipet tip for each sample and transfer 50 µl of each of your team's samples into the appropriately initialed three wells.

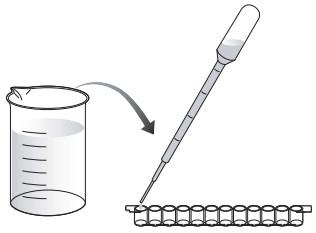


7. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
8. Wash the unbound sample out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make sure to avoid samples splashing back into wells.

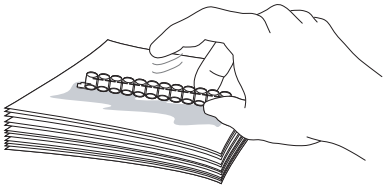


    b. Discard the top paper towel.

c. Use a fresh transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps.

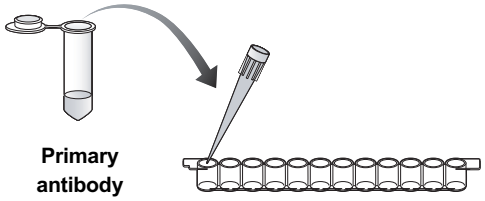


d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.

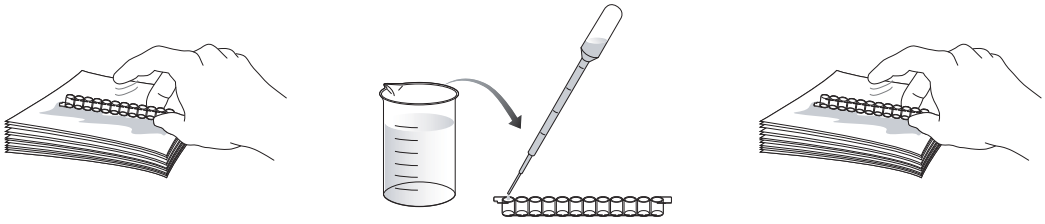


e. Discard the top 2–3 paper towels.

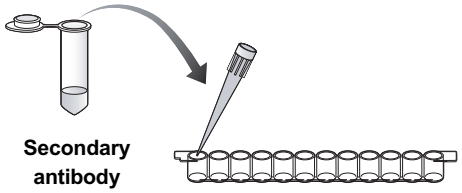
9. Repeat wash step 8.
10. Use a fresh pipet tip to transfer 50 µl of primary antibody (PA) from the green tube into all 12 wells of the microplate strip.



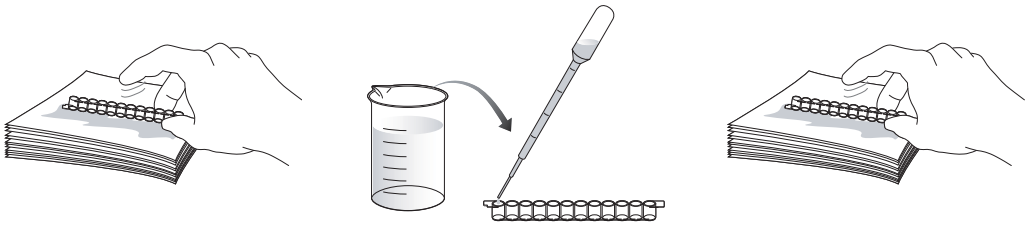
11. Wait 5 minutes for the primary antibody to bind.
12. Wash the unbound primary antibody out of the wells by repeating wash step 8 **two** times.



13. Use a fresh pipet tip to transfer 50 µl of secondary antibody (SA) from the orange tube into all 12 wells of the microplate strip.



14. Wait 5 minutes for the secondary antibody to bind.
15. Wash the unbound secondary antibody out of the wells by repeating wash step 8 **three** times.

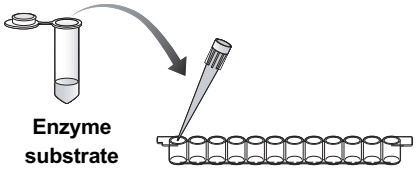


The secondary antibody is attached to an enzyme (HRP) that chemically changes the enzyme substrate, turning it from a colorless solution to a blue solution. Predict which wells of your experiment should turn blue and which should remain colorless and which wells you are not sure about.

_____

_____

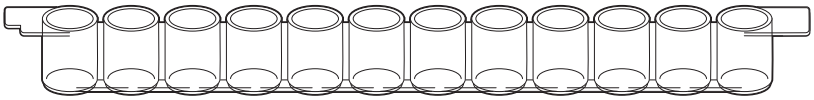
_____



16. Use a <u>fresh</u> pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.
17. Wait 5 minutes. Observe and record your results.

Results Section

Label the figure below with the same labels you wrote on the wells in step 5. In each of the wells, put a "+" if the well turned blue and a "–" if there is no color change.



Are you "infected" with the disease? YES/NO (circle one)

You can track the progress of the disease through the class by having each student record whether they tested positive or negative next to the names of the people with whom they shared "body fluids".

Class Results For Tracking Disease Transmission

Student's name	+ /–	Sharing partners (+ or –)	Student's name	+ /–	Sharing partners (+ or –)

Post-Lab Focus Questions

1. The samples that you added to the microplate strip contain many proteins and may or may not contain the disease antigen. What happened to the proteins in the plastic well if the sample contained the antigen? What if it did not contain the antigen?

2. Why did you need to wash the wells after every step?

3. When you added primary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?

4. When you added secondary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?

5. If the sample gave a negative result for the disease-causing agent, does this mean that you do not have the disease? What reasons could there be for a negative result when you actually do have the disease?

6. Why did you assay your samples in triplicate?

7. What antibody-based tests can you buy at your local pharmacy?

8. If you tested positive for disease exposure, did you have direct contact with one of the original infected students? If not, what conclusions can you reach about transmissibility of disease in a population?

Protocol II: Antigen Detection ELISA

Protocol II: Antigen Detection ELISA

Instructor's Guide

This activity simulates a test for the presence of an antigen in a sample. The protocol can be used to discuss how an antibody-based ELISA can detect disease agents in samples such as body fluids (before the body has had a chance to mount an immune response). Smallpox virus is one great real-world application of this protocol. If exposure is detected and treated with vaccine within 2–3 days, infected patients do not develop smallpox. Other disease-based scenarios include West Nile virus, HIV, SARS, and anthrax (see Appendix C). Other sources of disease-related antigens can include samples taken from farm animals, agricultural products, food, and water supplies.

Alternatively, you may choose to focus on non-disease-based applications for ELISA such as detecting the hormone hCG in a pregnancy test, illegal steroids in a drug test, bacterial toxins in a food safety test, or the presence of genetically modified organisms in non-GMO food — or any other scenario that may stimulate your students' interest. Over-the-counter kits that are based on the same principles as this ELISA activity include home pregnancy and ovulation tests and tests for the presence of illegal drugs like marijuana and cocaine.

To create a relevant and meaningful classroom context for this activity, the introductory pages to this manual as well as Appendices A and B provide background vocabulary and factual and conceptual lecture points. Appendix C contains useful information about specific diseases and classroom presentation scenarios.

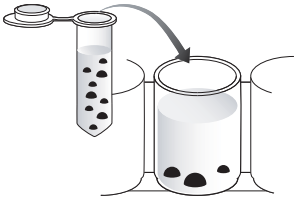
This ELISA relies on antibodies that are manufactured to detect particular antigens either in some body fluid or in samples from farm animals, food, or water supplies. Each student will be given a simulated sample to assay for the presence of the antigen. For more information about antibody manufacturing, see Appendix A.

Implementation Timeline

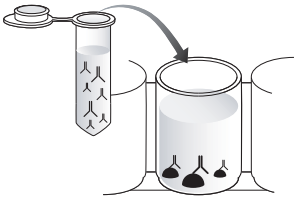
Day 1	Set the stage	Lecture and discussion
Day 2	Student ELISA lab	

Instructor's Laboratory Overview

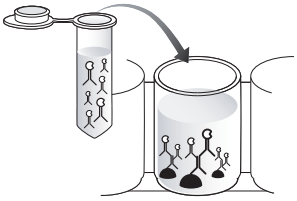
Step 1: Using a pipet, 50 µl of each student's sample (unknowns) along with positive and negative controls are added to the wells of the microplate strip and incubated for 5 minutes, allowing antigen, where present, to bind to the wells. The wells are rinsed with wash buffer (PBST: phosphate buffered saline containing 0.05% Tween 20) to block the unoccupied protein binding sites in the wells.



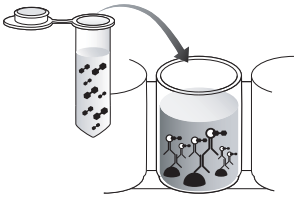
Step 2: Primary antibody (50 µl) is added to the wells and incubated for 5 minutes at room temperature. The primary antibody is an antibody that recognizes and binds to the antigen. The wells are rinsed with wash buffer to remove unbound antibody.

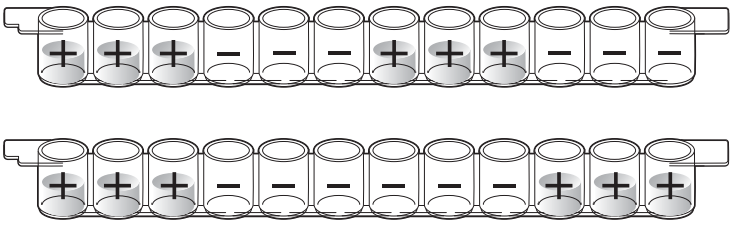


Step 3: Horseradish peroxidase (HRP)-labeled secondary antibody (50 µl) is added to the wells and incubated for 5 minutes at room temperature. The secondary antibody is antibody that recognizes and binds to the primary antibody. HRP is an enzyme that will oxidize a color-producing substrate. Wells are rinsed with wash buffer to remove unbound secondary antibody.



Step 4: The enzyme substrate (50 µl) is added to each well and students watch color development. If HRP is present (meaning that the antigen was present in the sample), the solution in the wells will turn blue within 5 minutes. If the antigen was not present in the sample, the wells will remain colorless.





Typical ELISA results.

Instructor's Advance Laboratory Preparation

This section is designed to help you prepare for the laboratory efficiently. We recommend that you read this section of the manual (Protocol II: Antigen Detection ELISA) in its entirety before beginning your preparation. In addition, if you are choosing to perform a scenario-based activity (for example, HIV testing), we recommend using the information given in Appendix C to help plan your lesson.

The most important thing for the students to do is to put the correct components in the assay wells in the correct order, so having the tubes clearly labeled and properly color-coded is crucial to a successful outcome.

Objectives

Step 1. Prepare buffers
Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody to make 50x stock solutions
Step 3. Dilute 50x stock solutions
Step 4. Dispense reagents for student workstations
Step 5. Set out student workstations

Time Required 1–3 hours

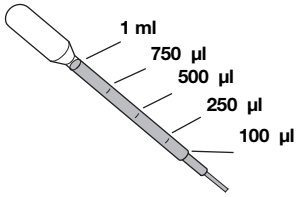
Preparation Timeframe

We recommend rehydrating and diluting the antigen and primary antibody no more than 3 days before the lesson, and the secondary antibody less than 24 hours before the lesson. We also suggest using sterile distilled water to prepare the 1x PBS to avoid contaminating rehydrated reagents. These reagents **must** be kept on ice or in the refrigerator if prepared over 4 hours before the lesson.

Note: If you are planning to use this kit for multiple lab sessions over a 1- or 2-week period, we strongly suggest using sterile water to prepare the PBS to avoid contaminating reagents. (Water can be sterilized by boiling it in a microwave oven for 5 minutes in a loosely capped bottle; after you remove the bottle from the microwave oven, let it cool, then secure the cap.) Dilute only as much concentrated antibody and antigen as required for each lab session. The rehydrated antibodies are 50x concentrates. Store the remaining concentrated antigen and antibodies in the refrigerator at 4°C. We do not recommend storing the concentrated antibody and antigen for more than 2 weeks, even at 4°C. Do not freeze the solutions.

Volume Measurements

This kit contains graduated disposable plastic transfer pipets (DPTPs) to use for preparing some of the reagents where volumes between 250 microliters (µl) and 5 milliliters (ml) are required. In addition adjustable- or fixed-volume micropipets are needed to measure 50 µl volumes. The illustration shows the marks on the DPTP corresponding to the volumes to be measured. Volumes over 1 ml will require multiple additions. For every step in this laboratory preparation, use a fresh DPTP or a fresh pipet tip.



Measuring liquids that contain detergents that foam (e.g., the wash buffer) requires that you read the volume at the interface of the liquid and the bubbles.

PROTOCOL II: Step-by-Step Instructor's Advance Preparation Guide

These instructions are for the setup of 12 student workstations of 4 students each.

Supplied Reagents	Quantity
Antigen, chicken gamma globulin, freeze-dried	1 vial
Primary antibody, rabbit anti-chicken polyclonal antibody, freeze-dried	1 vial
Secondary antibody, goat anti-rabbit antibody conjugated to HRP, freeze-dried	1 vial
HRP enzyme substrate (TMB)	1 bottle
10x phosphate buffered saline (PBS)	1 bottle
10% Tween 20	1 bottle

Required Reagents	
Distilled water, sterile is recommended, see note on page 40	1 L

Step 1. Prepare buffers.

We recommend you use a 100 ml and a 1 liter (L) graduated cylinder for preparing the buffer solutions. You will also need 1 L of distilled water.

Buffer	Volume	Reagent	Used for
1x PBS, 100 ml	90 ml	distilled water	• Rehydrating antigen, primary and secondary antibodies to make 50x reagent stock solutions • Diluting 50x antigen to make positive control and "infected" student samples • Negative control • Negative student samples
	10 ml	10x PBS	
Wash buffer, 900 ml	805 ml	Distilled water	• Dilution of 50x antibody stocks· • Plate washing
	90 ml	10x PBS	
	4.5 ml	10% Tween 20	

Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody.

Carefully remove the stoppers from the three freeze-dried reagents and use a fresh DPTP to add 0.5 ml 1x PBS to each. Close the stoppers and shake to mix. These solutions are 50x concentrates, or stock solutions. **NOTE: You must <u>not</u> use wash buffer in this step**.

Freeze-Dried reagent	Protocol for 50x Stock Solution	Used for
Antigen	Add 0.5 ml of 1x PBS to vial	• Positive control • "infected" student samples
Primary antibody	Add 0.5 ml of 1x PBS to vial	• Primary antibody
Secondary antibody	Add 0.5 ml of 1x PBS to vial	• Secondary antibody

Step 3. Dilute 50x stock reagents.

Label one 50 ml bottle or tube for each of the diluted solutions below. Use a fresh DPTP to add the contents of the appropriate 50x concentrated stock to the corresponding 50 ml bottle or tube.

Diluted solution	Volume	Reagent	Used for:
1x antigen, label one 50 ml bottle or tube	24.5 ml	1x PBS	positive control
	0.5 ml	50x antigen	
	NOTE: you must not add any buffer containing Tween 20 to the antigen, or the experiment will not work. • Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		
1x primary antibody, label one 50 ml bottle or tube	24.5 ml	Wash buffer	primary antibody
	0.5 ml	50x primary antibody stock	
	• Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		
1x secondary antibody, label one 50 ml bottle or tube	24.5 ml	Wash buffer	secondary antibody
	0.5 ml	50x secondary antibody stock	
	• Dilute the secondary antibody less than 24 hours before the start of the lesson. • Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		

Step 4. Dispense reagents for student workstations.

Tubes	Description	Label	Contents (Each Tube)
Violet tubes, 12	Positive controls	"+"	0.5 ml 1x antigen
Blue tubes, 12	Negative controls	"–"	0.5 ml 1x PBS
Green tubes, 12	Primary antibody	"PA"	1.5 ml 1x primary antibody solution
Orange tubes, 12	Secondary antibody	"SA"	1.5 ml 1x secondary antibody solution
Brown tubes, 12	Enzyme substrate	"SUB"	1.5 ml HRP enzyme substrate (TMB)
	Note: TMB is light sensitive, so it is important to use the dark tubes to store this reagent.		
Yellow tubes, # depends on # of students (48 max)	Positive student test sample(s)	50% of tubes	0.25 ml 1x antigen
	Negative student test sample(s)	50% of tubes	0.25 ml 1x PBS
	We recommend that you design the experiment so that 50% of your students will test positive and 50% will test negative. However, the final ratio is up to you. Make one student test sample for each student in your class as indicated above. Mix up the tubes before distributing.		

Step 5. Set out student workstations.

Student Workstation Checklist

One workstation serves 4 students.

Item (Label)	Contents	Number	(✔)
Yellow tubes	Student test samples (0.25 ml)	4	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipet		1	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

Stopping points: Although this procedure is designed to fit into a single lesson period, you may stop the procedure by adding wash buffer to the microplate wells at any stage after the addition of antigen and prior to the addition of enzyme substrate. Place the microplate strips and all the reagents in the refrigerator at 4°C overnight.

Setting Up The Activity to Test for a Specific Disease (e.g. HIV)

Appendix C provides information on a variety of diseases that can be diagnosed using ELISA. In addition, for each disease, we provide a table describing what the reagents for the activity represent in a real-world diagnostic ELISA. Below is an example of a diagnostic test to detect HIV viral proteins in a patient's blood sample.

Detecting p24 HIV-1 Capsid Protein

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Student samples	Yellow	1x antigen or 1x PBS	Sample derived from patient's blood
Primary antibody	Green	1x primary antibody	Anti-p24 capsid protein antibody from mouse
Secondary antibody	Orange	1x secondary antibody	Anti-mouse immunoglobulin antibody conjugated to HRP
Positive control	Violet	1x antigen	Heat-inactivated viral antigen (p24 protein)
Negative control	Blue	1x PBS	HIV negative human serum

Setting Up The Activity to Test for a Specific Antigen (e.g. pregnancy hormones)

The table below gives an example of how a diagnostic test for pregnancy can be simulated using protocol II.

Detecting Human Chorionic Gonadotropin Hormone

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Student samples	Yellow	1x antigen or 1x PBS	Patient's urine sample
Primary antibody	Green	1x primary antibody	Anti-hCG antibody from mouse
Secondary antibody	Orange	1x secondary antibody	Anti-mouse immunoglobulin antibody conjugated to HRP
Positive control	Violet	1x antigen	Purified human chorionic gonadotrophin (hCG) hormone
Negative control	Blue	1x PBS	Urine from a nonpregnant woman

Instructor's Answer Key and Discussion Points

Pre-lab Focus Questions

1. **How does the immune system protect us from disease?**

   The immune system includes physical barriers, such as the skin and mucous membranes that prevent pathogens from entering the body, and cellular responses, such as circulating macrophages that respond to foreign invaders. Our acquired immune system mounts a specific antibody response when the body is exposed to a foreign invader, and our immune cells attack the invader.

2. **How do doctors use the immune response to protect you from disease?**

   Doctors use the immune response when we are vaccinated against diseases. Our immune system remembers the pathogens to which we have been exposed, and the next time we are exposed to the pathogens our immune system attacks them more quickly and efficiently. Doctors take advantage of this priming effect by exposing us to inactivated pathogens (killed or weakened organisms that cannot make us sick) so that if we are later exposed to the live pathogen, our body will mount a strong and immediate antibody response, reducing or eliminating the chance that it will make us sick.

3. **How are the antibodies in your body made?**

   Antibodies in the body are made by immune cells called B lymphocytes, or B cells. Production of an antibody to a particular antigen increases when you are exposed to the antigen.

4. **How are antibodies that are used in ELISA made?**

   Antibodies used by scientists are usually made by immunizing animals. Polyclonal antibodies are purified from animal serum. Monoclonal antibodies are made by fusing B cells from immunized mice with immortalized cells in culture and collecting antibodies pumped out into growth medium. There are more technologically advanced methods of manufacturing antibodies involving recombinant DNA technology (discussed in Appendix A).

5. **Why is a rapid antigen detection test necessary?**

   In medicine, patients and doctors need test results quickly so they can make crucial decisions about the patient's care. Law enforcement and sports officials need rapid results from drug tests to help them decide whether or not to take action against someone suspected of abusing drugs. Environmental and food/water safety tests must yield rapid results because the population's health and safety may be at risk. In addition, in all the cases above, time spent waiting for test results could have an economic impact.

6. **What does ELISA stand for?**

   Enzyme-linked immunosorbent assay.

7. **Why are enzymes used in this immunoassay?**

   Enzymes provide a way to see whether the primary antibody has attached to its target (antigen) in the microplate well. Primary and secondary antibodies are invisible, so a detection method is necessary. The enzyme, HRP is linked to the secondary antibody. HRP reacts with a colorless substrate in a chemical reaction that turns blue. If the secondary antibody is present in the well, the color change indicates a positive result.

8. **Why do you need to assay positive and negative control samples as well as your experimental samples?**

   Controls are needed to make sure the assay is working correctly. If there are no positive controls and the sample is negative, we can't know if the sample was truly negative or if assay didn't work. Conversely, without a negative control, there is no way of knowing if all samples (positive or not) would have given a positive result.

Post-Lab Focus Questions

1. **Did your sample contain the antigen?**

   Students explain using data from their results.

2. **The samples that you added to the microplate strip contain many proteins and may or may not contain the antigen. What happened to the proteins in the plastic well if the sample contained the antigen? What if it did not contain the antigen?**

   In either case, all the proteins present in the sample bind to the plastic wells.

3. **Why did you need to wash the wells after every step?**

   Washing removes any proteins that have not bound to the plastic wells and any antibodies that have not bound to their targets, thus preventing unbound proteins (either antigen or antibodies) from giving false positive results.

4. **When you added primary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?**

   If it was positive for the antigen, the primary antibody bound the antigen. If it was negative, the primary antibody did not bind and was flushed out in the wash step.

5. **When you added secondary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?**

   If the sample contained antigen, the secondary antibody bound to the primary antibodies already bound to antigen in the wells. If the test sample did not contain antigen, primary antibody did not bind in the wells, so the secondary antibody had nothing to bind and was flushed out in the wash step.

6. **If the sample gave a negative result for the antigen, does this mean that the antigen is not present? What reasons could there be for a negative result when the antigen is actually present?**

   A negative result does not necessarily mean the antigen is not present. It could be a false negative. The ELISA may not be sensitive enough to detect very low levels of antigen. Another cause of false negatives is experimental error, such as putting a negative control into a well where you thought you were putting an experimental sample.

7. **Why did you assay your samples in triplicate?**

   Assaying the samples in triplicate is another control. If you do not get the same result in all triplicate wells, you have a problem with your experimental technique or you have made a pipetting error. In a clinical laboratory, the experiment would have to be repeated. If this error occurs in this activity, take the result of the two matching wells since this is probably correct.

8. **What antibody-based tests can you buy at your local pharmacy?**

   Test kits that are based on the same principles as the ELISA include home pregnancy and ovulation tests and tests for the presence of illegal drugs such as marijuana and cocaine.

Laboratory Quick Guide

Antigen Detection ELISA

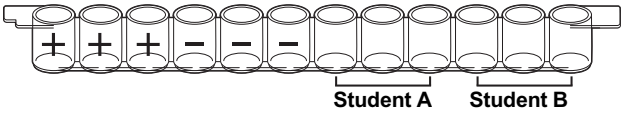
Student Workstation Checklist

One workstation serves 4 students.

Item (Label)	Contents	Number	(✔)
Yellow tubes	Student test samples (0.25 ml)	4	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipet		1	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

1. Label the yellow tubes with each student's initials.

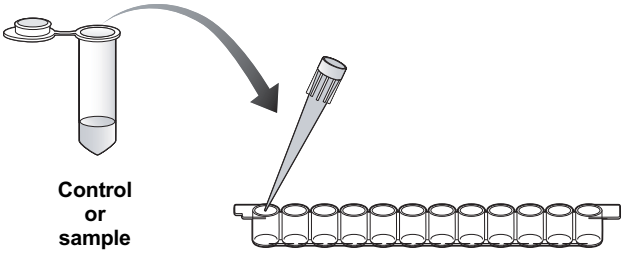
2. Label your 12-well strip. On each strip label the first 3 wells with a "+" for the positive controls and the next 3 wells with a "–" for the negative controls. Label the remaining wells with your and your partner's initials (3 wells each).



3. Use a fresh pipet tip to transfer 50 µl of the positive control (+) into the three "+" wells.

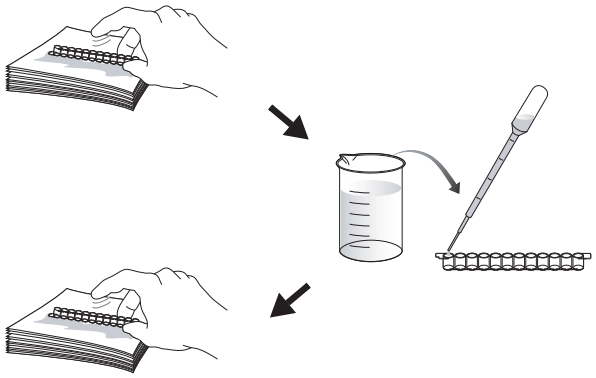
4. Use a fresh pipet tip to transfer 50 µl of the negative control (–) into the three "–" wells.

5. Transfer 50 µl of each of your team's samples into the appropriately initialed three wells, using a fresh pipet tip for each sample.



6. Wait 5 minutes while the proteins in the samples bind to the plastic wells.

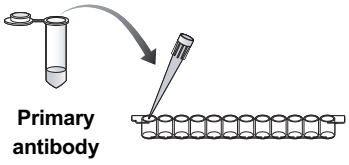
7. WASH:
    a. Tip the microplate strip upside down onto the paper towels, and gently tap the strip a few times upside down. Make sure to avoid splashing samples back into wells.
    b. Discard the top paper towel.
    c. Use your transfer pipet to fill each well with wash buffer, taking care not to spill over into neighboring wells. Note: the same transfer pipet is used for all washing steps.
    d. Tip the microplate strip upside down onto the paper towels and tap.
    e. Discard the top 2–3 paper towels.



8. Repeat wash step 7.

WASH

9. Use a <u>fresh</u> pipet tip to transfer 50 µl of primary antibody (PA) into all 12 wells of the microplate strip.

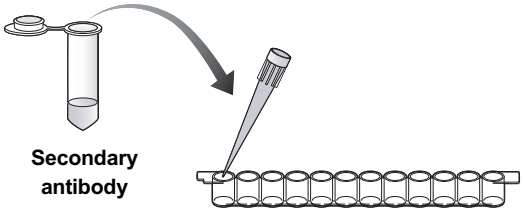


10. Wait 5 minutes for the antibodies to bind to their targets.

11. Wash the unbound primary antibody out of the wells by repeating all of wash step 6 **two** times.

WASH 2x

12. Use a <u>fresh</u> pipet tip to transfer 50 µl of secondary antibody (SA) into all 12 wells of the microplate strip.

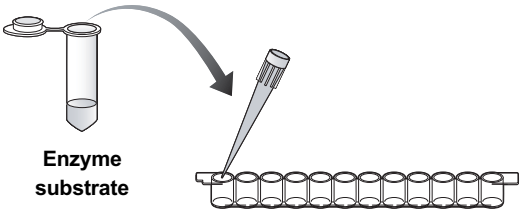


13. Wait 5 minutes for the antibodies to bind to their targets.

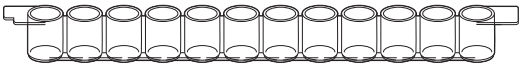
14. Wash the unbound secondary antibody out of the wells by repeating wash step 6 **three** times.

WASH 3x

15. Use a <u>fresh</u> pipet tip to transfer 50 µl of enzyme substrate (SUB) into all 12 wells of the microplate strip.



16. Wait 5 minutes. Observe and record the results.



Student Manual

Introduction

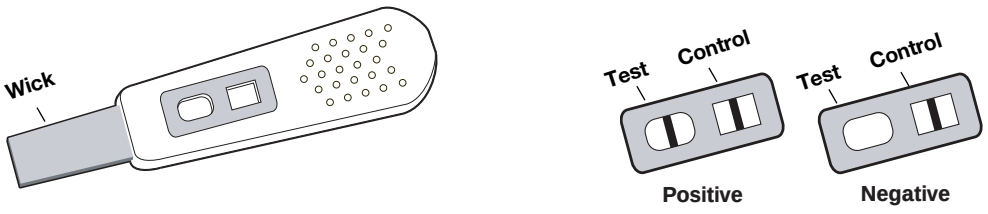
Immunology is the study of the immune system and how the body protects itself against disease. Over 100 years ago, biologists found that animals' internal immune systems respond to invasion by "foreign entities" or antigens. When an invader enters the body, it provokes an immune response that begins with the production of proteins called antibodies. Like magic bullets, antibodies seek out and attach themselves to invading entities (antigens), flagging the invaders for destruction by other cells of the immune system. The antigenic invaders may be any molecules foreign to the body, including components of infectious agents like bacteria, viruses, and fungi. Today, antibodies have become vital scientific tools, used in biotechnology research and to diagnose and treat disease. The number of different antibodies circulating in the blood has been estimated to be between 10^6 and 10^{11}, so there is usually an antibody ready to deal with any antigen. In fact, antibodies make up to 15% of your total blood serum protein. Antibodies are very specific; each antibody recognizes only a single antigen.

You are about to perform an ELISA (enzyme-linked immunosorbent assay). The ELISA relies on antibodies to detect the presence of antigens in liquid samples. Because they are antibody-based, ELISAs are called immunoassays. ELISAs can detect minute amounts of disease agents in samples such as body fluids (before the body has had a chance to mount an immune response). Smallpox virus is an example of a disease agent that can now be detected using an ELISA. If exposure is detected and treated with vaccine within 2–3 days, patients do not develop smallpox. Other applications for ELISA include testing for West Nile virus, HIV coat protein p24, SARS virus, anthrax spores, hormones such as hCG in pregnancy tests, illegal steroids in drug tests, bacteria in food safety tests, and the presence of genetically modified organisms contaminating non-GMO food.

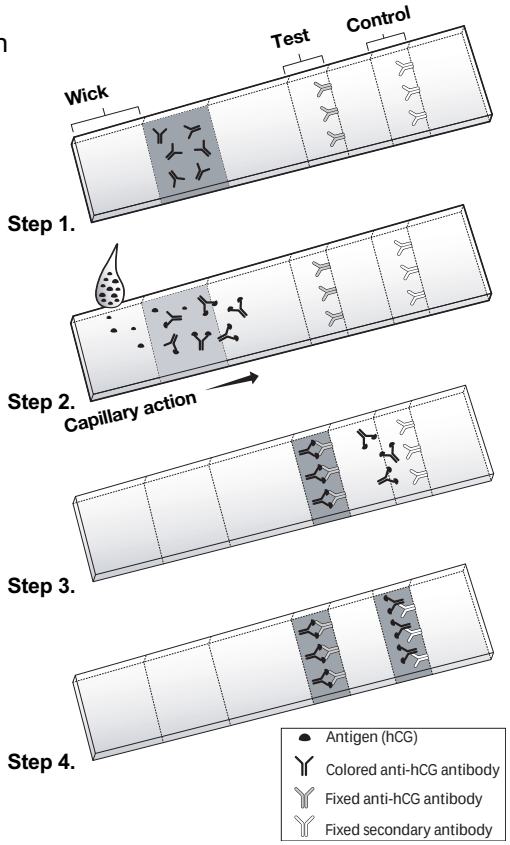
Where Is ELISA Used in the Real World?

With its rapid test results, the ELISA has had a major impact on many aspects of medicine and agriculture. ELISA is used for such diverse purposes as home pregnancy tests, disease detection in people, animals, and plants, detecting illegal drug use, testing indoor air quality, and determining if food is labeled accurately. For new and emerging diseases like severe acute respiratory syndrome (SARS), one of the highest priorities of the US Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) has been to develop an ELISA that can quickly and easily verify whether patients have been exposed to the virus.

Over-the-counter kits that are based on the same principles as this ELISA activity include home pregnancy and ovulation tests, and tests for the presence of illegal drugs like marijuana and cocaine.

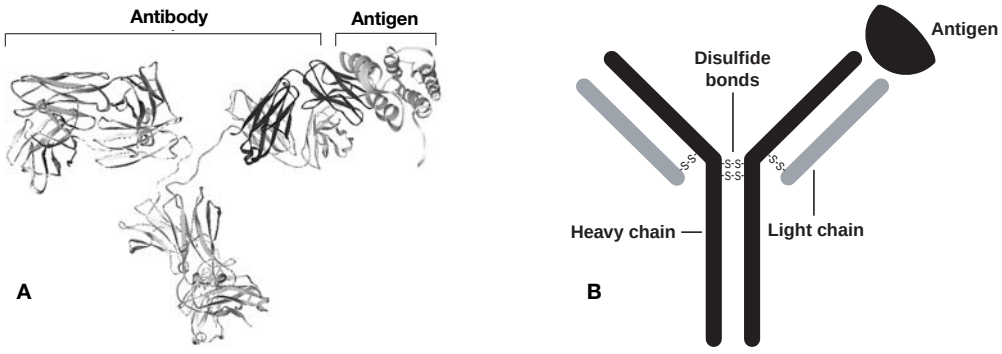


Some tests give positive or negative results in a matter of minutes. For example, home pregnancy dipstick tests detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization. The wick area of the dipstick is coated with anti-hCG antibody labeled with a pink compound (step 1). When the strip is dipped in urine, if hCG is present it will bind to the pink antibody, and the pink hCG-antibody complex will migrate up the strip via capillary action (step 2). When the pink complex reaches the first test zone, a narrow strip containing an unlabeled fixed anti-hCG antibody, the complex will bind and concentrate there, making a pink stripe (step 3). The dipsticks have a built-in control zone containing an unlabeled secondary antibody that binds unbound pink complex (present in both positive and negative results) in the second stripe (step 4). Thus, every valid test will give a second pink stripe, but only a positive pregnancy test will give two pink stripes.



How Are Antibodies Made?

When exposed to antigens, all mammals generate an immune response and produce antibodies, proteins that recognize and bind tightly to the specific antigens. Each antibody recognizes only a single antigen. Animals such as goats, rabbits, and mice can be injected with an antigen and, after a period of time, their serum will contain antibodies that specifically recognize that antigen. If the antigen was a disease-causing agent, the antibodies can be used to develop diagnostic tests for the disease. In an immunoassay, the antibodies used to recognize antigens like disease agents are called primary antibodies.



A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, (Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

Secondary antibodies recognize and bind to primary antibodies in an immunoassay. They are prepared by injecting antibodies produced by one species of animal into another species. This works because the antibodies produced by different species are different enough from each other that they will provoke an immune response. For example, if you want a secondary antibody that will recognize a human primary antibody, inject human antibodies into an animal like a rabbit. After the rabbit immune response, the rabbit serum will contain antibodies that recognize and bind to human antibodies. Secondary antibodies are frequently labeled to make them visible.

In this experiment, the secondary antibodies you will be working with are conjugated to an enzyme named horseradish peroxidase (HRP); HRP in the presence of its substrate, TMB, produces a blue color.

Controls in Immunoassays

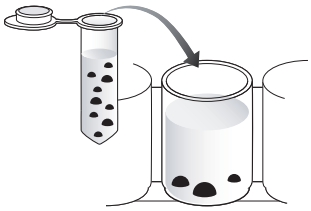
For any immunoassay to be valid, it must include both positive and negative controls, i.e., samples that will give known results. Controls are always run side by side with experimental samples. If you do not run a positive control and the experiment gives negative results, how can you be sure the results are truly negative? What if the assay simply did not work? If a positive sample gives a negative assay result, it is called a **false negative**. Conversely, if you do not run a negative control and the experiment gives all positive results, how can you be sure the results are truly positive? What if the assay was contaminated with antigen? If a negative sample gives a positive assay result, it is called a **false positive**.

Controls are also needed to guard against experimental error and to ensure that the assay is working correctly. There can be problems with reagents, which can degrade due to age or poor storage conditions. Operators can make mistakes by choosing the wrong reagents, making errors in dilutions or in pipetting, or failing to remove unbound reagents. Poor record keeping is another source of false assay results. Most of these possibilities can be checked for within the assay with the appropriate controls.

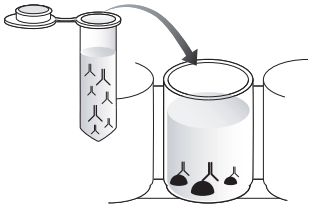
Now let's put this all together.

The main steps in this antigen detection ELISA are:

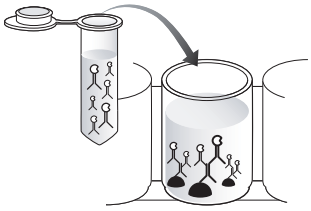
1. Add your sample and control samples to the wells in a microplate strip. Your samples contain many proteins and may or may not contain the antigen. Incubate for 5 minutes to allow all the proteins in the samples to bind to the plastic wells via hydrophobic interaction. This is called an immunosorbent assay because proteins adsorb (bind) to the plastic wells.



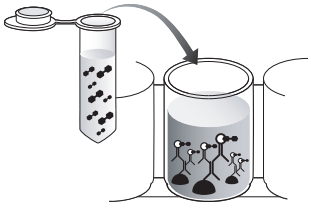
2. Add primary antibody to the wells and incubate. The antibodies will seek out the antigen from the many proteins bound to the well. If your sample contains the antigen, the antibodies will bind it tightly and remain in the well.



3. Detect the bound antibodies with HRP-labeled secondary antibody. If the primary antibodies have bound to the antigen, the secondary antibodies will bind tightly to the primary antibodies.



4. Add enzyme substrate to the wells, wait 5 minutes, and evaluate the assay results. If the antigen was present in your sample, the wells will turn blue. This is a positive diagnosis. If the wells remain colorless, the antigen was not present in your sample and the diagnosis is negative.



Pre-Lab Focus Questions

1. How does the immune system protect us from disease?

2. How do doctors use the immune response to protect you from disease?

3. How are the antibodies in your body made?

4. How are antibodies that are used in ELISA made?

5. Why is a rapid antigen detection test necessary?

6. What does ELISA stand for?

7. Why are enzymes used in this immunoassay?

8. Why do you need to assay positive and negative control samples as well as your experimental samples?

Laboratory Guide

Student Workstation Checklist

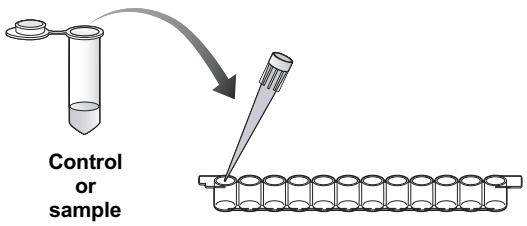
One workstation serves 4 students.

Items	Contents	Number	(✔)
Yellow tubes	Student samples (0.25 ml)	4	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipets		1	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

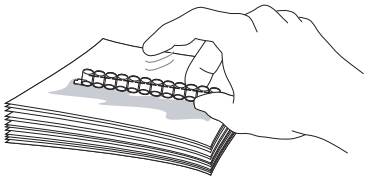
Laboratory Procedure

1. Label the yellow tubes with each student's initials.
2. Label the outside wall of each well of your 12-well strip. Two students may share a strip of 12 wells. On each strip label the first three wells with a "+" for the positive controls and the next three wells with a "–" for the negative controls. On the remaining wells write your and your partner's initials. For example, Florence Nightingale and Alexander Fleming would label their shared strip like this:

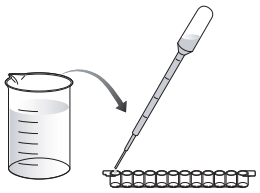
3. Bind the antigen to the wells:
    a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
    b. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
    c. Use a fresh pipet tip for each sample and transfer 50 µl of each of your team's samples into the appropriately initialed three wells.



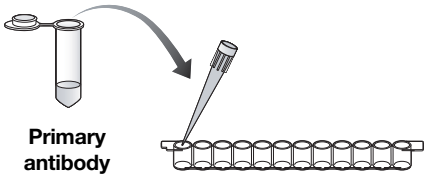
4. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
5. Wash the unbound sample out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make sure to avoid splashing sample back into wells.



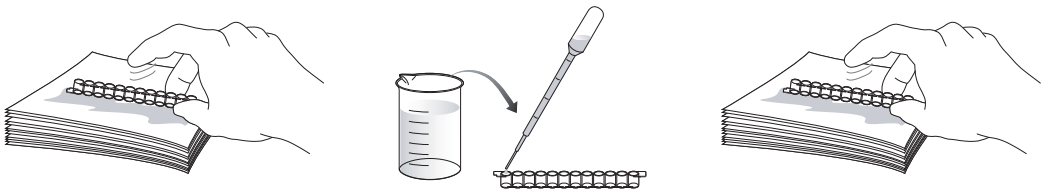
    b. Discard the top paper towel.
    c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps..



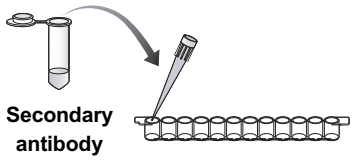
    d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.
    e. Discard the top 2–3 paper towels.
6. Repeat wash step 5.
7. Use a <u>fresh</u> pipet tip to transfer 50 µl of primary antibody (PA) from the green tube into all 12 wells of the microplate strip.



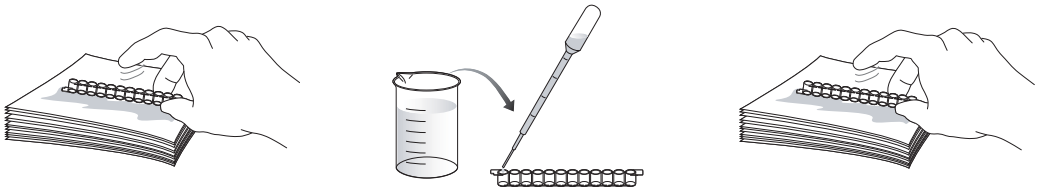
8. Wait 5 minutes for the primary antibody to bind.
9. Wash the unbound primary antibody out of the wells by repeating wash step 5 **two** times.



10. Use a fresh pipet tip to transfer 50 µl of secondary antibody (SA) from the orange tube into all 12 wells of the microplate strip.



11. Wait 5 minutes for the secondary antibody to bind.
12. Wash the unbound secondary antibody out of the wells by repeating wash step 4 **three** times.



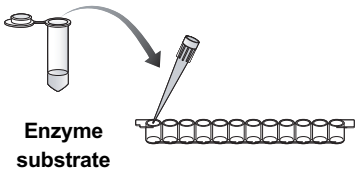
The secondary antibody is attached to an enzyme (HRP) that chemically changes TMB (the enzyme substrate), turning it from a colorless solution to a blue solution. Predict which wells of your experiment should turn blue and which should remain colorless and which wells you are not sure about.

_____

_____

_____

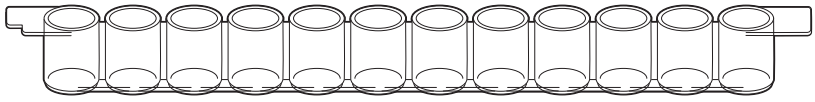
13. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



14. Wait 5 minutes. Observe and record your results.

Results Section

Label the figure below with the same labels you wrote on the wells in step 1. In each of the wells, put a "+" if the well turned blue and a "–" if there is no color change.



Is your sample positive? Explain your answer.

Post-Lab Focus Questions

1. Did your sample contain the antigen?

2. The samples that you added to the microplate strip contain many proteins and may or may not contain the antigen. What happened to the proteins in the plastic well if the sample contained the antigen? What if it did not contain the antigen?

3. Why did you need to wash the wells after every step?

4. When you added primary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?

5. When you added secondary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?

6. If the sample gave a negative result for the antigen, does this mean that the antigen is not present? What reasons could there be for a negative result when the antigen is actually present?

7. Why did you assay your samples in triplicate?

8. What antibody-based tests can you buy at your local pharmacy?

Protocol III: ELISA Antibody Test

Protocol III: ELISA Antibody Test

Instructor's Guide

This protocol simulates a diagnostic blood test for the detection of serum antibodies. With this protocol, students perform an ELISA to detect circulating antibodies in the blood as an indication of exposure to a disease-causing agent. Each student is provided with a simulated serum sample and asked to assay the sample for the presence of antibodies.

You may choose to focus on any of a variety of diseases such as HIV, West Nile virus (in people or animals), SARS, Lyme disease, trichinosis, tuberculosis, or any other disease that may stimulate your students' interest. This technique is useful for detection and diagnosis "post-infection" where the antigen itself is undetectable in the body. Once the body has mounted an immune response, antibodies are present in the blood serum and can be detected. For example, until very recently it was not possible to use ELISA to detect the HIV virus directly, but serum antibodies against the virus could be detected in vitro using ELISA. Hence, the ELISA antibody test was the only rapid way to diagnose HIV infection. Today, as the result of extensive investment into research on the biology of HIV/AIDS, it is possible to detect the HIV antigen in the blood directly by ELISA, allowing early treatment and extension of life.

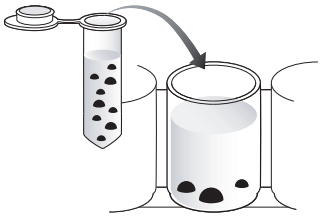
To create a relevant and meaningful classroom context for this activity, the introductory pages to this manual as well as Appendices A and B provide background vocabulary and factual and conceptual lecture points. Appendix C contains useful information about specific diseases and classroom presentation scenarios for this ELISA antibody test protocol. The assay can be personalized with students assaying their "own" simulated serum samples, or it can be performed as a role-playing activity where students assay patient samples in a clinical lab at a local hospital.

Implementation Timeline

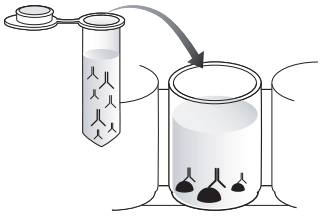
Day 1	Set the stage	Lecture and discussion
Day 2	Student ELISA lab	

Instructor's Laboratory Overview

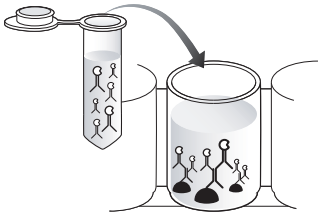
Step 1: Using a pipet, 50 µl of (simulated) purified disease antigen is added to the wells of the microplate strip and incubated for 5 minutes, allowing antigen to bind to the wells. The wells are rinsed with wash buffer (PBST: phosphate buffered saline containing 0.05% Tween 20) to block the unoccupied protein binding sites in the wells.



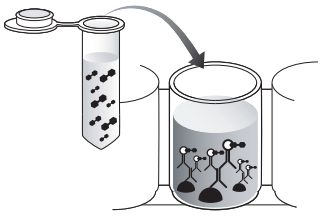
Step 2: Serum samples and positive and negative controls (50 µl) are added to the wells and incubated for 5 minutes at room temperature. In this protocol, the serum samples are actually primary antibodies and represent the primary antibodies in a patient's blood. If antibodies against the disease are present in the serum sample, they will bind to the purified disease antigen already bound in the wells. The wells are rinsed with wash buffer to remove unbound antibody.

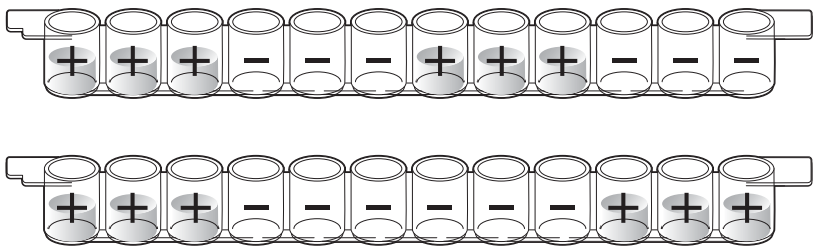


Step 3: Horseradish peroxidase (HRP)-labeled secondary antibody (50 µl) is added to the wells and incubated for 5 minutes at room temperature. The secondary antibody is antibody that recognizes and binds to the primary antibody. In this simulation, the secondary antibody represents an anti-human antibody. HRP is an enzyme that will oxidize a color-producing substrate. Wells are rinsed with wash buffer to remove unbound secondary antibody.



Step 4: The enzyme substrate (50 µl) is added to each well and students watch color development. If HRP is present (meaning that antibodies to the purified antigen were present in the serum sample), the solution in the wells will turn blue within 5 minutes, indicating a positive diagnosis. If there were no antibodies to the disease antigen, the wells will remain colorless, indicating a negative diagnosis.





Typical ELISA results.

Instructor's Advance Laboratory Preparation

This section is designed to help you prepare for the laboratory efficiently. We recommend that you read this section of the manual (Protocol III: ELISA Antibody Test) in its entirety before beginning your preparation. In addition, if you are choosing to perform a scenario-based activity (for example HIV testing), we recommend using the information given in Appendix C to help plan your lesson.

The most important thing for the students to do is to put the correct components in the assay wells in the correct order, so having the tubes clearly labeled and properly color-coded is crucial to a successful outcome.

Objectives

Step 1. Prepare buffers
Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody to make 50x stocks
Step 3. Dilute 50x stock solutions
Step 4. Dispense reagents for student workstations
Step 5. Set out student workstations

Time Required 1–3 hours

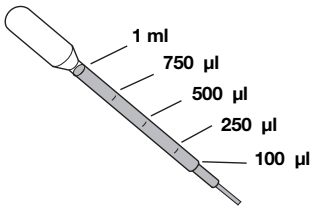
Preparation Timeframe

We recommend rehydrating and diluting the antigen and primary antibody no more than 3 days before the lesson, and the secondary antibody less than 24 hours before the lesson. We also suggest using sterile, distilled water to prepare the 1x PBS to avoid contaminating rehydrated reagents. These reagents must be kept on ice or in the refrigerator if prepared more than 4 hours before the lesson.

Note: If you are planning to use this kit for multiple lab sessions over a 1- or 2-week period, we strongly suggest using sterile water to prepare the PBS avoid contaminating reagents. (Water can be sterilized by boiling it in a microwave oven for 5 minutes in a loosely capped bottle; after you remove the bottle from the microwave oven, let it cool, then secure the cap.) Dilute only as much concentrated antibody and antigen as required for each lab session. The rehydrated antibodies are 50x concentrates. Store the remaining concentrated antigen and antibodies in the refrigerator at 4°C. We do not recommend storing the concentrated antibody and antigen for more than 2 weeks, even at 4°C. Do not freeze the solutions.

Volume Measurements

This kit contains graduated disposable plastic transfer pipets (DPTPs) to use for preparing some of the reagents where volumes between 250 microliters (µl) and 5 milliliters (ml) are required. In addition, adjustable- or fixed-volume micropipets are needed to measure 50 µl volumes. The illustration shows the marks on the DPTP corresponding to the volumes to be measured. Volumes over 1 ml will require multiple additions. For each step of the laboratory preparation use a fresh DPTP or a fresh pipet tip.



Measuring liquids that contain detergents that foam (e.g., the wash buffer) requires that you read the volume at the interface of the liquid and the bubbles.

PROTOCOL III: Step-by-Step Instructor's Advance Preparation Guide

These instructions are for the setup of 12 student workstations of 4 students each.

Supplied Reagents	Quantity
Antigen, chicken gamma globulin, freeze-dried	1 vial
Primary antibody, rabbit anti-chicken polyclonal antibody, freeze-dried	1 vial
Secondary antibody (goat anti-rabbit antibody conjugated to HRP, freeze-dried	1 vial
HRP enzyme substrate (TMB)	1 bottle
10x phosphate buffered saline (PBS)	1 bottle
10% Tween 20	1 bottle

Required Reagents	
Distilled water, sterile is recommended, see note on page 62	1 L

Step 1. Prepare buffers.

We recommend you use a 100 ml and a 1 liter (L) graduated cylinder for preparing the buffer solutions. You will also need 1 L of distilled water.

Buffer	Volume	Reagent	Used for
1x PBS, 100 ml	90 ml	distilled water	• Rehydrating antigen, primary and secondary antibodies to make 50x reagent stock solutions • Diluting 50x antigen
	10 ml	10x PBS	
Wash buffer, 900 ml	805 ml	Distilled water	• Diluting 50x primary antibody stock for positive control and positive student samples • Negative controls • Negative student serum samples • Dilution of 50x antibody stocks • Plate washing
	90 ml	10x PBS	
	4.5 ml	10% Tween 20	

Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody.

Carefully remove the stoppers from the three freeze-dried reagents and use a fresh DPTP to add 0.5 ml 1x PBS to each. Close the stoppers and shake to mix. These solutions are 50x concentrates, or stock solutions. **NOTE: You must <u>not</u> use wash buffer in this step**.

Freeze-Dried Reagent	Protocol for 50x Stock Solution	Used for
Antigen	Add 0.5 ml of 1x PBS to vial	• Purified antigen
Primary antibody	Add 0.5 ml of 1x PBS to vial	• Positive controls • Positive student serum samples
Secondary antibody	Add 0.5 ml of 1x PBS to vial	• Secondary antibody

Step 3. Dilute 50x stock reagents.

Label one 50 ml bottle or tube for each of the diluted solutions below. Use a fresh DPTP to add the contents of the appropriate 50x concentrated stock to the corresponding 50 ml bottle or tube.

Diluted Solution	Volume	Reagent	Used for
1x antigen, label one 50 ml bottle or tube	24.5 ml	1x PBS	Purified antigen
	0.5 ml	50x antigen	
	NOTE: you must not add any buffer containing Tween 20 to the antigen, or the experiment will not work. • Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		
1x serum (1x primary antibody), label one 50 ml bottle or tube	24.5 ml	Wash buffer	Positive controls
	0.5 ml	50x primary antibody stock	Positive student serum samples
	• Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		
1x secondary antibody, label one 50 ml bottle or tube	24.5 ml	Wash buffer	Secondary antibody
	0.5 ml	50x secondary antibody stock	
	• Dilute the secondary antibody less than 24 hours before the start of the lesson. • Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		

Step 4. Dispense reagents for student workstations.

Tubes	Description	Label	Contents (Each Tube)
Violet tubes, 12	Positive controls	"+"	0.5 ml 1x serum (1x primary antibody)
Blue tubes, 12	Negative controls	"–"	0.5 ml Wash buffer
Green tubes, 12	Purified antigen	"AG"	1.5 ml 1x antigen
Orange tubes, 12	Secondary antibody	"SA"	1.5 ml 1x secondary antibody solution
Brown tubes, 12	Enzyme substrate	"SUB"	1.5 ml HRP enzyme substrate (TMB)
	Note: TMB is light sensitive, so it is important to use the dark tubes to store this reagent.		
Yellow tubes, # depends on # of students (48 max)	Positive student serum sample(s)	50% of tubes	0.25 ml 1x serum (1x primary antibody)
	Negative student serum sample(s)	50% of tubes	0.25 ml Wash buffer
	We recommend that you design the experiment so that 50% of your students will test positive and 50% will test negative. However, the final ratio is up to you. Make one student test sample for each student in your class as indicated above. Mix up the tubes before distributing.		

Step 5. Set out student workstations.

Student Workstation Checklist

One workstation serves 4 students.

Item (Label)	Contents	Number	(✔)
Yellow tubes	Student test samples (0.25 ml)	4	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (AG)	Purified antigen (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipet		1	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

Stopping points: Although this procedure is designed to fit into a single lesson period, you may stop the laboratory activity by adding wash buffer to the microplate wells at any stage after the addition of antigen and prior to the addition of enzyme substrate. Place the microplate strips and all the reagents in the refrigerator at 4°C overnight.

Setting Up The Activity to Test for a Specific Disease (e.g. HIV)

Appendix C provides information on a variety of diseases that can be diagnosed using ELISA. In addition, for each disease, we provide a table describing what the reagents for the activity represent in a real-world diagnostic ELISA. Below is an example of a diagnostic test to detect antibodies towards HIV in a patient's blood sample.

Detecting antibodies to HIV*

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Purified antigen	Green	1x antigen	Purified HIV proteins
Student samples	Yellow	1x primary antibody or wash buffer	Serum sample from patient
Secondary antibody	Orange	1x secondary antibody	Anti-human immunoglobulin antibodies conjugated to HRP
Positive control	Violet	1x antigen	Serum from an HIV negative patient spiked with HIV antibodies
Negative control	Blue	1x PBS	Serum from an HIV negative patient

* Note: Tests to detect anti-HIV antibodies in saliva and urine are also used.

Instructor's Answer Key and Discussion Points

Pre-Lab Focus Questions

1. **How does the immune system protect us from disease?**

   The immune system includes physical barriers, such as the skin and mucous membranes that prevent pathogens from entering the body, and cellular responses, such as circulating macrophages that respond to foreign invaders. Our acquired immune system mounts a specific antibody response when the body is exposed to a foreign invader, and our immune cells attack the invader.

2. **How do doctors use the immune response to protect you from disease?**

   Doctors use the immune response when we are vaccinated against diseases. Our immune system remembers the pathogens to which we have been exposed, and the next time we are exposed to the pathogens our immune system attacks them more quickly and efficiently. Doctors take advantage of this priming effect by exposing us to inactivated pathogens (killed or weakened organisms that cannot make us sick) so that if we are later exposed to the live pathogen, our body will mount a strong and immediate antibody response, reducing or eliminating the chance that it will make us sick.

3. **What is an example of a disease that attacks the human immune system?**

   Diseases that attack the immune system include autoimmune diseases (e.g., rheumatoid arthritis, lupus, asthma, eczema, SCID) and AIDS. An extensive list can be found in Appendix A.

4. **What problems can prevent the immune system from working properly?**

   Problems with the immune system fall into three categories: hypersensitivity, immunodeficiency, and autoimmune diseases. Hypersensitivity occurs when the immune system overreacts to an antigen; hypersensitivity reactions include anaphylactic reactions, allergies, and contact sensitivity (e.g., reaction to poison ivy). Immunodeficiency means that an individual cannot mount an effective immune response. Immunodeficiency may be genetic (e.g., SCID or "bubble boy" disease) or induced by a disease (e.g., immunodeficiency from HIV infection) or by immunosuppressive drugs (e.g., drugs given after organ transplant to prevent rejection). Autoimmune disease results from the immune system inappropriately mounting an immune response to itself, (for example, diseases like lupus, rheumatoid arthritis, multiple sclerosis (MS), insulin-dependent diabetes, and celiac disease).

5. **Why is it important to be able to detect antibodies in people who don't appear sick?**

   It is important because people may be disease carriers even though they are not sick themselves. Typhoid fever is an example of a disease that has chronic carriers; up to 5% of individuals infected with typhoid fever excrete the bacteria for up to a year. For example, in the historical case of Typhoid Mary, Mary Mallon infected 47 people with typhoid fever over the course of 15 years, even though she was never ill herself.

West Nile virus (WNV) is another good example of why it is important to detect antibodies in people who aren't sick. WNV is spread by mosquitoes. Most people who are infected with West Nile have either no symptoms or very mild symptoms. To get a true picture of the epidemiology of West Nile virus, it is necessary to test for antibodies against WNV in many more individuals than just the few who develop symptoms. For example, if one person on a street becomes ill from WNV, testing all of his neighbors may show that dozens were infected with the virus but did not get sick. Information on infection rates, not just on rates of illness, is necessary to get an accurate picture of the disease.

For a disease like HIV/AIDS, early detection of infection is crucial. People infected with HIV may have no symptoms at all for many years, yet if they begin treatment prior to the onset of disease, development of symptoms may be postponed indefinitely. In addition, during the asymptomatic phase of the infection, individuals infected with HIV and unaware of their infection could pass the virus on to other people with whom they have intimate contact.

6. **What does ELISA stand for?**

   Enzyme-linked immunosorbent assay.

7. **Why are enzymes used in this immunoassay?**

   Enzymes provide a way to see whether the primary antibody has attached to its target (antigen) in the microplate well. Primary and secondary antibodies are invisible, so a detection method is necessary. The enzyme horseradish peroxidase (HRP) is linked to the secondary antibody. HRP reacts with a colorless substrate in a chemical reaction that turns blue. If the secondary antibody is present in the well, the color change indicates a positive result.

8. **Why do you need to assay positive and negative control samples as well as your experimental samples?**

   Controls are needed to make sure the experiment worked. If there are no positive controls and the sample is negative, we can't know if the sample was truly negative or if the assay didn't work. Conversely, without a negative control, there is no way of knowing if all samples (positive or not) would have given a positive result.

Post-Lab Focus Questions

1. **Did your serum have antibodies to the disease?**

   Students explain using data from their results.

2. **If you tested positive for antibodies, does this mean that you have been exposed to the disease?**

   A positive result does not necessarily mean that you have been exposed to the disease.

3. **What reasons could there be for a positive test when you actually do not have the disease?**

   It could be a false positive. Not all assays are specific for a single disease agent. For example, the ELISA for exposure to Lyme disease, which tests for IgG and IgM against the bacteria that cause the disease, has only 72% specificity, and a positive ELISA must be confirmed by a more specific test such as a western blot.

Another cause of false positives is past exposure to a disease. For example, most adults have been exposed to Epstein-Barr virus (EBV) (the cause of many, but not all, cases of mononucleosis), and some people maintain levels of antibodies to EBV for years after clearing the disease. If they present to their physician with a fever and sore throat, they may test positive for EBV, but EBV may not be the cause of their symptoms.

A third cause of false positives is experimental error, such as putting a positive control into a well where you thought you were putting an experimental sample.

4. **Why did you assay your samples in triplicate?**

   Assaying the samples in triplicate is another control. If you do not get the same result in all triplicate wells, you have a problem with your experimental technique or you have made a pipetting error. In a clinical laboratory, the experiment would have to be repeated. If this error occurs in this activity, take the result of the two matching wells since this is probably correct.

5. **When you added serum samples to the wells, what happened to the serum antibodies if the sample was positive? What if it was negative?**

   If the sample was positive, serum antibodies that recognize the purified antigen in the wells bound to the antigen. If the sample was negative, no antibodies bound.

6. **Why did you need to wash the wells after every step?**

   Washing removes any proteins that have not bound to the plastic wells and any antibodies that have not bound to their targets, thus preventing unbound proteins (either antigen or antibodies) from giving false positive results.

7. **When you added secondary antibody, what happened if your serum sample was positive? What if it was negative?**

   If the sample was positive, the secondary antibody bound to the serum antibodies that were bound to the purified antigen adsorbed to the wells. If the sample was negative, there were no serum antibodies bound in the wells and therefore the secondary antibody had nothing to bind to and was washed away.

8. **What antibody-based tests can you buy at your local pharmacy?**

   Test kits that are based on the same principles as the ELISA include home pregnancy and ovulation tests, and tests for the presence of illegal drugs such as marijuana and cocaine.

Laboratory Quick Guide

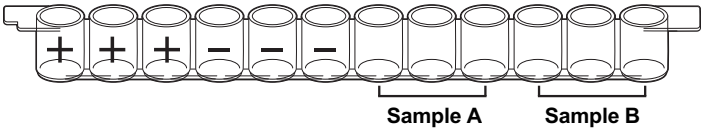
ELISA Antibody Test

Student Workstation Checklist

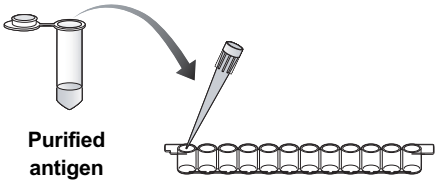
One workstation serves 4 students.

Item (Label)	Contents	Number	(✔)
Yellow tubes	Student test samples (0.25 ml)	4	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (AG)	Purified antigen (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipet		1	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

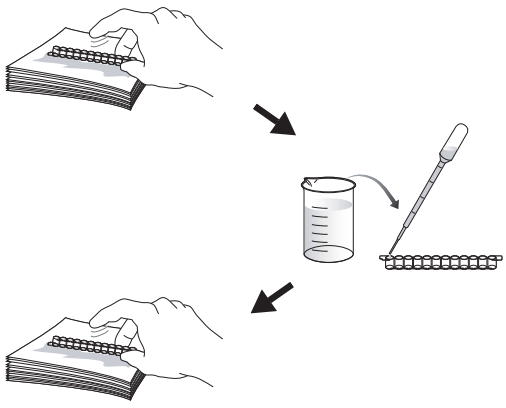
1. Label the yellow tubes (if necessary) to identify the samples being tested.
2. Label your 12-well strip. On each strip label the first 3 wells with a "+" for the positive controls and the next 3 wells with a "–" for the negative controls. Label the remaining wells to identify the samples being tested (3 wells each).



3. Use a fresh pipet tip to transfer 50 µl of purified antigen (AG) into all 12 wells of the microplate strip.
4. Wait 5 minutes for the antigen to bind to the plastic wells.



5. WASH:
   a. Tip the microplate strip upside down onto the paper towels, and gently tap the strip a few times upside down. Make sure to avoid splashing sample back into wells.
   b. Discard the top paper towel.
   c. Use your transfer pipet to fill each well with wash buffer, taking care not to spill over into neighboring wells. Note: the same transfer pipet is used for all washing steps.



d. Tip the microplate strip upside down onto the paper towels and tap.

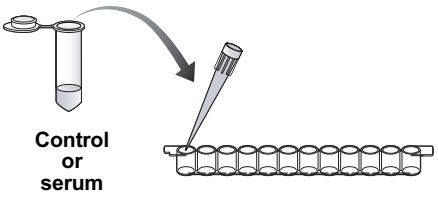
e. Discard the top 2–3 paper towels.

6. Repeat wash step 5.

WASH

7. Use a fresh pipet tip to transfer 50 µl of the positive control (+) into the three "+" wells.

8. Use a fresh pipet tip to transfer 50 µl of the negative control (–) into the three "–" wells.



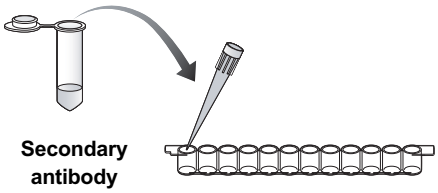
9. Transfer 50 µl of each of your team's serum samples into the appropriately initialed three wells, using a fresh pipet tip for each serum sample.

10. Wait 5 minutes for the antibodies to bind to their targets.

11. Wash the unbound primary antibody out of the wells by repeating all of wash step 5 **two** times.

WASH 2x

12. Use a fresh pipet tip to transfer 50 µl of secondary antibody (SA) into all 12 wells of the microplate strip.

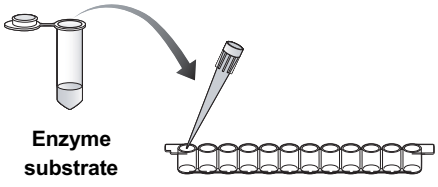


13. Wait 5 minutes for the antibodies to bind to their targets.

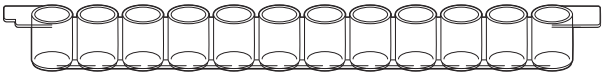
14. Wash the unbound secondary antibody out of the wells by repeating wash step 5 **three** times.

WASH 3x

15. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) into all 12 wells of the microplate strip.



16. Wait 5 minutes. Observe and record the results.



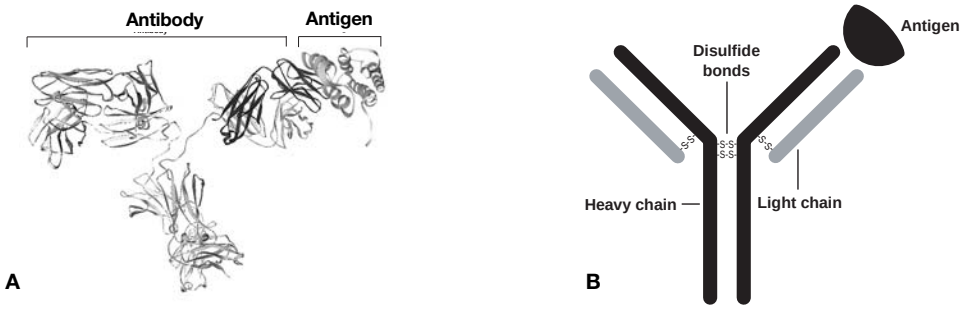
Student Manual

Introduction

You are about to perform an ELISA or enzyme-linked immunosorbent assay, a test that detects antibodies in your blood to determine if you have been exposed to a disease.

When you are exposed to a disease agent, your body mounts an immune response. Molecules that cause your body to mount an immune response are called antigens, and may include components of infectious agents like bacteria, viruses, and fungi. Within days, millions of antibodies — proteins that recognize the antigen and bind very tightly to it — are circulating in your bloodstream. Like magic bullets, antibodies seek out and attach themselves to their target antigens, flagging the invaders for destruction by other cells of the immune system.

Over 100 years ago, biologists found that animals' immune systems respond to invasion by "foreign entities", or antigens. Today, antibodies have become vital scientific tools, used in biotechnology research and to diagnose and treat disease. The number of different antibodies circulating in the blood has been estimated to be between 10^6 and 10^{11}, so there is usually an antibody ready to deal with any antigen. In fact, antibodies make up to 15% of your total blood serum protein. Antibodies are very specific; each antibody recognizes only a single antigen.



A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, (Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

How Are Antibodies Made?

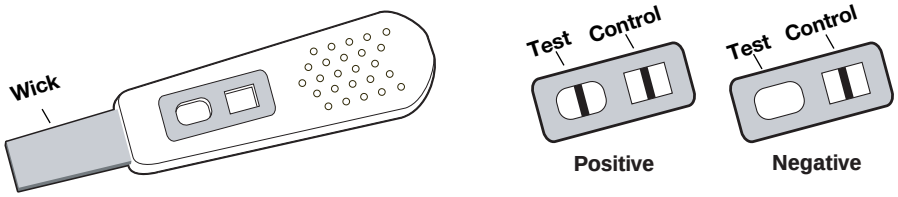
Scientists have learned to use the immune response of animals to make antibodies that can be used as tools to detect and diagnose diseases. The study of the immune system is called "immunology". Animals such as chickens, goats, rabbits, and sheep can be injected with an antigen and, after a period of time, their serum will contain antibodies that specifically recognize that antigen. If the antigen was a disease agent, the antibodies can be used to develop diagnostic tests for the disease. In an immunoassay, the antibodies used to recognize antigens like disease agents are called primary antibodies; primary antibodies confer specificity to the assay.

Other kinds of antibody tools, called secondary antibodies, are made in the same way. In an immunoassay, secondary antibodies recognize and bind to the primary antibodies, which are antibodies from another species. Secondary antibodies are prepared by injecting antibodies made in one species into another species. It turns out that antibodies from different species are different enough from each other that they will be recognized as

foreign proteins and provoke an immune response. For example, to make a secondary antibody that will recognize a human primary antibody, human antibodies can be injected into an animal like a rabbit. After the rabbit mounts an immune response, the rabbit serum will contain antibodies that recognize and bind to human antibodies. The 2° antibodies used in this experiment are conjugated to the enzyme HRP which produces a blue color in the presence of its substrate, TMB. These antibody and enzyme tools are the basis for the ELISA.

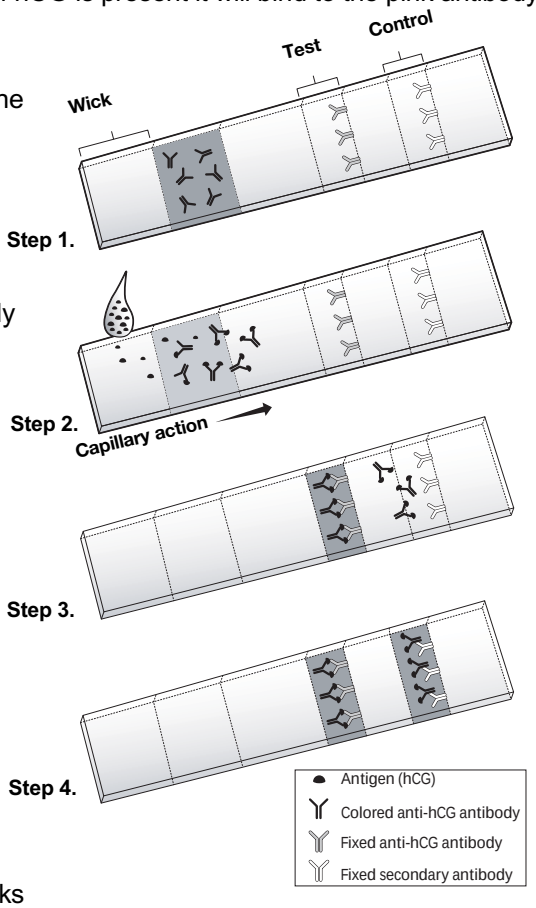
Where Is ELISA Used in the Real World?

With its rapid test results, the ELISA has had a major impact on many aspects of medicine and agriculture. ELISA is used for such diverse purposes as pregnancy tests, disease detection in people, animals, and plants, detecting illegal drug use, testing indoor air quality, and determining if food is labeled accurately. For new and emerging diseases like severe acute respiratory syndrome (SARS), one of the highest priorities of the US Centers for Disease Control (CDC) and the World Health Organization (WHO) has been to develop an ELISA that can quickly and easily verify whether patients have been exposed to the virus. Over-the-counter kits that are based on the same principles as the ELISA include home pregnancy and ovulation tests, and tests for the presence of illegal drugs like marijuana and cocaine.



Some tests give positive or negative results in a matter of minutes. For example, home pregnancy dipstick tests detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization.

The wick area of the dipstick is coated with anti-hCG antibody labeled with a pink compound (step 1). When the strip is dipped in urine, if hCG is present it will bind to the pink antibody, and the pink hCG-antibody complex will migrate up the strip via capillary action (step 2). When the pink complex reaches the first test zone, a narrow strip containing an unlabeled,fixed anti-hCG antibody, the complex will bind and concentrate there, making a pink stripe (step 3). The dipsticks have a built-in control zone containing an unlabeled secondary antibody that binds unbound pink complex (present in both positive and negative results) in the second stripe (step 4). Thus, every valid test will give a second pink stripe, but only a positive pregnancy test will give two pink stripes.

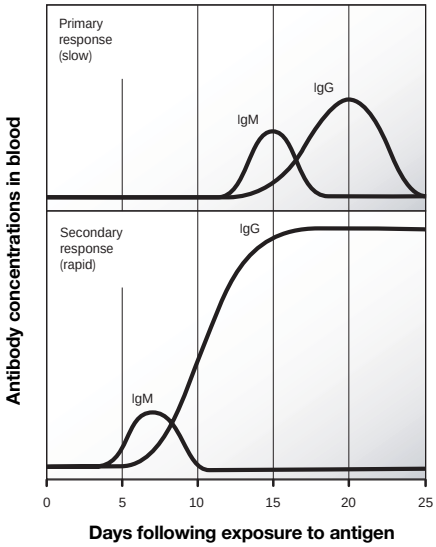


The Immune Response

Following exposure to a disease, the immune system produces antibodies directed against the disease antigens. The typical immune response follows a predictable course. First, the body produces a type of antibody called immunoglobulin M, or IgM. Within a week of disease exposure, IgM can be detected in the blood. Approximately 3 weeks after that, IgM levels drop and levels of another immunoglobulin, IgG, begin to rise. If there is no further disease exposure, the levels of IgG drop rapidly after a few days.

The second time the body is exposed to a disease, the immune response is stronger and faster than the first time. This is the principle behind vaccination (causing the primary response) and booster shots (causing the secondary response). By exposing you to a disease agent that can't harm you (for example, an injection of inactivated influenza virus), your doctor is ensuring that when you are exposed to active flu virus, your immune system will respond rapidly to the invader.

Rate of Seroconversion:



This ELISA protocol is designed to detect the presence of antibodies circulating in the blood that have been produced in response to exposure to a specific disease. The technique is based on the fact that antibodies produced by your body in response to a disease antigen will bind tightly to that antigen — even in a test tube. All that is needed is a source of pure antigen to capture the antibody of interest and a way to visualize the result.

Controls in Immunoassays

For any immunoassay to be valid, it must include both positive and negative controls, i.e., samples that will give known results. Controls are always run side by side with actual samples. If you do not run a positive control and the experiment gives negative results, how can you be sure the results are truly negative? What if the assay simply did not work? If a positive sample gives a negative assay result, it is called a **false negative**. Conversely, if you do not run a negative control and the experiment gives all positive results, how can you be sure the results are truly positive? What if the assay was contaminated with antigen? If a negative sample gives a positive assay result, it is called a **false positive**.

Why Do We Need Controls?

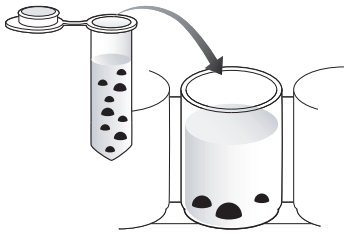
Control samples are necessary to be sure your ELISA is working correctly. Many diagnostic assays give a percentage of false positive or false negative results, so confirmation of diagnosis by a second type of assay is important. For example, immunoassays for antibodies to human immunodeficiency virus (HIV) may give either false positive or false negative results. False positives can result from recent vaccinations, and false negatives can result from immunosuppression (e.g., from drugs given after transplants) or from administering the test too soon after infection with HIV. (Antibodies against HIV do not appear until some weeks after HIV infection; the appearance of antibodies is called seroconversion.) Because of this, positive HIV ELISA tests are always confirmed by performing a western blot.

Controls are also needed to guard against experimental errors. There can be problems with reagents, which can degrade due to age or poor storage conditions. Technicians can make mistakes by choosing the wrong reagents, making errors in dilutions or in pipetting, or failing to remove unbound reagents. Poor record keeping is another source of false assay results. Most of these possibilities can be checked for within the assay with the appropriate controls.

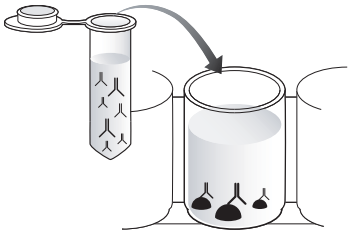
Now let’s put this all together.

The main steps in the ELISA antibody test are:

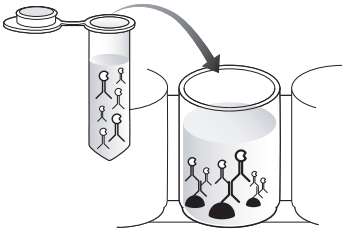
1. Add purified disease antigen to the wells of a microplate strip. Incubate for 5 minutes to allow proteins to bind to the plastic wells via hydrophobic interaction. This is called an immunosorbent assay because proteins adsorb (bind) to the plastic wells.



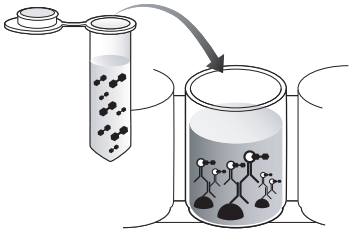
2. Add your serum sample and control samples to the wells and incubate. Serum contains millions of different types of antibodies, but only if your serum contains antibodies that were produced in response to the disease will antibodies bind to the antigen in the wells.



3. Detect the serum (primary) antibodies with HRP-labeled secondary antibody. If serum antibodies have bound to the antigen, the secondary antibodies will bind tightly to the serum antibodies.



4. Add enzyme substrate to the wells, wait 5 minutes, and evaluate the assay results. If the primary antibody was present in your serum sample, the wells will turn blue. This is a positive diagnosis. If the wells remain colorless, the primary antibody was not present in your serum sample, and the diagnosis is negative.



Pre-Lab Focus Questions

1. How does the immune system protect us from disease?

2. How do doctors use the immune response to protect you from disease?

3. What is an example of a disease that attacks the human immune system?

4. What problems can prevent the immune system from working properly?

5. Why is it important to be able to detect antibodies in people who don't appear sick?

6. What does ELISA stand for?

7. Why are enzymes used in this immunoassay?

8. Why do you need to assay positive and negative control samples as well as your experimental samples?

Laboratory Guide

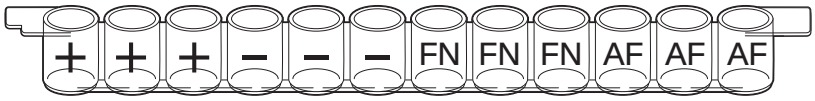
Student Workstation Checklist

One workstation serves 4 students.

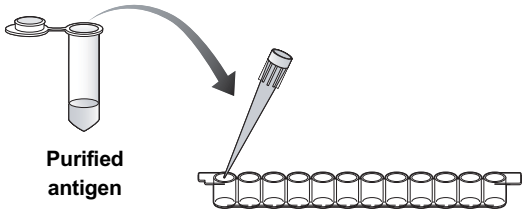
Items	Contents	Number	(✔)
Yellow tubes	Student samples (0.25 ml)	4	❑
Green tube (AG)	Purified antigen (1.5 ml)	1	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipet		1	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

Laboratory Procedure

1. The yellow tubes contain the serum samples that will be tested for the presence of antibodies. Label each yellow tube to identify the sample being tested.
2. Label the outside wall of each well of your 12-well strip. Two students may share a strip of 12 wells. On each strip label the first three wells with a "+" for the positive controls and the next three wells with a "–" for the negative controls. Label the remaining wells to identify the samples being tested. For example, Florence Nightingale and Alexander Fleming would label their shared strip like this:

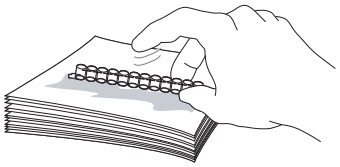


3. Use a pipet to transfer 50 µl of the purified antigen (AG) from the green tube into all 12 wells.

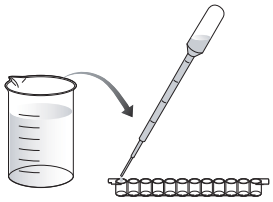


4. Wait 5 minutes while the antigen binds to the plastic wells.

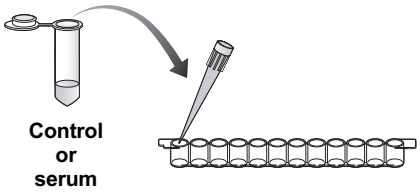
5. Wash unbound antigen out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make sure to avoid splashing sample back into wells.



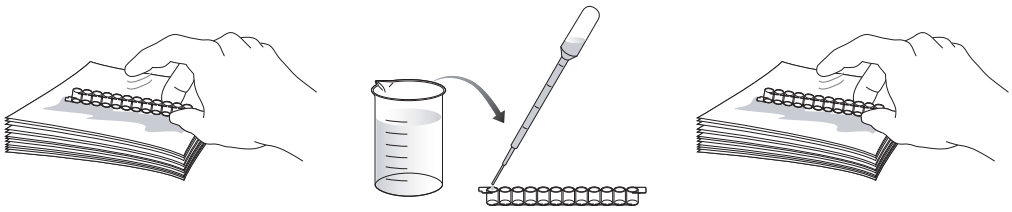
    b. Discard the top paper towel.
    c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer, taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps.



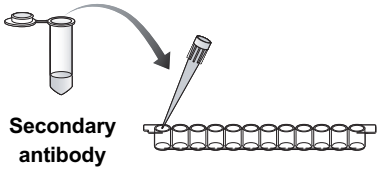
    d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.
    e. Discard the top 2–3 paper towels.
6. Use a fresh pipet tip to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.



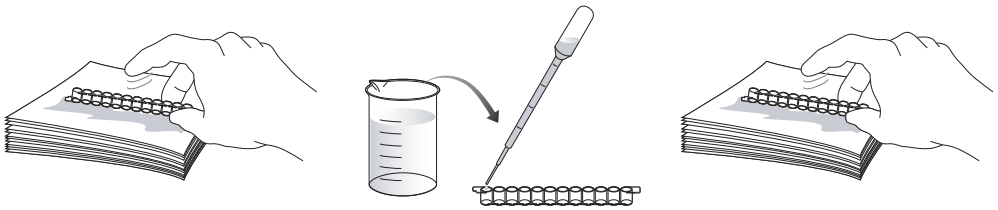
7. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
8. Use a fresh pipet tip to transfer 50 µl of each student's serum sample into the appropriately initialed three wells.
9. Wait 5 minutes to allow the serum antibodies in the samples to bind to the antigen.
10. Wash the samples out of the wells by repeating wash step 5.



11. Use a fresh pipet tip to transfer 50 µl of secondary antibody (SA) from the orange tube into all 12 wells of the microplate strip.



12. Wait 5 minutes for the secondary antibody to bind to the primary antibody.
13. Wash the unbound secondary antibody out of the wells by repeating wash step 5 **two times**.



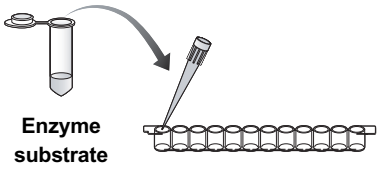
The secondary antibody is attached to an enzyme (HRP) that chemically changes the enzyme substrate, turning it from a colorless solution to a blue solution. Predict which wells of your experiment should turn blue, which should remain colorless, and which wells you are not sure about.

_____

_____

_____

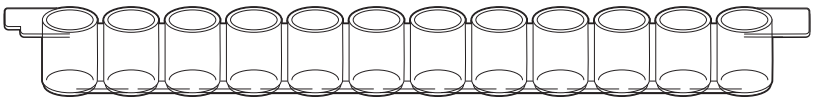
14. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



15. Wait 5 minutes. Observe and record your results.

Results Section

Label the figure below with the same labels you wrote on the wells in step 1. In each of the wells, put a "+" if the well turned blue and a "–" if there is no color change.



Is your sample positive? Explain your results.

Post-Lab Focus Questions

1. Did your serum have antibodies to the disease?

2. If you tested positive for antibodies, does this mean that you have been exposed to the disease?

3. What reasons could there be for a positive test when you actually do not have the disease?

4. Why did you assay your samples in triplicate?

5. When you added serum samples to the wells, what happened to the serum antibodies if the sample was positive? What if it was negative?

6. Why did you need to wash the wells after every step?

7. When you added secondary antibody, what happened if your serum sample was positive? What if it was negative?

8. What antibody-based tests can you buy at your local pharmacy?

Appendix A: Immunological Concepts

Immunity

Immunology is the study of the immune system. The body protects itself from infection using physical and chemical barriers, antibodies that circulate in the blood, and immune cells that attack foreign substances and invading microorganisms. Some types of immune cells adapt to "remember" (recognize) specific invaders, in case of future attacks.

A person is born with certain immunological defenses against pathogens. This is called **innate immunity** and includes circulating macrophages and natural killer cells. These defenses do not change with exposure to pathogens and do not have much specificity for particular pathogens.

Passive immunity is the acquisition of antibodies from an external source, for example, antibodies passed from mother to infant, or certain postexposure vaccines such as that for rabies. Passive immunity lasts only a few weeks, and also does not change with multiple exposures.

Acquired or adaptive immunity is a specific response to specific foreign substances. Although individuals (except for those individuals who are immune-compromised) are born with the ability to respond to these invaders, the system must be activated by an initial contact with the invader. The initial contact, or immunization, begins a cascade of events that allows the body to mount a specific response on subsequent exposure to the invader, hence the term acquired immunity, as initial contact is necessary to acquire the immunity. Acquired immunity is split into two categories: **humoral immunity** involves production of antibodies that circulate in the bloodstream and lymph and bind specifically to foreign antigens, and **cell-mediated immunity** involves the production of T lymphocytes (T cells) that bind and destroy infected cells.

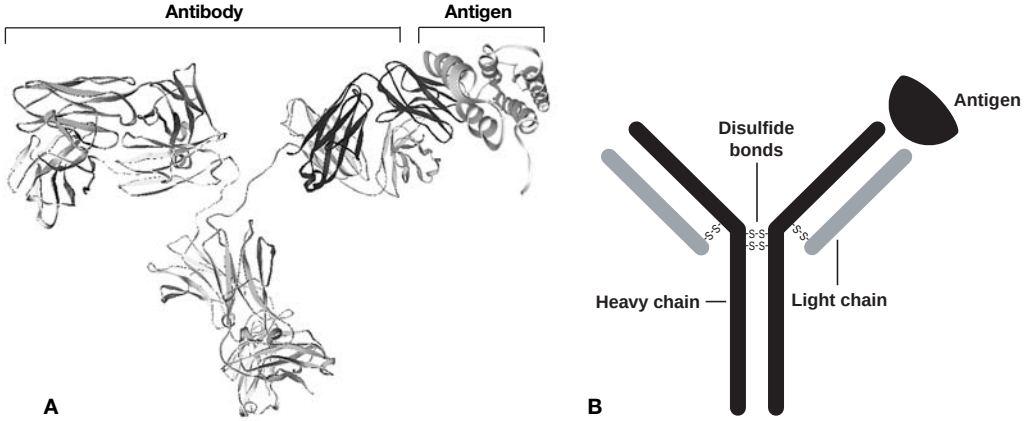
Acquired immunity is the basis for the series of vaccinations that we undergo as we grow up. In the 1790s, long before we had any understanding of the immune system, it was discovered that inoculation with pus from a cowpox lesion prevented infection with smallpox, a disease related to cowpox. The US Centers for Disease Control (CDC) currently recommends childhood vaccination against 12 diseases: measles, mumps, rubella (German measles), diphtheria, tetanus (lockjaw), pertussis (whooping cough), polio, *Haemophilus influenzae* type b (Hib disease), hepatitis B, varicella (chicken pox), hepatitis A, and pneumococcal disease. For travelers abroad, additional vaccinations are recommended (or required, in the case of the US military). The recommendations are based on the traveler's destination. For example, the CDC recommends that travelers to tropical South America be vaccinated against hepatitis A, hepatitis B, rabies (if the traveler will be exposed to animals), typhoid, and yellow fever, plus booster doses for tetanus, diphtheria, and measles.

Components of the Acquired Immune Response

In an immune response, an invasion by something foreign to the body (an **antigen**) generates **antibody** production by B lymphocytes (B cell). Each B lymphocyte generates a unique antibody that recognizes a single shape on an antigen called an **epitope** and thus helps the **immune cells** (including B cells, T cells, and macrophages) to recognize and attack foreign invaders. Everyone (except those who are immune-compromised) has circulating antibodies and lymphocytes that collectively recognize a huge number of antigenic substances.

Antigens can be microorganisms (e.g., viruses and bacteria), microbial products (e.g., toxins produced by some bacteria, or protein components of the microbes), foreign proteins, DNA and RNA molecules, drugs, and other chemicals.

Antibodies are proteins also called immunoglobulins (Ig), that are produced by B cells and can remain attached to B cells or become free floating. There are five classes of immunoglobulins: IgG, IgM, IgA, IgE, and IgD. IgG is the most abundant (Parham) in the internal body fluids, comprising about 15% of total serum protein in adults, and each IgG molecule can bind two antigen molecules. IgM is also in serum and is responsible for the primary immune response. IgA is found in external secretions such as tears, saliva, milk, and mucosal secretions of the respiratory, genital, and intestinal tracts and is a first line of defense against invading microorganisms. IgA is also the only antibody passed from mother to infant. IgD may be involved in regulating the immune response, and IgE is a primary component in allergic reactions.



A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

Epitopes are the specific parts of antigens that are recognized by antibodies. Each antibody recognizes a single epitope, thus multiple antibodies may recognize and bind to different epitopes on a single antigen. For example, an HIV virus particle (virion) has many potential epitopes on its surface that may be recognized by many different antibodies. One particular antibody may recognize the amino terminus of p24, an HIV capsid protein, while another may recognize the carboxy terminus of p24.

Immune cells are the soldiers of the acquired immune response. Macrophages serve two primary functions: 1) removing foreign cells and molecules from the blood, and 2) processing antigens and presenting them on their cell surfaces. Macrophages present antigenic epitopes on their cell surfaces to be recognized by T cells. The T cells draw more immune cells to the site of infection, causing inflammation. Both B cells and T cells are lymphocytes (white blood cells), and each recognizes a single specific epitope. T cells mature in the thymus, and B cells mature in the bone marrow. B cells produce antibodies; the number of different circulating antibodies has been estimated to be between 10^6 and 10^{11}, so there is usually an antibody ready to deal with any antigen. The huge number and diversity of different antibodies are possible because B cells have the ability to rearrange their DNA to make different antibody genes. Like macrophages, B cells present antigenic

epitopes on their surface to attract T cells. T cells have two main functions: they stimulate the proliferation of B cells that have bound to an antigen, and they kill whole cells that are infected by a virus to prevent the virus infecting other cells.

Why We Need an Immune System

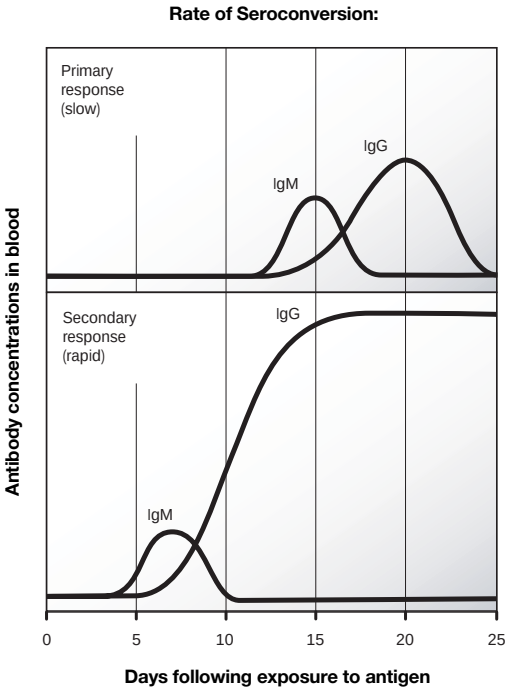
Even bacteria have a rudimentary innate immune system; they make restriction enzymes that destroy foreign DNA from bacterial viruses (bacteriophages), and they protect their own DNA by labeling it as "self" through methylation. Our immune system is at work every day, protecting us from thousands of potential threats, but it is so efficient that we usually don't notice it. Disease can result from infection, genetic defect, or environmental toxins. **Infection** is an invasion by and multiplication of pathogenic (disease-causing) microorganisms. The infection can be 1) transmitted from person to person, like a cold or the flu, 2) transmitted from animals to people (called zoonosis), like rabies or psittacosis, or 3) contracted from the environment, like parasites contracted from water or soil.

The CDC and World Health Organization (WHO) state that **infectious diseases** are the leading cause of death worldwide. Organisms that can cause disease are called **pathogens** and include bacteria, viruses, fungi, infectious proteins called prions, and parasites. Infectious diseases spread in a variety of ways:

Pathogen Spread Through:	Examples
Exchange of body fluids	• HIV, SARS, Epstein-Barr virus (EBV), sexually transmitted diseases
Food	• Foodborne agents like *E. coli* O157:H7, which causes diarrheal disease; prions, which cause Creutzfeldt-Jakob disease (mad cow disease in cattle); or nematodes, which cause trichinosis
Water	• Waterborne agents like the bacteria that cause cholera or the protozoa that cause giardiasis
Inhalation	• Microorganisms like the viruses that cause the flu or the bacteria that cause tuberculosis
Absorption through the skin	• Nematodes like hookworms
Vector transfer (vectors are organisms such as ticks or mosquitoes that carry pathogens fromo one host to another).	• Malaria, West Nile virus, dengue fever, and yellow fever (mosquito vector) • Lyme disease and Rocky Mountain spotted fever (both tick vectors) • Plague (flea vector) • Some diseases, such as Ebola hemorrhagic fever, are presumed to have vectors, but the vectors have not yet been identified

Immune Response

When immunized with a foreign substance (either by vaccination or through natural exposure), an individual mounts an immune response, called the **primary response**. Within 1–2 weeks, there is a risein antibody production directed against the antigen (termed seroconversion), predominated by the IgM class of antibodies. IgM production is usually followed by production of IgG, and after that antibody levels decrease.



The second time that the individual is exposed to the antigen, be it weeks or years after immunization, the immune response is larger and much more rapid. In the **secondary response**, IgM is produced in detectable amounts in a matter of days, followed by a large production of IgG. Other classes of immunoglobulin may also be produced. IgG is generated in much greater quantities, and persists in the blood for a much longer time than in the primary response. Antibody production may continue for months or even years.

Problems With the Immune System

We depend on our immune system to protect us from disease, but when the immune system fails to function correctly, it can cause severe health problems. These problems fall in to three basic categories: hypersensitivity, immunodeficiency, and autoimmune diseases.

Hypersensitive reactions occur when the immune system overreacts to an antigen. The immune system functions are normal in a hypersensitive reaction, just exaggerated in scope, and this can result in illness or even death. There are four types of hypersensitive reactions: 1) anaphylactic reactions or immediate hypersensitivity, generally called allergies, such as food, dust mite, and pollen allergies (the antigen that causes the reaction is called an allergen); 2) cytotoxic reactions, such as transfusion reactions and Rh incompatibility reactions; 3) immune complex reactions, such as farmer's lung, a disease caused by inhaling mold spores; and 4) delayed-type hypersensitivity, such as contact sensitivity (e.g., poison ivy dermatitis and contact dermatitis after exposure to chemicals or environmental agents ranging from metallic nickel to cosmetics).

Immunodeficiency means that an individual is unable to mount an effective immune response, resulting in increased vulnerability to opportunistic infections. There are two types of immunodeficiency: 1) Primary immunodeficiency has a genetic basis. Severe combined immunodeficiency (SCID, "bubble boy" disease) is an example of primary immunodeficiency. Treatments for primary immunodeficiency may include gene therapy. 2) Secondary immunodeficiency has an external cause and is more common than primary immunodeficiency. Secondary immunodeficiency may be caused by an infection, as in the case of HIV/AIDS, by drug treatments, such as immunosuppressive drugs given after organ transplant, or by other health factors, such as poor nutrition, stress, or aging.

Autoimmune disease results from the immune system making a mistake and mounting an immune response against one's own body. Some examples of autoimmune disease include systemic lupus erythematosus (lupus, SLE), rheumatoid arthritis, multiple sclerosis (MS), insulin-dependent diabetes mellitus (IDDM), and celiac disease.

Detecting Infectious Diseases

Infectious diseases are diagnosed by observing symptoms and performing laboratory tests. Diagnostic tests may look for the microorganism itself or some part of it (e.g., bacterial or viral antigens), microbial products (e.g., bacteria toxins), or reactions of the body to the disease agent. The latter may include testing for signs of an immune response to the disease agent (e.g., antibodies) or for indications of effects of the disease agent on the body (e.g., abnormal enzyme activity or protein levels). In the last decade, tests to detect microbial RNA and DNA have become common.

Laboratory tests cover a wide variety of methods, some of which have been in use for decades and others, like the tests for RNA and DNA from disease agents, which are very new. Depending on the test and putative diagnosis, laboratory tests may look for signs of disease in most body fluids, including blood, urine, stool samples, cerebrospinal fluid, and saliva. In the US, the Food and Drug Administration regulates laboratory tests.

The first tests for detecting and identifying microorganisms from clinical samples used antisera directed against specific microbes. The antibodies were labeled with a fluorescent tag, and the microorganisms could be detected with microscopy when the antibodies bound to them. Other early diagnostic tests include: 1) culture methods, in which microorganisms from clinical samples are grown on different culture media and their growth and appearance observed (frequently takes weeks to get results); 2) identification of microbe-specific antibodies in serum by immunoassays such as ELISA; and 3) agar diffusion assays, in which antisera and antigens are placed in holes in agar plates. Both diffuse into the agar, and where antibodies encounter antigens for which they are specific, they bind. Upon antibody-antigen binding, a visible precipitation band forms. Many of these tests are still in use.

Current diagnostic tests include:

Test Type	Description	Examples
Immunofluorescence assay (IFA)	Specific microorganisms detected with fluorescently labeled antibodies	• *E. coli* O157:H7 • Identifying respiratory viruses
Agglutination	Visible precipitates appear when antibodies and specific antigens come in contact	• Gram-positive bacteria, such as *Staphylococcus aureus* (e.g., Staph A) • Fungi, such as *Cryptococcus neoformans* and Candida species
Immunochromatography tests	Card or dipstick-based immunoassays	• *E. coli* O 157:H7 • *Legionella* • *Mycoplasma pneumoniae*
Microplate tests	ELISA or RIA (radioimmunoassays) used to detect microbial antigens, microbial products, and antibodies against the microorganisms and their products. RIA uses radioactive labels to replace the enzymes used in ELISA	• *E. coli* O157:H7 • *Legionella* • Influenza viruses • HIV antigen • HIV antibodies • *Giardia*
Molecular methods	Detection of microbial RNA or DNA; also used to detect microbial drug resistance (AST); may use PCR	• *Mycobacterium tuberculosis* (TE) • HIV • *Chlamydia trachomatis* • Cytomegalovirus (CMV) • AST (antimicrobial susceptibility testing)
Microscopy	Visual identification based on staining with specific reagents or on physical characteristics	• Electron microscopy of Ebola virus • Light microscopy of parasites such as protozoa, helminthes, etc.

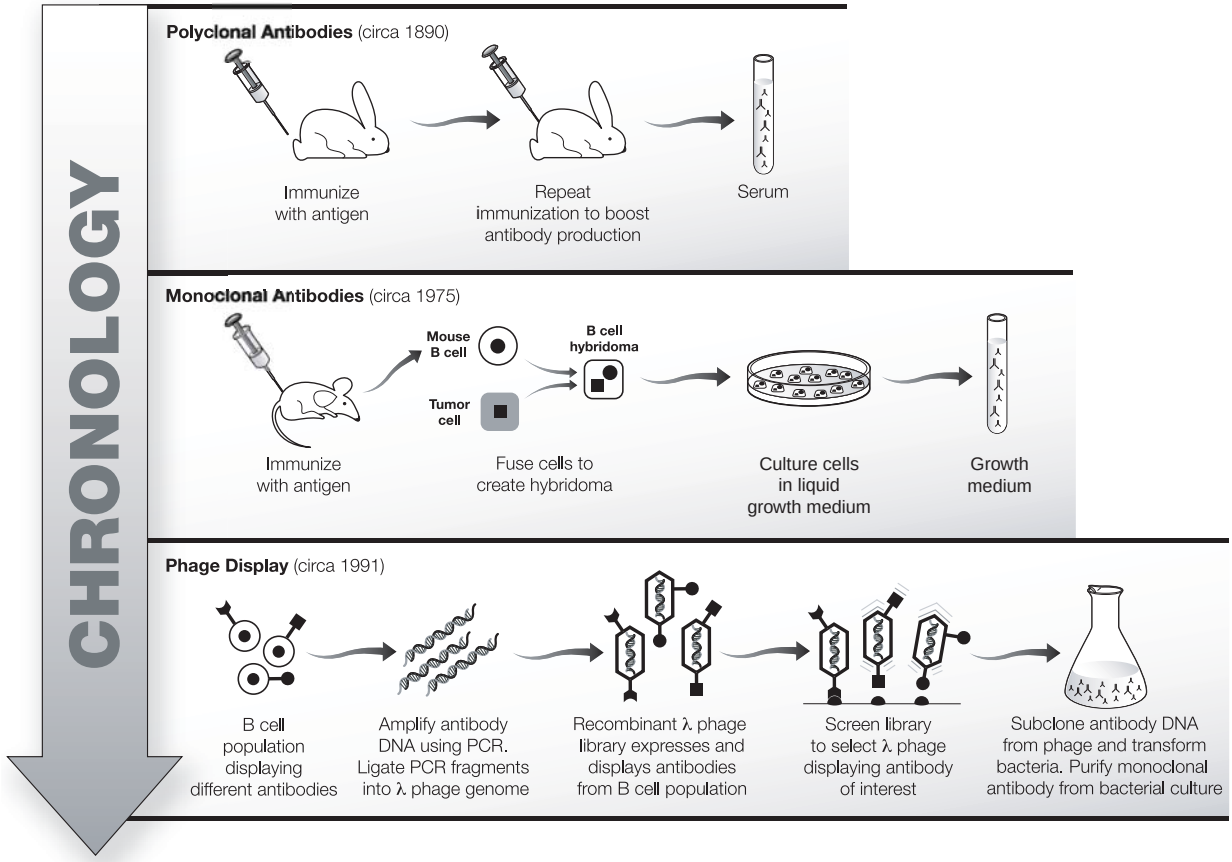
Boosting the Immune System With Vaccination

Doctors use the immune response to give us resistance to infectious diseases before we are exposed to them. Through vaccination, we are exposed to non-harmful forms of the pathogen that invoke an immune response. We also frequently need booster shots to invoke the secondary response to maintain the antibody levels in our blood. Vaccines used in immunization may be of several types:

1) **Live attenuated vaccines** are weakened (attenuated) microbes, that are nonpathogenic. Using current technology, deletion or inactivation of microbial genes weakens the pathogens so they can be used in vaccines; previously, less pathogenic strains were selected from natural populations. Examples of live vaccines include those against polio (Sabin type), measles, mumps, and smallpox.
2) **Killed or inactivated vaccines** are made of microbes killed by heat or chemicals. Killed vaccines are much safer than live vaccines, particularly for individuals with compromised immune systems, but they do not usually provoke as strong an immune response as do live vaccines. Examples of killed vaccines include those against rabies, cholera, polio (Salk type), and influenza.
3) **Subunit vaccines** are made from pieces of microbes. They consist of one or more antigens from either the disease agent or a microbial product, and they may be derived from the organisms or engineered using molecular biology. Examples of subunit vaccines include those against hepatitis B, anthrax, and tetanus.
4) **DNA vaccines** are a recent approach to vaccine development. DNA that codes for microbial antigens is cloned into a vector, and the naked DNA is injected into the patient. The DNA is taken up by cells, transcribed, and translated, and the resulting antigenic protein elicits an immune response. No DNA vaccines are yet available, but some are in clinical trials.
5) **Antibody vaccines** are another innovation in vaccine development. The ability to construct human monoclonal antibodies using recombinant DNA technology means that antibodies prepared against specific antigens may be used safely in humans. For example, a human monoclonal antibody against an antigen involved in anthrax infection may soon be in clinical trials.
6) **Postexposure vaccinces (immunotherapy)** are used to treat a disease. Some immunotherapies have been used for years (e.g., administering immune serum globulin after exposure to hepatitis and administering equine antivenin for snakebite), but there are not many other current vaccine-based immunotherapies. Probably the best known is postexposure rabies vaccination, consisting of 5 doses of rabies vaccine over 30 days. If the vaccine regimen is begun promptly after exposure, it is 100% effective in preventing disease. Smallpox vaccination also provides protection even when administered 2–3 days postexposure. If the smallpox vaccine is administered as late as 5 days after exposure, it may prevent smallpox from being fatal, although it will not prevent the disease.

Tapping Nature's Toolkit: Manufacturing Antibodies

Antibodies used in research can be manufactured in the laboratory, both in vivo and in vitro. In vivo techniques have been in use for over 100 years. There are two types of traditionally produced antibodies: **polyclonal antibodies** and, in the last 30 years, **monoclonal antibodies**. Currently, antibody production is being revolutionized by recombinant DNA technology and, while most antibodies are still produced by traditional methods using animals or animal cells, techniques for making antibodies using recombinant DNA technology are becoming more common.



Timeline of antibody production technology.

Polyclonal Antibodies

Polyclonal antibodies are generated by immunizing an animal (usually a rabbit, goat, or sheep) and obtaining serum. For example, purified HIV gp120 protein can be injected into a goat, which will then generate antibodies directed against the many epitopes of gp120. (Remember that the goat will produce many different antibodies to the multiple epitopes of an antigen.) Blood containing the antibodies is drawn from the goat and the cells of the blood are removed, leaving the serum. The product is **antiserum** towards gp120, and the antiserum can be used directly or the antibodies can be purified from it. The antibodies are called polyclonal because the antibodies are from many (*poly*) B cell clones (*clonal*) in the goat's blood. Polyclonal antiserum has the advantage of being simple and inexpensive to produce, but the disadvantage is that no two batches, even made in the same animal, will be exactly the same.

Monoclonal Antibodies

For many antibody applications such as diagnostic tests, polyclonal antibodies are too variable. In these cases, one antibody type from a single B cell clone is preferable. B cell clones producing single antibodies can be isolated from the spleens of immunized mice, but these cells die after a few weeks in the laboratory, limiting production of the large amounts of antibody generally needed for research and commercial applications. However, B cells can be made to live (and produce antibodies) indefinitely if they are fused with tumor-like immortal cells. The fusion generates hybrid cells (a hybridoma cell line), which can be cultured indefinitely; the monoclonal antibodies generated by the hybrid cells can be collected and purified from the growth medium with almost no batch-to-batch variability.

Genetically Engineering Antibodies

The ability of antibodies to act like magic bullets and home in on their targets makes them ideal candidates for medical therapies. For example, an antibody that recognizes a tumor antigen can be attached to a chemotherapy drug or radioactive molecule and be used to deliver the drug specifically to targeted tumor cells, sparing the patient many of the side effects of conventional chemotherapy or radiation treatment. However, traditional antibodies made in animals are seen by the human immune system as foreign and elicit an immune response that results in their destruction. Recombinant DNA technology can be used to produce antibodies that look human to the human immune system and so can be used as therapeutic agents in people. (For example, Herceptin is a “humanized” antibody used to treat breast cancer.) Using genetic engineering to manufacture antibodies also obviates the sacrifice of laboratory animals. Two of the methods used to engineer antibodies are described below.

Hybridoma Immortalization

Recombinant DNA technology allows the antigen recognition site from a known mouse monoclonal antibody to be camouflaged within a human antibody by combining part of the mouse gene with the human antibody gene. Bacteria transformed with this DNA are capable of producing humanized monoclonal antibodies indefinitely, with the added bonus that culturing bacteria requires much less time and expense then the culture of a mouse hybridoma cell line.

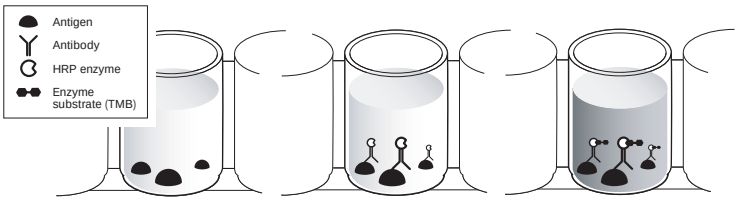
Phage Display

Novel antibodies to antigens are being generated using modern biotechnology. Libraries of billions of potentially useful antibodies are being created by inserting shuffled antibody genes from billions of human B cells into the genomes of bacteriophage lambda (bacteriophages, or phages, are viruses that infect bacteria; lambda phage is a specific species of phage), so that the lambda phages display the binding sites from human antibodies on their surfaces. This **phage library** is screened to find a phage that binds to a specific antigen. The phage can then be used directly as an antibody would be used. Alternatively, the DNA from the selected phage can be cloned into a human antibody gene and transformed into bacteria. Large amounts of the antibody can then be produced for therapeutic use. Phage display is on the cutting edge of immunotherapy.

Labeling and Detecting Antibodies

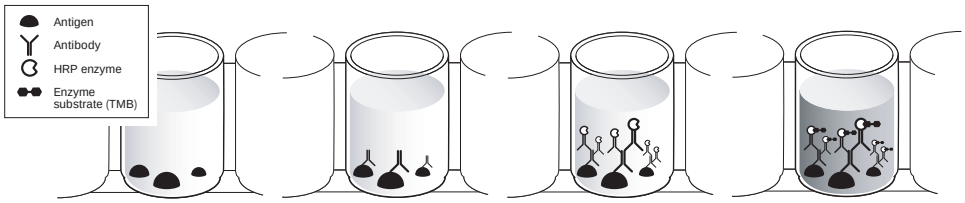
Antibodies are used in diagnosis and research as labeling tools. As labels they have to be made visible, so antibodies are covalently linked (or conjugated) to chemical labels that emit detectable signals. Detection systems can be low-tech or high-tech, and the detection system determines the type of label used. For example, a fluorescently labeled antibody allows you to localize an antigen in a cell using a high-tech fluorescent microscope. Antibodies are also linked to enzymes that oxidize a chromogenic (color-producing) substrate, producing visible color only where the enzyme-linked antibody has bound. Enzyme-linked antibodies are commonly used in western blots, microscopy, and ELISA.

Antibody targets or antigens can be detected directly by labeling the antibody specific for the antigen and looking for signal.



Direct detection of antibodies.

However, labeling every type of antibody scientists might wish to use is time-consuming and costly. Thus, a more common method to visualize antigens is called indirect detection. This technique relies on the use of polyclonal secondary antibodies. Secondary antibodies recognize primary antibodies. The primary antibody binds specifically to the antigen, and the secondary antibody binds specifically to the primary antibody. The indirect method means that only one type of enzyme-linked secondary antibody is needed to visualize all antibodies produced in one type of animal (e.g., in rabbits), reducing time and cost. Indirect detection adds a bonus, since the primary antibody is effectively an antigen to the secondary antibody. The primary antibody has many different epitopes and so is bound by multiple secondary antibodies. Thus, more labels accumulate around the antigen, amplifying the signal.



Indirect detection of antibodies.

Secondary antibodies are produced by injecting the antibodies of one animal into a different species of animal. For example, if the primary antibody is a mouse monoclonal antibody, secondary antibodies are generated by immunizing a goat with any mouse antibody. Goat polyclonal anti-mouse IgG is purified from the goat serum and linked to an enzyme for detection. Secondary antibodies are commercially available, either unlabeled or with a wide variety of fluorescent or enzymatic labels for many applications.

Putting Antibodies to Use

Antibodies have been used for decades as research tools, but in recent years the expansion of technology to produce antibodies has yielded a myriad of new applications that take advantage of the specificity of antibody binding. The basis of all immunoassays is the specific binding of an antibody to its antigen, and there are many ways that binding can be utilized. Here are some of those uses:

Immunostaining localizes antigens in organelles, cells, tissues, or whole organisms, and can also be used to distinguish one cell type from another. For example, pathologists can identify cancer cells using immunostaining. Cancer cells frequently look identical to normal cells under the microscope, but when they are immunostained, variations in the amount and kinds of cell surface proteins (antigens) are revealed. Studying this information helps diagnose cancer, and it can help in our understanding of how cancer cells cause harm.

Immunostaining tissues or organisms can tell us in what cell types a protein is normally found, which can help us understand the protein's function. For instance, immunostaining of plant seedlings at different stages of maturation allows us to follow how a protein's abundance and localization change as the plant grows. Antibodies for immunostaining are labeled with either fluorescent molecules or enzymes that produce colored signals upon addition of a substrate.

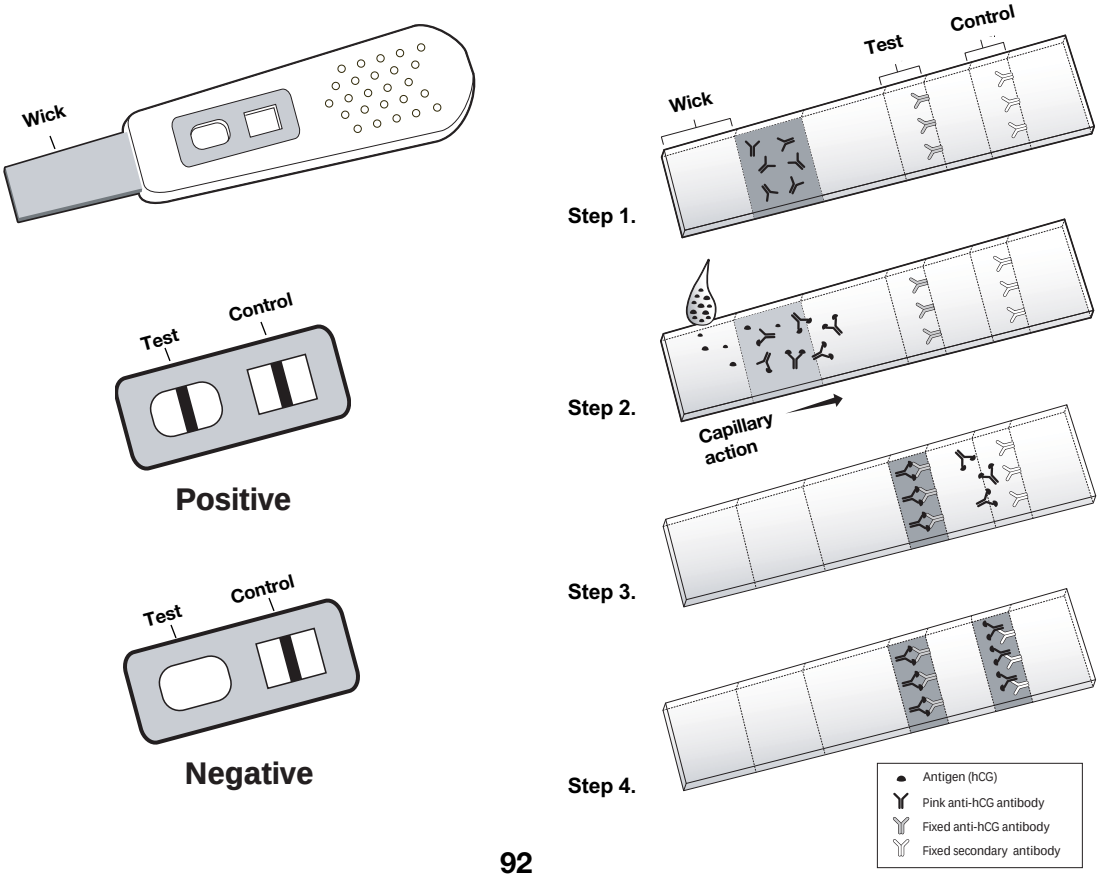
A special application of immunostaining is fluorescence-activated cell sorting (FACS), in which a population of cells is stained with a fluorescently labeled antibody and then physically separated into labeled and unlabeled cells. The cell sorter uses lasers to detect the fluorescent labels and an electrostatic charge to sort the cells in solution. Cell sorters can separate as many as 30,000 cells per second!

Immunoblotting or western blotting tells us about a protein's size and relative abundance in a given sample. In western blotting, an antibody picks out a specific protein from a complex sample (usually lysed cells or tissue) that has been separated by size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins separated in SDS-PAGE gels are transferred (electroblotted) from the gel to the surface of a nylon or nitrocellulose membrane using an electrical current. The membrane is probed with a primary antibody that is specific for the protein of interest, and then an enzyme-linked secondary antibody is used to visualize the protein. The enzyme oxidizes a colorimetric substrate, producing a colored band on the membrane. Alternatively, the oxidized substrate may emit light (chemiluminescent substrate) that is detected as a band on photographic film. The size of the protein is determined by comparing the position of the band to the position of known protein standards that are run alongside it on the SDS-PAGE gel. The abundance of the protein is determined by comparing band intensity to known amounts of protein standards run on the same gel.

A modification of immunoblotting is called **dot blotting**, in which a sample is spotted onto a membrane directly rather than being blotted from a gel. Dot blotting is used for rapid

screening of a large number of samples. This technique provides a rapid determination of whether a particular protein or antigen is present, as many samples may be spotted on a membrane and processed simultaneously, but dot blotting provides no information about the size of the protein.

There are many varieties of **dipstick tests**, including home pregnancy tests, tests for illegal drug use (e.g., marijuana, cocaine, and methamphetamines), and tests for infectious agents (e.g., HIV, plague, *E. coli* O157, and *Legionella*). These immunochromatography assays give positive or negative results in a matter of minutes and are called sandwich immunoassays because they rely on the use of multiple antibodies. One of the antibodies is labeled with a colored compound, such as colloidal gold, which produces a pink band on the test strip in positive assays. For example, home pregnancy dipstick tests detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization. The wick area of the dipstick is coated with mouse monoclonal anti-hCG antibody labeled with colloidal gold, a pink compound (step 1). When the strip is dipped in urine, if hCG is present it will be bound by the pink antibody and the pink hCG-antibody complex will migrate up the strip via capillary action (step 2). When the complex reaches the test zone, a narrow strip containing a fixed unlabeled goat polyclonal anti-hCG, the pink complex will bind and concentrate there, making a pink stripe (step 3). The dipstick tests have a built-in control zone containing polyclonal goat anti-mouse IgG. The unbound pink mouse anti-hCG (present in both positive and negative results) will continue to migrate up the strip, past the test zone, and bind in the control zone, giving a second (or first, if the test is negative for pregnancy) pink stripe (step 4). If no pink stripe appears in the control zone, the test did not function properly.



Appendix B: Glossary

3,3',5,5'-tetramethylbenzidine (TMB): A soluble colorimetric substrate, oxidized to a blue color by horseradish peroxidase and frequently used in ELISA assays.

Acquired immunity: A specific response to specific foreign substances that adapts with multiple exposures. Also called adaptive immunity.

Antibody: Immunoglobulin protein formed in response to a challenge of the immune system by a foreign agent. Antibodies bind to specific antigens.

Antigen: Any agent that provokes an acquired immune response and is bound specifically by either antibodies or T cells.

Antiserum: Blood serum containing antibodies raised against a specific antigen.

Autoimmune disease: Disease that results from the immune system making a mistake and mounting an immune response against one's own body. Examples are systemic lupus erythematosus (lupus, SLE), rheumatoid arthritis, and multiple sclerosis (MS).

Bacteriophage: A virus that infects bacteria; also called a phage. Can be used to introduce foreign DNA into a bacterial genome.

Chromogenic: Color-producing. Substrates that produce a colored product when acted upon by an enzyme are termed chromogenic substrates; for example, 3,3',5,5'-tetra-methylbenzidine (TMB) produces a blue product when oxidized by horseradish peroxidase.

Clone: In the context of molecular biological techniques, "to clone" means to obtain a fragment of DNA from a genome and ligate it into another piece of DNA, such that the ligated DNA now has an identical copy of that gene fragment. In the context of cell biology, "a clone" is a cell or group of cells that are all derived through cell division from the same parent cell and thus have identical genetic data.

Conjugate: A substance formed by the covalent bonding of two types of molecules, such as horseradish peroxidase linked to ("conjugated to") an antibody.

Enzyme: A protein with catalytic activity. The molecule that an enzyme acts on is called its substrate. Enzymes are classified (and frequently named) on the basis of the reactions that they catalyze. For example, a peroxidase oxidizes its substrate.

Epitope: A specific site on an antigen that is recognized by an antibody. Also called antigenic determinant.

Genetically modified organism (GMO): An organism whose genetic material (DNA) has been altered in a way that does not occur naturally by mating or natural recombination.

Horseradish peroxidase (HRP): An enzyme frequently used to label secondary antibodies. HRP oxidizes substrates (e.g., TMB) for colorimetric detection.

Immune cell: Any cell of the immune system, including lymphocytes (B and T cells) and macrophages.

Immunodeficiency: Weakening or defects of the immune response such that an individual is unable to mount an effective immune response. May have a genetic basis, result from a disease or other health factor, or be caused by immunosuppressive drugs.

Immunogen: Any agent that provokes an immune response. Immunogens that provoke a response from the acquired immune system are called antigens.

Immunoglobulin (Ig): General term for all types of antibodies.

Immunology: The study of the immune system, the body system that protects the body from foreign substances, cells, and tissues by producing an immune response.

Innate immunity: The immunity with which a person is born. Includes cells such as circulating macrophages that respond to foreign invaders. Also called nonadaptive immunity.

Ligate: To connect pieces of DNA together, for example, inserting a fragment of an antibody gene into a phage genome.

Lymphocyte: Type of white blood cell. Component of the immune system, includes T cells (thymus-derived) and B cells (bone marrow-derived).

Macrophage: A type of white blood cell that binds and engulfs foreign materials and antigens in a process called phagocytosis. Macrophages serve two primary functions: 1) removing foreign cells and molecules from the blood; and 2) processing antigens and presenting them on their cell surfaces.

Microplate: Molded plastic plate consisting of multiple small wells, usually in a 96-well format.

Opportunistic infections: Infections that occur as a result of deficiencies in the immune system, e.g., diseases like oral candidiasis and tuberculosis that occur in immunodeficient AIDS patients.

Passive immunity: The acquisition of antibodies from an external source, e.g., antibodies passed from mother to infant, or certain postexposure vaccines such as that for rabies. Passive immunity lasts only a few weeks and does not change with multiple exposures.

Pathogens: An organism that can cause disease. Pathogens include bacteria, viruses, fungi, infectious proteins called prions, and parasites.

Phage display: A method of producing novel antibodies to specific antigens using recombinant DNA technology and bacterial viruses (bacteriophages).

Primary antibody: In an immunoassay, the antibody that binds a specific antigen, conferring specificity to the assay.

Secondary antibody: In an immunoassay, the antibody that recognizes the primary antibody, which is from a different species. Secondary antibodies are frequently labeled for easy detection.

Serum (plural sera): The clear fluid obtained when the solid components (e.g., red and white blood cells) are removed from whole blood.

Seroconversion: The development of detectable antibodies in the blood directed against an infectious agent. For example, after a person is exposed to HIV, no antibodies against HIV can be detected in the blood for approximately 6 weeks. Once seroconversion has occurred, antibodies against HIV can be detected (e.g., by ELISA).

Subclone: To transfer an already cloned fragment of DNA into another carrier vector, such as an expression plasmid.

Substrate: The target molecule for an enzyme.

TMB: see 3,3',5,5'-tetramethylbenzidine.

Vaccination: The process of inducing acquired immunity by deliberately stimulating an immune response with a nonpathogenic form of a disease agent. Also called immunization or immunoprophylaxis. The term vaccination originated from the Latin word for cow (*vacca*) because the first vaccination used cowpox to vaccinate against smallpox.

Vector: An organism that carries pathogens from one host to another. Vectors are frequently arthropods, e.g., ticks or mosquitoes.

Zoonosis (plural zoonoses): An infection transmitted to humans from an animal host, e.g., SARS and rabies.

Appendix C: Disease Descriptions

HIV/AIDS

Protocol Fit:

- I: ELISA for Tracking Disease Outbreaks
- II: Antigen Detection ELISA
- III: ELISA Antibody Test

Name of pathogen	Human immunodeficiency virus or HIV
Type of organism	Virus
Infectious agent	Virus
Method of spread	Exchange of body fluids Sharing needles (illicit drug use; developing nations) Mother to child during pregnancy Blood transfusion or organ donation (rarely, since all donated blood and organ donors are tested) Not spread through casual contact
Incubation	2 months to over 10 years
Symptoms	Flu-like symptoms within 1–2 months of exposure During asymptomatic period (2 months to >10 years), the immune system declines, and symptoms may include lack of energy, weight loss, frequent fevers and sweats, persistent or frequent yeast infections (oral or vaginal), persistent skin rashes or flaky skin, and short-term memory loss At onset of AIDS, opportunistic infections produce symptoms which may include coughing, shortness of breath, seizures, lack of coordination, difficult or painful swallowing, mental symptoms such as confusion and forgetfulness, severe and persistent diarrhea, fever, vision loss, nausea, abdominal cramps, weight loss, extreme fatigue, severe headaches, and coma.
Infectivity	From time of exposure
Diagnosis	In the first 4–8 weeks after exposure, virus can be detected by ELISA against viral protein p24 or by western blot. After 4–8 weeks, antibodies against HIV can be detected by ELISA.
Treatment	Nucleoside reverse transcriptase (RT) inhibitors, e.g., AZT Protease inhibitors Combination drug treatments Treatments for opportunistic infections
Mortality	Varies widely with geographic location of patients
History as a pathogen	Earliest known case in 1959. HIV may have spread from an animal host to man. The disease was described and named acquired immune deficiency syndrome (AIDS) in 1982; HIV, the cause of AIDS, was isolated in 1983.

Suggested scenarios for the classroom: A role-playing exercise in which students act as scientists working in a clinical laboratory of the local hospital, performing HIV tests on patient samples. There are ELISAs for the HIV antigen (HIV capsid protein p24) and for serum antibodies against HIV. This can be further expanded to include biographies of the patients to aid discussion on lifestyle behavior and risk of HIV infection.

Simulation for detecting p24 HIV capsid protein.

The table below gives an example of how a diagnostic test to detect HIV virions in a patient's blood sample can be simulated using protocols I and II.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Students samples	Yellow	1x antigen or 1x PBS	Sample derived from patient's blood
Primary antibody	Green	1x primary antibody	Anti-p24 capsid protein antibody from mouse
Secondary antibody	Orange	1x secondary antibody	Anti-mouse immunoglobulin antibody conjugated to HRP
Positive control	Violet	1x antigen	Heat-inactivated viral antigen (p24 protein)
Negative control	Blue	1x PBS	HIV-negative human serum

Simulation for detecting HIV antibodies.

The table below gives an example of how a diagnostic test to detect antibodies to HIV in a patient's serum sample can be simulated using protocol III.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Purified antigen	Green	1x antigen	Purified HIV proteins
Student samples	Yellow	1x primary antibody or wash buffer	Serum sample* from patient
Secondary antibody	Orange	1x secondary antibody	Anti-human immunoglobulin antibodies conjugated to HRP
Positive control	Violet	1x antigen	Serum from an HIV-negative patient spiked with HIV antibodies
Negative control	Blue	1x PBS	Serum from an HIV-negative patient

*Note: Tests to detect anti-HIV antibodies in saliva and urine are also used.

Smallpox

Protocol Fit:

- I: ELISA for Tracking Disease Outbreaks
- II: Antigen Detection ELISA
- III: ELISA Antibody Test

Name of pathogen	*Variola major*, smallpox virus
Type of organism	DNA virus in genus *Orthopoxvirus*
Infectious agent	Virus (only a few virus particles needed for infection)
Method of spread	Person to person via aerosols from infected individual. Can also be spread through infected clothing, bedding, etc. Intentional dissemination of aerosolized virus; if not exposed to UV light, virus may be viable for ≥24 hours under optimal conditions.
Incubation	12–14 days
Symptoms	High fever, malaise, headache, backache, abdominal pain, rash
Infectivity	Most infectious at onset of rash; contagious until rash is totally gone.
Diagnosis	Initially by symptoms. Virus identification by electron microscopy. Identification of viral DNA by molecular biological methods. Development of ELISA against IgG, IgM, and antigen high priority with CDC and many companies (smallpox was eradicated before current immunoassays were developed).
Treatment	Postexposure vaccination within 2–3 days of exposure protects against the disease. Postexposure vaccination within 4–5 days of exposure may prevent a fatal outcome. After 4–5 days postexposure, supportive treatment only. Experimental treatment with antiviral drugs.
Mortality	Up to 30%
History as a pathogen	Used for biological warfare by British in North America in the 18th century. Army distributed blankets that had been used by smallpox patients. Mortality in some Native American tribes was 50%. In 1796, Jenner discovered cowpox vaccine effective against smallpox. Global immunization effort eradicated smallpox in 1977, and routine vaccination ceased. May have been weaponized in the Soviet Union in the 1980s

Suggested Scenario for the Classroom Using Protocols I & II: Bioterrorism on a Field Trip

Your students have been on a field trip. While on public transportation (aircraft, bus, subway, train), it is possible that they have been exposed to smallpox, deliberately released in aerosol form. (Smallpox virus, if not exposed to UV light, may survive for ≥24 hours in cool, dry locations.)

It is important to determine as soon as possible which students have been exposed. Vaccination within 2–3 days of exposure can prevent smallpox, but the vaccine is in short supply. Also, vaccination can have nasty side effects, so no one should be vaccinated unnecessarily. Vaccination of exposed individuals is essential to prevent further spread of the disease.

To determine which students have been exposed, perform an ELISA to detect the virus in samples of their body fluid.

Students who test positive for the virus should undergo immediate vaccination.

Simulation for detecting smallpox virus.

The table below gives an example of how a diagnostic text to detect smallpox virus in patient samples can be simulated using protocols I and II.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Students samples	Yellow	1x antigen or 1x PBS	Sample from patient's lesion
Primary antibody	Green	1x primary antibody	Anti-*variola* antibody from mouse
Secondary antibody	Orange	1x secondary antibody	Anti-mouse immunoglobulin antibody conjugated to HRP
Positive control	Violet	1x antigen	Sample from lesion of chickenpox patient spiked with *variola* proteins
Negative control	Blue	1x PBS	Sample from lesion of chickenpox patient

Suggested Scenario for the Classroom Using Protocol III ELISA Antibody Test: Are prior smallpox immunizations in healthcare providers still current?

In the event of a smallpox outbreak, healthcare providers will be needed to tend to the sick and this will put them at risk of becoming infected. It has been proposed that teams of healthcare workers should be vaccinated in preparation for a smallpox attack. However, since the smallpox vaccine can have detrimental side effects, the ethics of this proposition has been questioned; i.e., should someone assume such a risk in order to prepare themselves for a situation that has not yet occurred? A partial solution to this dilemma is to test older healthcare providers who may have already been given the smallpox vaccination. In this scenario, your students act as lab technicians testing serum samples from healthcare providers for the presence of anti-smallpox antibodies to determine whether they still have immunity to the disease. The smallpox vaccine was routinely given in the U.S. until 1972, was recommended for health care providers until 1976, and was administered in the military until 1990.

Simulation for detecting smallpox antibodies.

The table below gives an example of how a diagnostic test to detect antibodies to smallpox virus in a patient's serum sample can be simulated using protocol III.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Purified antigen	Green	1x antigen	Purified smallpox proteins
Student samples	Yellow	1x primary antibody or wash buffer	Patient's serum sample
Secondary antibody	Orange	1x secondary antibody	Anti-human immunoglobulin antibodies conjugated to HRP
Positive control	Violet	1x antigen	Serum from smallpox patient
Negative control	Blue	1x PBS	Serum from a patient without smallpox

- Note: At press time, an ELISA to detect smallpox had not yet been developed, although it is a high priority with the CDC. Thus, these scenarios are hypothetical.

Trichinosis

Protocol Fit:

- III: ELISA Antibody Test

Name of pathogen	*Trichinella spiralis* and 4 other *Trichinella* species
Type of organism	Nematode
Infectious agent	Larvae
Method of spread	Eating larvae encysted in muscle (frequently in undercooked pork)
Incubation	The larval cyst wall is digested in the stomach, and the larvae penetrate the lining of the small intestine. In <8 days, larvae mature into adults and mate. Over the next 1–4 months, they produce up to 1,500 new larvae. The new larvae travel to striated muscle where they eventually form cysts and become dormant. Encysted larvae remain viable for many years.
Symptoms	Early symptoms (during maturation of larvae) may include nausea, cramps, and diarrhea. In mild cases, there may be few symptoms. Later symptoms include fever, aching joints, and muscle soreness. In severe cases, infection may cause coordination, heart, and breathing problems, and death from trichinosis. When death occurs, it is usually because of inflammation of heart tissue. Once the larvae are fully encysted, symptoms may end.
Infectivity	Encysted larvae may be passed in feces; however, infection occurs only from eating infected meat.
Diagnosis	Muscle biopsy (frequently on tongue). ELISA to detect anti-*Trichinella* antibodies.
Treatment	CDC recommends that treatment begin as soon as possible. Treat symptoms and let infection run its course. Aspirin and other painkillers are used for muscle pains; steroids may be used for more severe symptoms. No treatment for encysted larvae. Experimental treatment with mebendazole, an anthelmintic (a drug for treatment of worm infections). Mebendazole works by keeping the worm from absorbing sugar. This gradually causes loss of energy and death of the worm.
Mortality	Approximately 1%
History as a pathogen	Occurs worldwide, but most common in Europe and the US In a 2-1/2 year period in the 1990s, there were over 10,000 human cases reported worldwide, from sources including pork, wild boar, horsemeat, and wild game.
Prevention	Larvae are killed by thoroughly cooking meat or by freezing meat. Smoking meat may not kill larvae.

Suggested scenarios for the classroom: In the weeks following a barbecue at which roast pig was the main dish, many attendees complain of nausea and cramps. You begin to wonder if the pig was cooked well enough and suspect trichinosis. ELISA to detect anti-*Trichinella* antibodies confirms that some attendees have contracted trichinosis. (Antibodies appear in the blood 3–5 weeks after infection.)

Simulation for detecting antibodies to *trichinella.*

The table below gives an example of how a diagnostic test to detect antibodies to *Trichinella* species in a patient's serum sample can be simulated using protocol III.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Purified antigen	Green	1x antigen	Extraction of *Trichinella spiralis* larvae
Student samples	Yellow	1x primary antibody or wash buffer	Serum sample from patient
Secondary antibody	Orange	1x secondary antibody	Anti-human immunoglobulin antibodies conjugated to HRP
Positive control	Violet	1x antigen	Serum from a trichinosis-positive patient
Negative control	Blue	1x PBS	Serum from a trichinosis-negative patient

West Nile Virus

Protocol Fit:

- I: ELISA for Tracking Disease Outbreaks
- II: Antigen Detection ELISA
- III: ELISA Antibody Test

Name of pathogen	West Nile virus (WNV)
Type of organism	RNA virus; flavivirus (virus in the family Flaviviridae)
Infectious agent	Virus
Method of spread	Bite from an infected mosquito. Transmission cycle is: 1) mosquito bites infected bird or animal; 2) virus circulates in mosquito's blood; 3) virus enters mosquito's salivary glands; and 4) mosquito injects virus into a human or animal when it bites. No evidence that WNV can be spread person to person or animal to person. Very rarely, transmission via transplanted organs from an infected individual, transmission by transfusion of blood products, or mother-to-child (across the placenta) transmission.
Incubation	3 to 14 days
Symptoms	Most people infected with WNV will have no illness. Approximately 20% of infected people will have West Nile fever with mild symptoms including fever, headache, body aches, skin rash, and swollen lymph glands. Less than 1% of infected people will have West Nile encephalitis (inflammation of the brain) or meningitis (inflammation of the lining of the brain and spinal cord) with severe symptoms including headache, high fever, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, and paralysis.
Infectivity	Not infectious from person to person.
Diagnosis	Initial diagnosis based on clinical suspicion (flu-like symptoms and presence of the virus in birds in the area). ELISA to detect anti-WNV IgM in serum or cerebrospinal fluid, called MAC-ELISA; IgM is detectable in 90% of cases within 8 days of infection. ELISA for West Nile virus or viral antigens in cerebrospinal fluid, tissue, blood, or other body fluids. ELISA for anti-WNV IgG in serum. Note: ELISA reagents are not available commercially, but may be obtained from the CDC.
Treatment	Supportive treatment only (IV fluids, respiratory support, treatment for secondary infections).
Mortality	Among those hospitalized with severe symptoms, mortality rate ~10% (rate highest among those >70 years old).
History as a pathogen	Isolated in Uganda in 1937; characterized in Egypt in the 1950s. Outbreaks in Africa, West Asia, Europe, and Middle East in the 1990s. First appeared in the United States in 1999. In 2003, human cases were reported in 45 states (up from 10 states in 1999–2001).

Prevention	Avoid mosquito bites by using insect repellent containing DEET (N,N-diethyl-*meta*-toluamide), wearing long-sleeved clothes and long pants treated with insect repellents, staying indoors at peak mosquito biting times (dawn, dusk, and in the early evening), and eliminating standing water sources to reduce the number of places available for mosquitoes to lay their eggs.

Suggested scenarios for the classroom: Epidemiological study. In order to understand the characteristics of the West Nile virus, an emerging disease in the United States, it is important to understand how infectious and how pathogenic the disease is. On one street in your town, there have been two severe cases of WNV. You survey the street and find that three other people report having had flu-like symptoms. To determine the epidemiology of the West Nile virus, you test serum samples from everyone in the neighborhood to see how many have been exposed (you may test for IgM, which indicates recent exposure, or you may test for the viral antigen itself). Once you know how many have been exposed, you can determine how many people actually get sick from WNV. Note: According to the CDC, WNV-IgM can persist in serum for 12 months or longer.

Simulation for detecting West Nile virus.

The table below gives an example of how a diagnostic test to detect West Nile virus in a patient's serum sample can be simulated using protocols I and II.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Student samples	Yellow	1x antigen or 1x PBS	Sample derived from patient's blood
Primary antibody	Green	1x primary antibody	Anti-West Nile virus antibody from mouse
Secondary antibody	Orange	1x secondary antibody	Anti-mouse immunoglobulin antibodies conjugated to HRP
Positive control	Violet	1x antigen	Heat-inactivated viral antigen
Negative control	Blue	1x PBS	West Nile virus-negative human serum

Simulation for detecting antibodies to West Nile virus.

The table below gives an example of how a diagnostic test to detect antibodies to West Nile virus in patient's serum sample can be simulated using protocol III.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Purified antigen	Green	1x antigen	Purified West Nile virus proteins
Student samples	Yellow	1x primary antibody or wash buffer	Serum sample from patient
Secondary antibody	Orange	1x secondary antibody	Anti-human immunoglobulin antibodies conjugated to HRP
Positive control	Violet	1x antigen	Serum from a patient with West Nile virus
Negative control	Blue	1x PBS	West Nile virus-negative human serum

Severe Acute Respiratory Syndrome (SARS)*

Protocol Fit:

- I: ELISA for Tracking Disease Outbreaks
- II: Antigen Detection ELISA
- III: ELISA Antibody Test

Name of pathogen	Coronavirus (SARS-CoV)
Type of organism	Virus
Infectious agent	Virus
Method of spread	Close person-to-person contact. Infected individual spreads infectious droplets by coughing or sneezing; infection occurs when individuals touch contaminated surfaces and then touch their eyes, nose, or mouth. There is no evidence of airborne transmission
Incubation	Onset of symptoms within 10 days of exposure.
Symptoms	Initial symptoms are fever >100.4°F and other flu-like symptoms. Respiratory illness, including dry cough and breathing difficulties, may develop after 2–7 days.
Infectivity	Patients are most infectious at around day 10 of the illness while they are showing respiratory symptoms (coughing and sneezing).
Diagnosis	Initial diagnosis by symptoms and epidemiology; *i.e.*, Has the patient either been in contact with another SARS patient or traveled in an area in which SARS is known to be prevalent during the previous 10 days.? Laboratory tests for diagnosis include: 1) ELISA to detect the SARS-CoV in patient specimens. 2) PCR to detect the SARS-CoV in patient specimens. 3) Detection of SARS-CoV RNA by reverse transcription PCR (RT-PCR). 4) ELISA that detects SARS-CoV antibodies (both IgG and IgM). Antibodies can be detected within 21 days of onset of illness. Note: ELISA reagents are not available commercially, but may be obtained from the CDC.
Treatment	No specific treatments are yet recommended for SARS. Symptoms are being treated with standard treatments for pneumonia. Tests of antiviral agents are ongoing.
Mortality	As of May 2003, the mortality rate is approximately 8%.
History as a pathogen	Was first recognized in February 2003. Evidence suggests that SARS may have arisen in rural China, possibly in the fall of 2002. Report in Science in September 2003 that the virus had been found in palm civets (*Paguma larvata*, carnivores closely related to meerkats and mongeese) in a live animal market in Guangdong, China. The same study found evidence of infection in a raccoon-dog (*Nyctereutes procyonoides*) and in humans working in the market. This is evidence of transmission of the virus between species, but does not tell us what animal is the reservoir of infection. SARS spread around the world via international air travel.

*SARS is an emerging infection and investigations into the virus, its origins, epidemiology, etc., are ongoing. Information is current as of press time, but see the CDC and WHO web sites (www.cdc.gov and www.who.int/en) for the most recent information.

Suggested scenarios for the classroom:

Simulation for detecting SARS virus using Protocol I or II:

A few of your students have been on a field trip. It is later determined that they rode on public transportation (aircraft, bus, subway, train) with a health care worker who has SARS, and one or two of the students may have contracted the disease. The remainder of the class may also have been exposed. Some have had contact with several of their classmates since their exposure, plus SARS may be spread by inhalation. Perform an ELISA to determine the spread of SARS within your classroom. It is important to determine as soon as possible which students have been exposed so that they may be isolated. (Quarantine of exposed individuals is currently the best way to prevent the spread of this very infectious virus.) To determine which students have been exposed, perform an ELISA to detect the SARS virus in samples of their body fluid.

The table below gives an example of how a diagnostic test to detect SARS coronavirus in a patient sample can be simulated using protocols I and II.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Student samples	Yellow	1x antigen or 1x PBS	Sample derived from patient's blood
Primary antibody	Green	1x primary antibody	Anti-SARS-CoV antibody from mouse
Secondary antibody	Orange	1x secondary antibody	Anti-mouse immunoglobulin antibody conjugated to HRP
Positive control	Violet	1x antigen	Heat-inactivated viral antigen
Negative control	Blue	1x PBS	SARS-negative human serum

Simulation for detecting antibodies to SARS using Protocol III:

The entire class has been on a field trip and it is later determined that they stayed at the same hotel as a SARS-infected person. Since SARS can be spread by contact with respiratory droplets, the class may have been exposed. It is important to determine as soon as possible which students have been exposed so that they can be isolated. (Quarantine of exposed individuals is currently the best way to prevent the spread of this very infectious virus.) To determine which students have been exposed, perform an ELISA to detect the SARS virus or antibodies to the SARS virus in samples of their body fluid or serum, respectively.

The table below gives an example of how a diagnostic test to detect antibodies to SARS coroniavirus in a patient's serum sample can be simulated using protocol III.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Purified antigen	Green	1x antigen	Extracts from SARS-CoV-infected, cultured human cells
Student samples	Yellow	1x primary antibody or wash buffer	Serum sample from patient
Secondary antibody	Orange	1x secondary antibody	Anti-human immunoglobulin antibodies conjugated to HRP
Positive control	Violet	1x antigen	Serum from a SARS patient
Negative control	Blue	1x PBS	SARS-negative human serum or serum from a patient with a respiratory disease that is not SARS

Lyme Disease or Lyme Borreliosis

Protocol Fit:

- III: ELISA Antibody Test

Name of pathogen	*Borrelia bergdorferi*
Type of organism	Bacterium (spirochete)
Infectious agent	Bacterium
Method of spread	Lyme disease is spread in the eastern US through the bite of the black-legged tick, *Ixodes scapularis*; on the west coast, it is spread by the western black-legged tick, *Ixodes pacificus*. Transmission of the bacteria usually does not occur until the tick has been attached for 36–48 hours, so finding and removing ticks can prevent infection. Animal reservoir in the US is the white-footed deer mouse (*Peromyscus leucopus*).
Incubation	3–32 days after tick bite
Symptoms	Bull's eye rash (erythema migrans) appears around tick bite in 75% of patients, typically 7–14 days after bite. Other early symptoms are flu-like (malaise, chills, fever, headache, body aches). About 60% of patients develop arthritis within weeks to months. Less common symptoms include neurological and myocardial abnormalities.
Infectivity	Not infectious from person to person.
Diagnosis	Bull's eye around bite location is very diagnostic. ELISA for antibodies (IgG and/or IgM) against *B. bergdorferi*. Since the current assays have 89% sensitivity and 72% specificity, clinical diagnosis must be confirmed by a more specific test, such as a western blot. (If there is no bull's eye rash, a positive ELISA is most likely a false positive.) Presence of antibodies indicates exposure, not active disease, as the antibodies may be present for years after exposure.
Treatment	Early-stage Lyme disease is treated with a 3–4 week course of oral antibiotics. Later disease may be treated with intravenous antibiotics for >4 weeks.
Mortality	Rarely fatal.
History as a pathogen	Reports in Europe of a bull's eye rash (erythema migrans) associated with a tick bite date to the early 1900s. The disease was recognized in the US in 1975 because of a cluster of cases around Lyme, Connecticut.

Suggested scenario for the classroom: Your class went on an outdoor field trip last fall, and a number of students have been suffering from flu-like symptoms and joint pain. Several remember having a red bull's eye mark and wonder if they had been bitten by ticks on the outing. Perform an ELISA to determine if they have antibodies to *B. bergdorferi* in their blood.

Simulation for detecting antibodies to *B. bergdorferi.*

The table below gives an example of how a diagnostic test to detect antibodies to *B. bergdorferi* species in a patient's serum sample can be simulated using protocol III.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Purified antigen	Green	1x antigen	*B. bergorferi* purified proteins
Student samples	Yellow	1x primary antibody or wash buffer	Serum sample from patient
Secondary antibody	Orange	1x secondary antibody	Anti-human immunoglobulin antibodies conjugated to HRP
Positive control	Violet	1x antigen	Serum from patients with Lyme disease
Negative control	Blue	1x PBS	Serum negative for Lyme disease

Appendix D: Lesson Extensions

Investigations Into Specific Diseases

There is much information on infectious diseases on the Internet, particularly on the CDC and WHO web sites (see appendix E for other websites). Web searches will also uncover web sites for companies with newly developed diagnostic tests for infectious diseases. As a student assignment, have your students fill out a disease table on a different disease, either one that they chose or one that is assigned.

As a secondary lesson, this activity can be used to help students distinguish between "good" and "bad" information on the Internet. There are many web sites containing unproven treatments and incorrect information about medical issues, as there are no controlling agencies to prevent spurious information from being posted on the Web.

Disease name: _____

Name of pathogen	
Type of organism	
Infectious agent	
Method of spread	
Incubation	
Symptoms	
Infectivity	
Diagnosis	
Treatment	
Mortality	
History as a pathogen	

References used:

Quantitative ELISA Laboratory Exercise

While ELISA gives a definitive qualitative (yes/no) answer, a major strength lies in that it can also give quantitative (how much?) information. This lesson extension provides a protocol to adapt this kit for use as a quantitative ELISA to assay either antigen or antibody levels. The following protocol is written to quantitate levels of antigen with protocol II as a basis, but it can easily be adapted to quantitate levels of antibody in serum with protocol III as a basis. It can be adapted for protocol I, but the results will probably not be in the linear range and therefore will not be very accurate.

This extension activity uses serial dilution to generate dilutions of known antigen concentration. As the concentration of antigen or antibody increases, so does the intensity of the blue color in the wells. The blue color absorbs light at a specific wavelength, and this absorbance can be measured with a microplate reader. Students compare their test samples to the dilution series to calculate (or estimate) the concentration of the test samples. Using the Model 680 microplate reader with a 655 nm filter (Bio-Rad catalog #168-1002EDU), students can read the absorbance values of their wells, generate a standard curve, and calculate the concentration of their samples. If no microplate reader is available, the students can visually match the intensity of their samples with the samples in their dilution series and estimate the concentration of antigen in their samples.

Instructor's Advance Preparation

We strongly recommend that you familiarize yourself with the basic protocols in this kit prior to performing this lab. It is especially important to read the information and notes on p. 40 concerning reagent stability.

The instructor's advance preparation is similar to that of protocol II, but includes two extra tubes at the workstation: one with a sample of known concentration and one tube of PBS. This protocol also has only two student test samples per workstation of up to four students. The two will have different levels of antigen in them and can be labeled either as "A" and "B" or with hypothetical patient initials. You will also need two clean 15 ml test tubes or equivalent containers.

Quantitative ELISA: Step-by-Step Instructor's Advance Preparation Guide

These instructions are for the setup of 12 student workstations of 4 students each.

Supplied Reagents	Quantity
Antigen (chicken gamma globulin), freeze-dried	1 vial
Primary antibody (rabbit anti-chicken polyclonal antibody), freeze-dried	1 vial
Secondary antibody (goat anti-rabbit antibody conjugated to horseradish peroxidase(HRP)), freeze-dried	1 vial
HRP enzyme substrate (TMB)	1 bottle
10x phosphate buffered saline (PBS)	1 bottle
10% Tween 20	1 bottle
Required Reagents	
Distilled water, sterile is recommended, see note on page 40	1 L

Step 1. Prepare buffers.

We recommend you use a 100 ml and a 1 liter graduated cylinder for preparing the buffer solutions. You will also need 1 liter of distilled water.

Buffer	Volume	Reagent	Used for
1x PBS, 100 ml	90 ml	Distilled water	• Rehydrating antigen, primary and secondary antibodies to make 50x reagent stock solutions • Diluting 50x antigen to make positive control and sample of known concentration • Diluting 1x stocks to make student samples • Negative control • Diluting sample of known concentration during experiment to make serial dilutions for standard curve
	10 ml	10x PBS	
Wash buffer, 900 ml	805 ml	Distilled water	• Dilution of 50x antibody stocks· • Plate washing
	90 ml	10x PBS	
	4.5 ml	10% Tween 20	

Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody.

Carefully remove the stoppers from the three freeze-dried reagents and use a fresh pipet to add 0.5 ml 1x PBS to each. Close the stoppers and shake to mix. These solutions are 50x concentrates, or stock solutions. **NOTE: You must <u>not</u> use wash buffer in this step**.

Freeze-Dried Reagent	Protocol for 50x Stock Solution	Used for
Antigen	Add 0.5 ml of 1x PBS to vial	• Positive control • Student samples A and B
Primary antibody	Add 0.5 ml of 1x PBS to vial	• Primary antibody
Secondary antibody	Add 0.5 ml of 1x PBS to vial	• Secondary antibody

Label one 30 ml bottle each for 1x antigen, 1x primary antibody and 1x secondary antibody. Label one 15 ml tube for student sample A and student sample B. Use a fresh DPTP to add the contents of the appropriate stock to the corresponding receptacle.

Diluted Solution	Volume	Reagent	Used for
1x Antigen, label one 30 ml bottle	24.5 ml	1x PBS	• Positive control • Concentrated reagent for student samples A and B • Sample of known concentration
	0.5 ml	50 x antigen stock	
	NOTE: you must not add any buffer containing Tween 20 to the antigen, or the experiment will not work. • Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		
1x Primary antibody, label one 30 ml bottle	24.5 ml	Wash buffer	• Primary antibody
	0.5 ml	50 x primary antibody stock	
	• Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		
1x Secondary antibody, label one 30 ml bottle	24.5 ml	Wash buffer	• Secondary antibody
	0.5 ml	50x secondary antibody stock	
	• Dilute the secondary antibody less than 24 hours before the start of the lesson. • Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		
125 ng/ml antigen (A), label one 15 ml tube	8.75 ml	1x PBS	• Student sample A
	1.25 ml	1x antigen	
	• Close the tube cap and shake to mix.		
25 ng/ml antigen (B), label one 15 ml tube	9.75 ml	1x PBS	• Student sample B
	0.25 ml	1x antigen	
	• Close the tube cap and shake to mix.		

Step 4. Dispense reagents for student workstations.

Tubes	Description	Label	Contents (Each Tube)	
Violet tubes, 12	Positive controls	"+"	0.5 ml	1x antigen solution
Blue tubes, 12	Negative controls	"–"	0.5 ml	1x PBS
Green tubes, 12	Primary antibody	"PA"	1.5 ml	1x primary antibody solution
Orange tubes, 12	Secondary antibody	"SA"	1.5 ml	1x secondary antibody solution
Brown tubes, 12	Enzyme substrate	"SUB"	1.5 ml	HRP enzyme substrate (TMB)
	Note: TMB is light sensitive, so it is important to use the dark tubes to store this reagent.			
Yellow tubes, 12	Sample of known concentration	"1,000 ng/ml AG"	0.25 ml	1x antigen
Yellow tubes, 12	1x PBS	"PBS"	1 ml	1x PBS
Yellow tubes, 12	125 ng/ml antigen	"A"	0.25 ml	125 ng/ml antigen
Yellow tubes, 12	25 ng/ml antigen	"B"	0.25 ml	25 ng/ml antigen

Step 5. Set out student workstations.

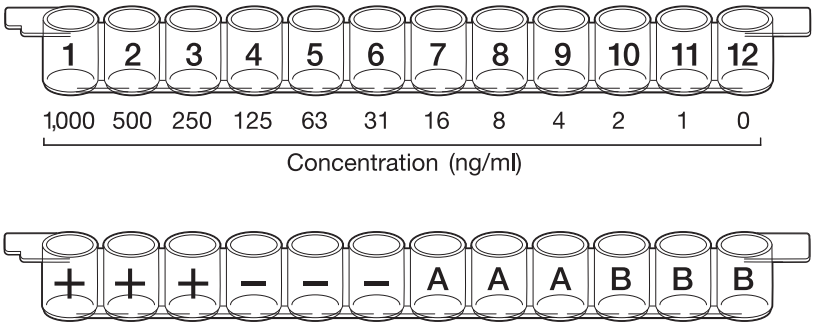
Student Workstation Checklist

One workstation serves 4 students.

Item (Label)	Contents	Number	Check
Yellow tubes (A and B)	Student samples (0.25 ml)	2	❑
Yellow tube (1,000 ng/ml AG)	1x antigen (0.25 ml)	1	❑
Yellow tube (PBS)	1x PBS (1 ml)	1	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable-volume micropipet		1	❑
Yellow tips		9	❑
Disposable plastic transfer pipet		1	❑
70–80 ml wash buffer in beaker	1x PBS with 0.05% Tween 20	1	❑
Black marking pen		1	❑
Waste container		1	❑
Large stack of paper towels		1	❑

Student Lab Procedure

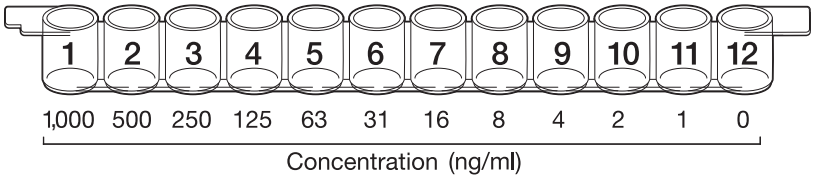
1. Label the outside wall of each well on one 12-well strip with the numbers 1–12. Label the first three wells of a second 12-well strip with a "+" for the positive controls, the next three wells with a "–" for the negative controls, the next three wells with the initials of one of your sample tubes, and the last three wells with the initials of the second sample tube.



2. Use a pipet to add 50 µl of PBS from the yellow tube labeled "PBS" to wells labeled #2 through #12.
3. Add 100 µl from the yellow tube labeled "1,000 ng/ml AG" to the well labeled #1.
4. Perform serial dilution from well #1 through well #11 in the following manner:
   a. Pipet 50 µl out of well #1 and add it to well # 2. Pipet up and down gently three times to mix the sample in well #2.
   b. Using the same pipet tip, transfer 50 µl from well # 2 to well # 3 and mix the sample in well # 3.
   c. Using the same pipet tip, transfer 50 µl from well # 3 to well # 4 and mix the sample in well # 4.
   d. Repeat this transfer and mixing step, moving to the next well each time. STOP when you reach well # 11; discard the 50 µl of solution from well #11 into a waste container.
5. In the second microplate strip, use a fresh pipet tip to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
6. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
7. Use a fresh pipet tip to transfer 50 µl of each of your team's samples into the appropriately initialed three wells.
8. Wait 5 minutes to allow the proteins in the samples to bind to the plastic wells, then proceed exactly as in protocol II, starting at step 5 on page 56.

Analysis of Results

The concentration of 1x antigen is 1 microgram per milliliter (µg/ml) or 1,000 nanograms per ml (ng/ml). Note: If adapting this protocol for a quantitative ELISA antibody test, the concentration of primary antibody in 1x serum is also 1 g/ml. Thus, the concentrations of protein in the wells of the dilution series are:



If using a microplate reader, insert the microplate strips firmly back into the strip holder in the correct orientation, secure the plate into the microplate reader, close the lid, and read using a 655 nm filter.

Microplate readers measure the amount of light at a specific wavelength (in this case, 655 nm) that is absorbed by the liquid in the wells of the microplate. The absorption of light by the liquid is directly related to the intensity of the colored product in the wells, which in turn is determined by the amount of enzyme activity in the wells. The amount of enzyme activity is governed by the amount of antigen that originally bound to the wells.

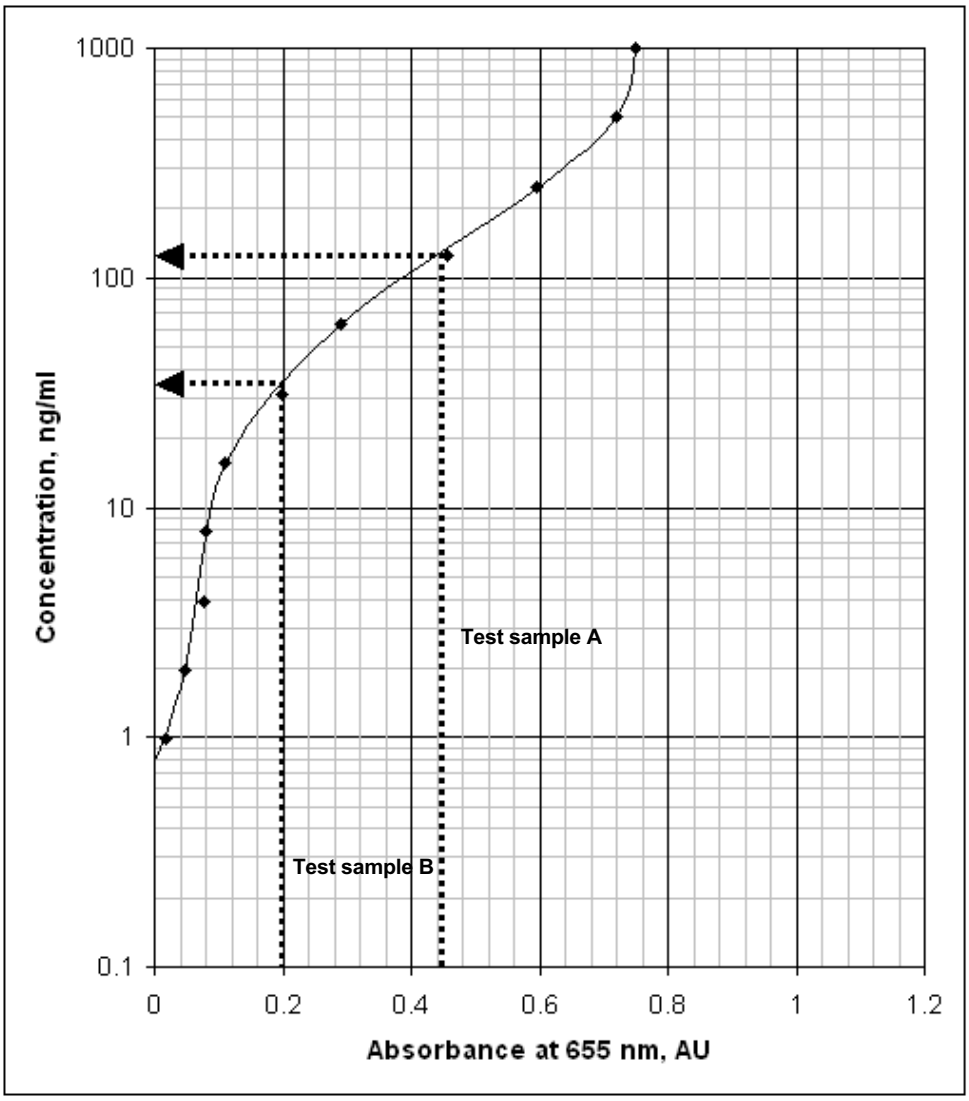
Create a standard curve by plotting the known concentrations of each well on the y-axis and the corresponding absorbance values from the microplate reader on the x-axis (See example on next page). (Note: The unit of measurement for absorbance is absorbance units, or AU.) Since the resulting curve will be logarithmic, you will need to linearize it by plotting the data on semilog graph paper. A sheet is provided on the next page.

Calculate the concentrations of the test samples by drawing vertical lines from their absorbance values on the x-axis to the standard curve. Read horizontally from the points where the vertical lines intersect with the standard curve to the concentration values on the y-axis.

Please note that the substrate – enzyme reaction loses correlation between antigen concentration and absorbance value when unoxidised substrate becomes scarce either due to too concentrated antigen (see 1000 ng/ml datapoint on example graph on next page) or after too long a reaction time. Thus microplates should be read within 15 min of adding substrate.

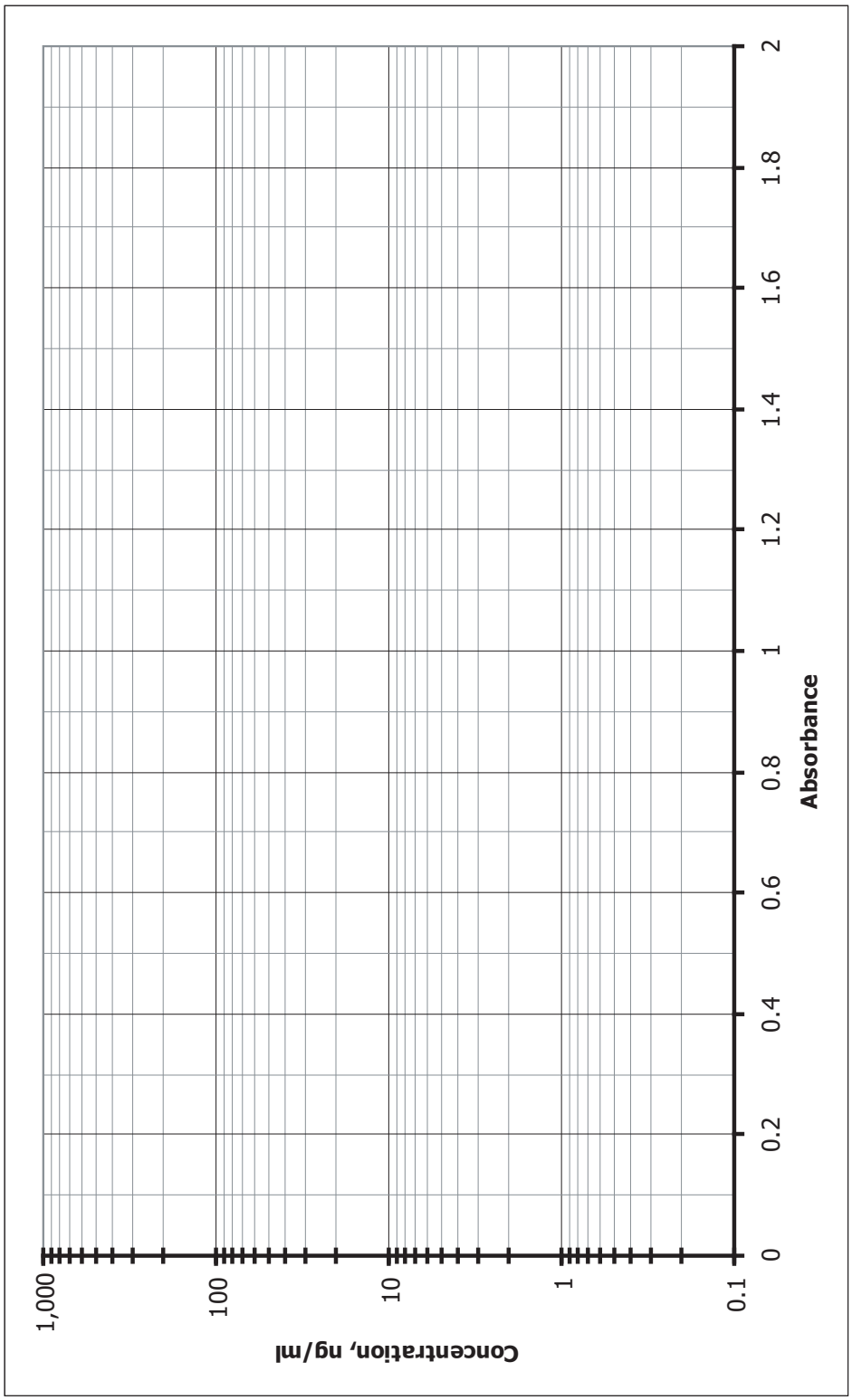
Note: The enzymatic reaction can be stopped after 5 min by adding 0.18 M sulfuric acid to the wells. The blue solution will turn yellow. The yellow solution is read using a microplate reader with a 450 nm filter. Stopping the run after 5 min produces a more linear reading of diluted samples.

If no microplate reader is available, visually compare the intensity of the blue color in test wells to the intensities of the dilution series of known concentrations. Identify which wells of known concentration most closely match the test wells and, from this, approximate the concentration of antigen in the test samples.



An example of a standard curve from a dilution series from 1,000 ng/ml to 1 ng/ml, read at 655 nm. Test sample A had an absorbance of 0.456 AU, and test sample B had an absorbance of 0.208 AU. Thus, their concentrations were 125 ng/ml and 25 ng/ml, respectively.

Semilog graph paper



Possible Scenarios for Quantitative ELISA Assays

Protocol II: Antigen Detection ELISA

Monitoring viral load is crucial in treating HIV/AIDS infections. Antiretroviral therapies are directed at keeping the viral load at or near undetectable levels. Some antiretroviral agents currently in use include efavirenz, indinavir, nelfinavir, ritonavir, zidovudine, didanosine, and stavudine. Treatments normally include a combination of two or more antiretroviral drugs. (For more information, search on the CDC or WHO web sites for "using retroviral agents".)

As a classroom scenario, evaluate new combinations of antiretroviral drugs by assaying patient samples for the presence of HIV. A good combination of drugs will reduce the viral load, as indicated by little or no detectable HIV antigen in the samples. Poor drug combinations will result in a high concentration of HIV antigen in the samples.

Instructor's preparation: The two samples of unknown concentration ("A" and "B") are samples from patients who have been treated with novel combinations of antiretroviral drugs. Your students will determine which is the more efficacious treatment by testing for HIV antigen concentration in the samples. The drug combination that results in lower viral load in the patient is interpreted as a more effective treatment for HIV.

For further discussion, consider what other factors are important in evaluating a new drug treatment, for example, side effects, cost of treatment, interactions with other drugs used to treat HIV/AIDS, etc.

Protocol III: ELISA Antibody Test

Anthrax is a disease caused by *Bacillus anthracis*, a bacterium that forms spores. There are three types of anthrax: skin (cutaneous), lung (inhalation), and digestive (gastrointestinal). All three types are treated with antibiotics. Cutaneous anthrax is not as dangerous as inhalation or gastrointestinal anthrax; even without treatment, most patients will survive cutaneous anthrax. Gastrointestinal and inhalation anthrax are much more dangerous; over 25% of patients die, even with antibiotic treatment.

Anthrax infection is unusual in humans; historically, anthrax is primarily a disease of herbivores. However, after the bioterrorism attack of 2001 in the US, during which anthrax spores were sent through the mail and 22 people were infected, many researchers have been looking for ways to detect anthrax infections. A research group at the CDC has developed a quantitative ELISA for detecting antibodies against anthrax protective antigen, a protein component of anthrax. The ELISA can detect seroconversion, an increase in serum antibodies against anthrax protective antigen, between the time initial symptoms appear and 2–4 weeks later.

As a classroom scenario, evaluate two serum samples from a single patient for the presence of anti-anthrax IgG. The patient is suspected of having anthrax. The first sample was drawn from the patient a week after symptoms developed, and the second sample was drawn 3 weeks later. If the patient indeed has anthrax, there should be a rise in the concentration of antibodies against anthrax.

Instructor's preparation: The two samples of unknown concentration ("A" and "B") are samples from a patient who may have anthrax. The "B" sample is the serum drawn early in the illness and the "A" sample is the serum drawn later in the infection. An increase in the concentration of antibodies indicates exposure to anthrax.

Appendix E: Bibliography and Useful Web Sites

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Referenced Web Sites (Disease Information)

http://www.cdc.gov/health/default.htm
http://ww.who.int/health_topics/infectious_diseases/en/
http://www.niaid.nih.gov/dmid/
http://www.merck.com/pubs/mmanual/
The Merck Veterinary Manual (online, http://www.merckvetmanual.com/mvm/index.jsp)
The Merck Manual of Diagnosis and Therapy (online, http://www.merck.com/pubs/mmanual/)

Useful Web Sites

http://www.who.int/en/	World Health Organization (WHO)
http://www.cdc.gov/	Centers for Disease Control and Prevention (CDC)
http://www.niaid.nih.gov/	National Institute of Allergy and Infectious Diseases (NIAID)

http://www.usamriid.army.mil/
http://www.fda.gov/cdrh/clia/index.html
http://www.bioterry.com/History_of_Biological_Terrorism.asp

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Web site *www.bio-rad.com* ***USA*** *800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 11 5044 5699*
Canada *905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00*
France *01 47 95 69 65* ***Germany*** *089 31 884 0* ***Greece*** *30 210 9532 220* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 459 6100* ***India*** *91 124 4029300*
Israel *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666*
New Zealand *64 9 415 2280* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04*
Singapore *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55*
Taiwan *886 2 2578 7189* ***Thailand*** *800 88 22 88* ***United Kingdom*** *020 8328 2000*

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