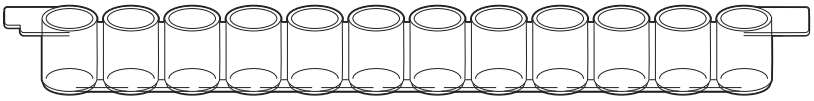
General Introduction to This ELISA Kit

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.

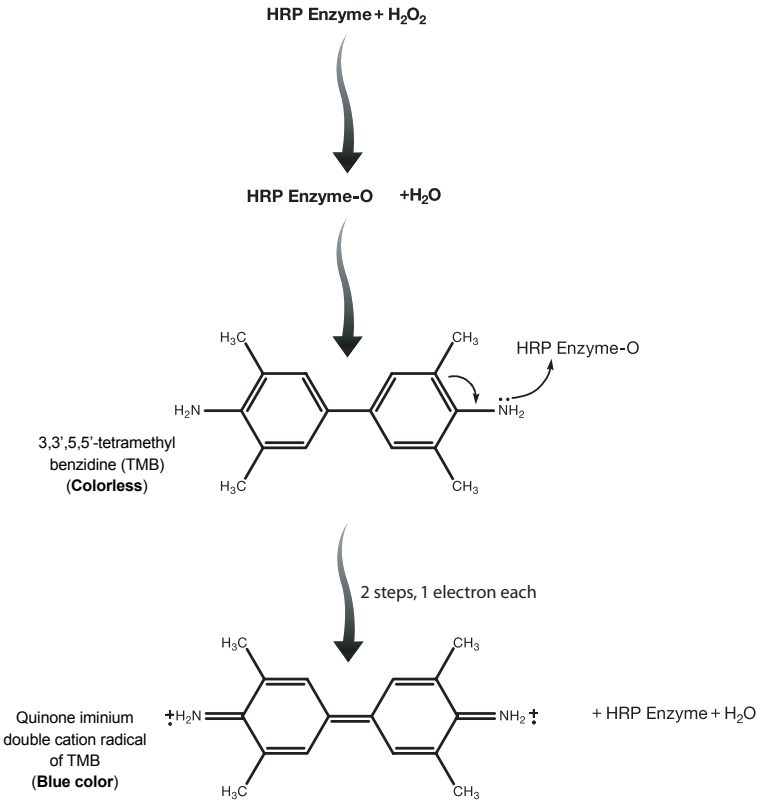
Incubation times: The rate of binding depends on the incubation temperature and the concentrations of the reagents. This kit has been optimized so that each incubation can be performed for 5 minutes at room temperature. Exceeding this time or temperature will cause an increase in color intensity and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

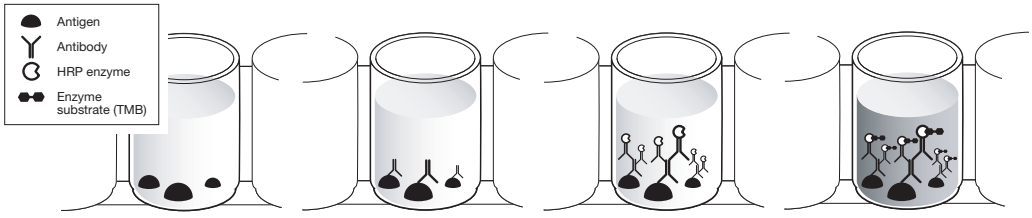
In an ELISA like those in Protocols I and II (in which antigen concentration is the experimental variable), an appropriate negative control would be wells with antigen omitted. Any color product in those wells would be the result of either 1) nonspecific binding of the antibodies, or 2) experimental 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).

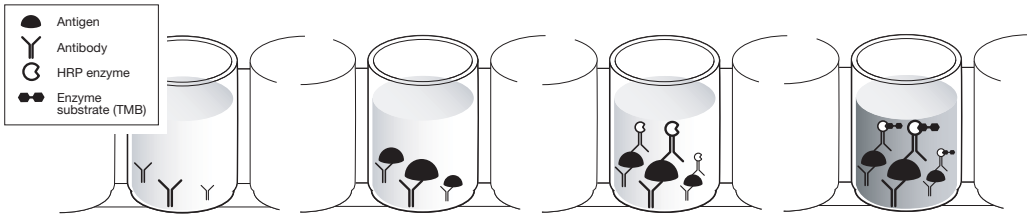
A commercial ELISA kit to test for antibodies to HIV-2, from Bio-Rad's Clinical Diagnostics Group.

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.