Student Manual

Introduction

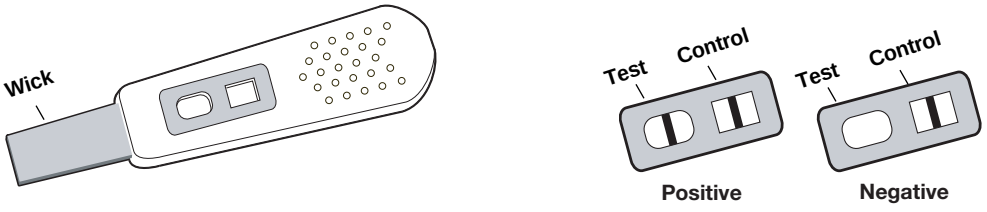
Immunology is the study of the immune system and how the body protects itself against disease. Over 100 years ago, biologists found that animals' internal immune systems respond to invasion by "foreign entities" or antigens. When an invader enters the body, it provokes an immune response that begins 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.

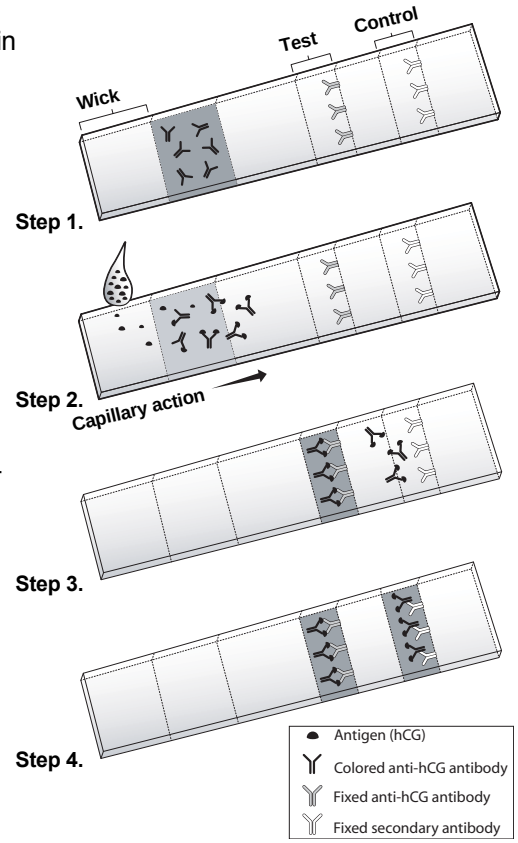
Where Is ELISA Used in the Real World?

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.

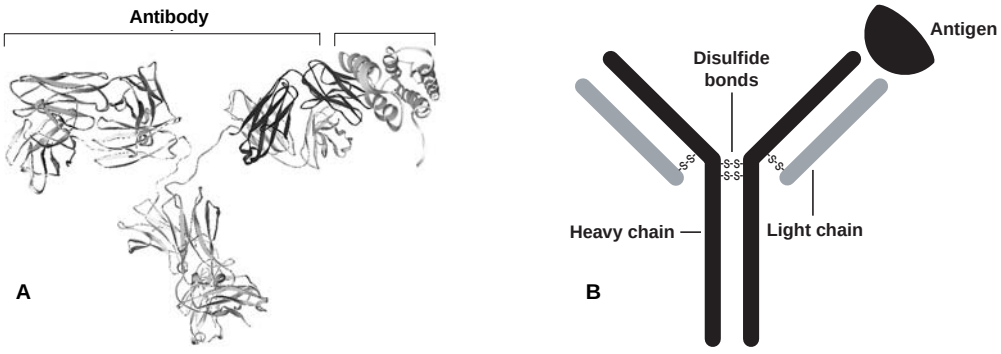


Some tests give positive or negative results in a matter of minutes. For example, home pregnancy dipstick tests detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization. The wick area of the dipstick is coated with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

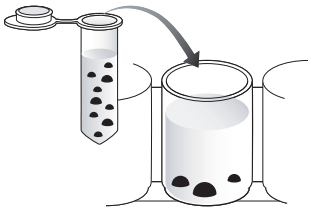
For any immunoassay to be valid, it must include both positive and negative controls, i.e., samples that will give known results. Controls are always run side by side with experimental samples. If you do not run a positive control and the experiment gives negative results, how can you be sure the results 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.

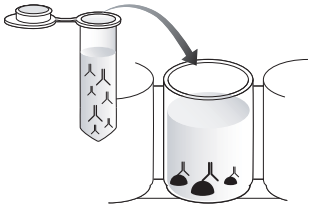
Now let's put this all together.

The main steps in this antigen detection ELISA are:

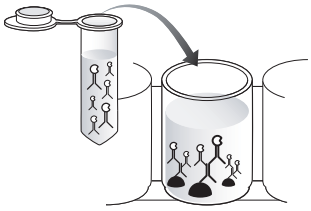
1. Add your sample and control samples to the wells in a microplate strip. Your samples contain many proteins and may or may not contain the antigen. Incubate for 5 minutes to allow all the proteins in the samples to bind to the plastic wells via hydrophobic interaction. This is called an immunosorbent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



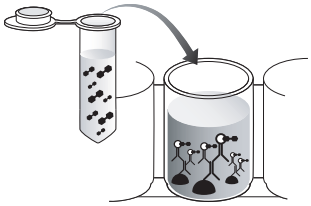
2. Add primary antibody to the wells and incubate. The antibodies will seek out the antigen from the many proteins bound to the well. If your sample contains the antigen, the antibodies will bind it tightly and remain in the well.



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

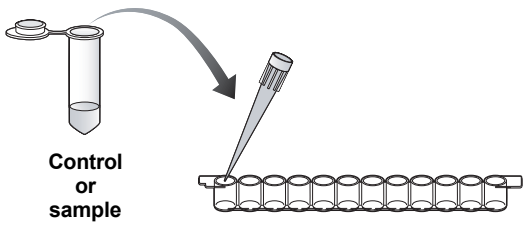
One workstation serves 4 students.

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	❑

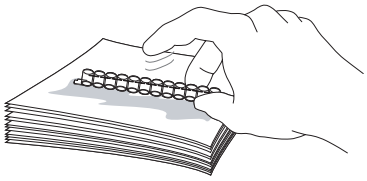
Laboratory Procedure

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:

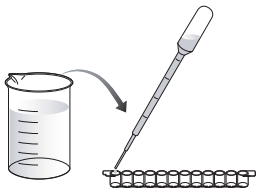
3. Bind the antigen to the wells:
    a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
    b. Use a <u>fresh</u> pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
    c. Use a <u>fresh</u> pipet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



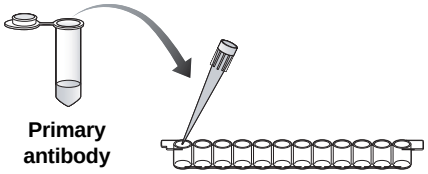
4. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
5. Wash the unbound sample out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make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



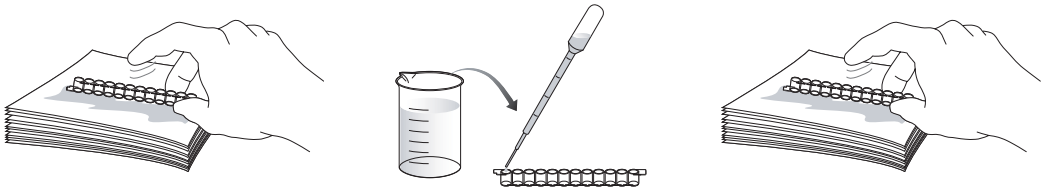
    b. Discard the top paper towel.
    c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps..



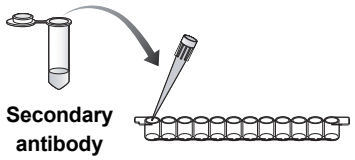
    d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.
    e. Discard the top 2–3 paper towels.
6. Repeat wash step 5.
7. Use a <u>fresh</u> pipet tip to transfer 50 µl of primary antibody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



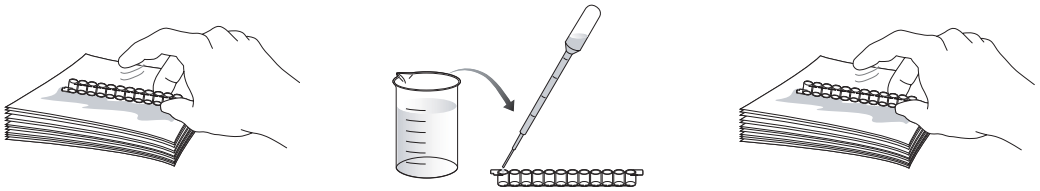
8. Wait 5 minutes for the primary antibody to bind.
9. Wash the unbound primary antibody out of the wells by repeating wash step 5 **two** times.



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.

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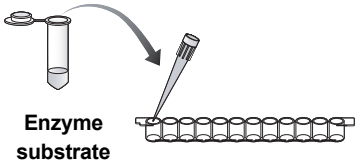
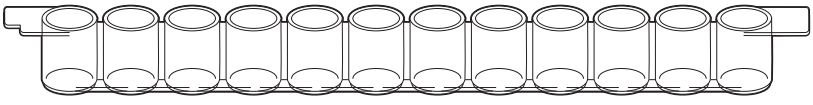
13. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



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?

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.

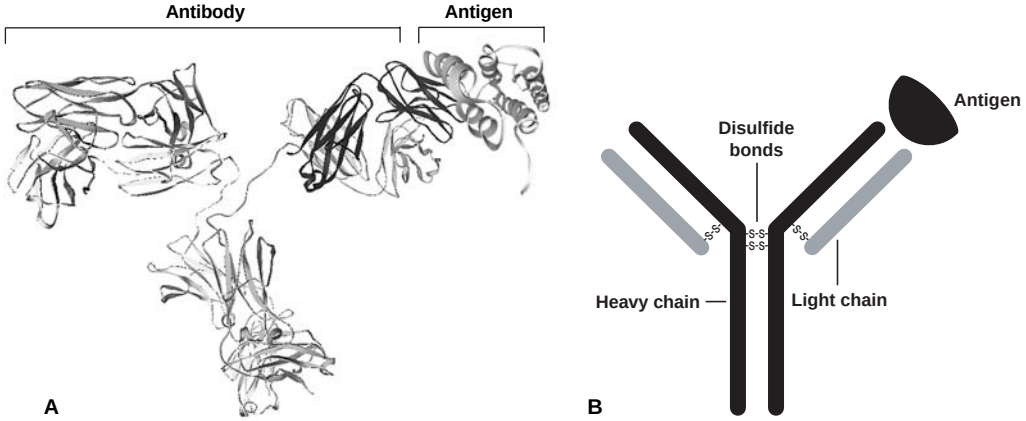
Acquired immunity is the basis for the series of vaccinations that we undergo as we grow up. In the 1790s, long before we had any understanding of the immune system, it was discovered that inoculation with pus from a cowpox lesion prevented infection with smallpox, a disease related to cowpox. The US 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

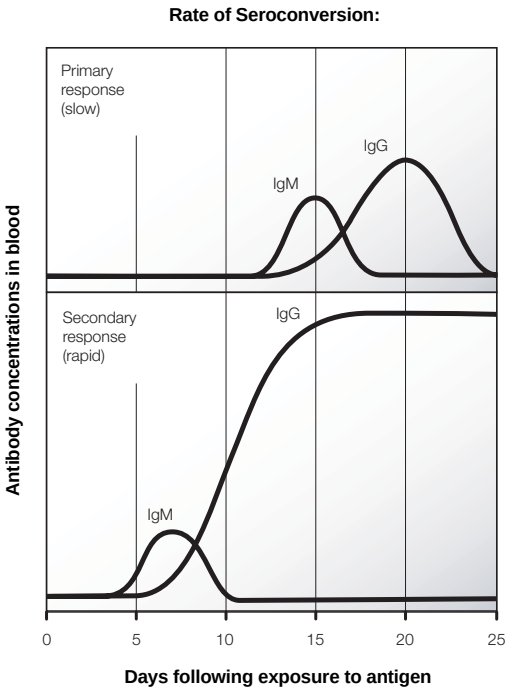
Even bacteria have a rudimentary innate immune system; they make restriction enzymes that destroy foreign DNA from bacterial viruses (bacteriophages), and they protect their own DNA by labeling it as "self" through methylation. Our immune system is at work every day, protecting us from thousands of potential 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.

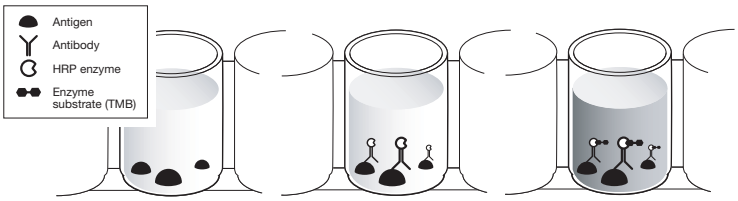
Laboratory tests cover a wide variety of methods, some of which have been in use for decades and others, like the tests for RNA and DNA from disease agents, which are very new. Depending on the test and putative diagnosis, laboratory tests may look for signs of disease in most body fluids, including 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.

Labeling and Detecting Antibodies

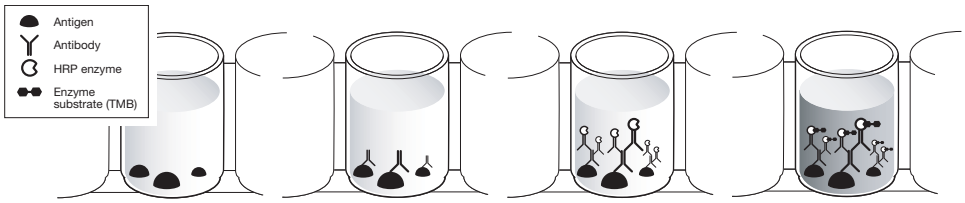
Antibodies are used in diagnosis and research as labeling tools. As labels they have to be made visible, so antibodies are covalently linked (or conjugated) to chemical labels that emit detectable signals. Detection systems can be low-tech or high-tech, and the detection system determines the type of 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.