Biol 465
Immunology

Lab 4: Practical Application of Antigen Recognition: Pregnancy testing

Today's lab is really the second time we have applied antigen-antibody reaction in a diagnostic application. The agglutination of RBCs for ABO blood testing was a quick and easy to visualize antibody-antigen interactions in the lab. However, this kind of testing is typically done in a lab by professionals and includes controls to insure safety in blood donation. The home pregnancy test is for all practical purposes a simple, observable antigen-antibody reaction. Since it uses both an immunological activity and an observable color change it is referred to as an **immunochromatographic** test.

Modern pregnancy tests

Most commercially available pregnancy tests look for the presence of the beta subunit of human chorionic gonadotrophin (hCG) in the blood or urine. hCG is found in blood and urine of a pregnant woman after implantation of the embryo (6-12 days after fertilization). hCG is only found in humans during pregnancy so it is an ideal indicator of pregnancy. The fact that it is secreted so early in pregnancy also makes it a preferred antigen in immunological tests. Although there are several variations on the pregnancy test, all are based on detection of a specific antigen (e.g. hCG) , by a specific antibody (produced in an animal which has been immunized with the antigen). How that antigen-antibody binding is visualized varies from test to test, but most tests rely on **lateral flow** of the body sample, usually urine.

In a **lateral flow test**, the test sample is applied at one end of a test strip and flows toward the other end by controlled absorption or capillary action. The test strip will have indicator substances at various positions along the test strip. As the urine passes the indicator substances, a color either appears (or not) according to the specific test design and the presence of pregnancy antigens.

Immunology behind the tests: The most sophisticated tests actually use a series of 2 or 3 antibodies, one of which becomes bound to hCG and then bound by antibodies raised against this antibody. These types of assays are called "sandwich assays" since the antibodies end piling up with each binding another in succession.
http://www.whfreeman.com/kuby/content/anm/kb07an01.htm

Other tests use 1 or more antibodies which are specific for different regions of the hCB protein. http://student.ccbcmd.edu/courses/bio141/labmanua/lab17/preg_flash.html

Visualizing the test results: The results of a pregnancy test must be easy to interpret since they are commercially available for the public. Modern tests work in one of 2 ways, typically. Both methods require that the antibodies used be linked to something else. Most tests use immobilized indicator regions so the results come out in one spot on the test strip.

1. **The enzyme-linked immunosorbant assay (ELISA) method.** In this case, the antibody(ies) are linked to an enzyme. Lateral flow of urine/hCG and bound antibodies brings the enzyme in proximity to a bound (fixed) substrate. The enzyme and substrate mix, resulting in a chemical reaction that produces a color change in the substrate. The color change ultimately means that hCG was present in the urine.

2. **Physical migration of pigmented particles.** In this case, hCG binds to antibodies which have some type of pigmented particle attached (sometimes red latex beads). Within the test strip is another fixed substance (often another antibody!) which results in the stoppage of flow and the collection of the pigmented particles in a concentrated area. The user notes a line where the pigment deposits.

Controls for the tests: Most pregnancy tests include some type of control to make sure the test was done properly and that the results are indicative of the woman's pregnancy status. Although a positive result can rarely be due to anything but the presence of hCG, a negative result could come from any number of failures in the test. Usually the control tests two aspects:

1. The complete flow of urine to the important parts of the test strip.
2. The presence/activity of the antibodies used in the test.

   View again paying special attention to the control aspect of the test.

   http://www.whfreeman.com/kuby/content/anm/kb07an01.htm

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

Student Manual

Introduction

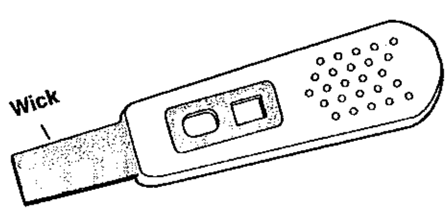
Immunology is the study of the immune system and how the body protects itself against disease. Over 100 years ago, biologists found that animals' internal immune systems respond to invasion by "foreign entities" or antigens. When an invader enters the body, it provokes an immune response that begins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

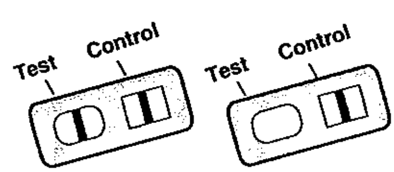
You are about to perform an ELISA (enzyme-linked immunosorbent assay). The ELISA relies on antibodies to detect the presence of antigens in liquid samples. Because they are antibody-based, ELISAs are called immunoassays. ELISAs can detect minute amounts of disease agents in samples such as body fluids (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.

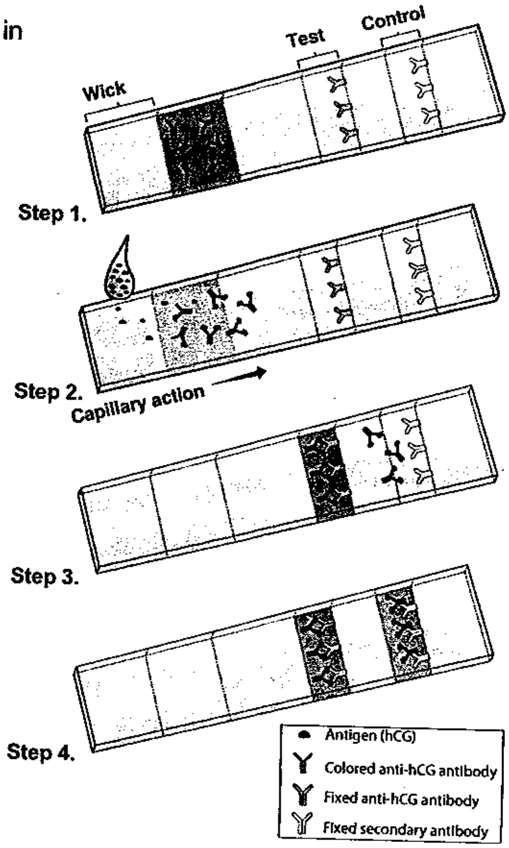
Over-the-counter kits that are based on the same principles as this ELISA activity include home pregnancy and ovulation tests, and tests for the presence of illegal drugs like marijuana and cocaine.





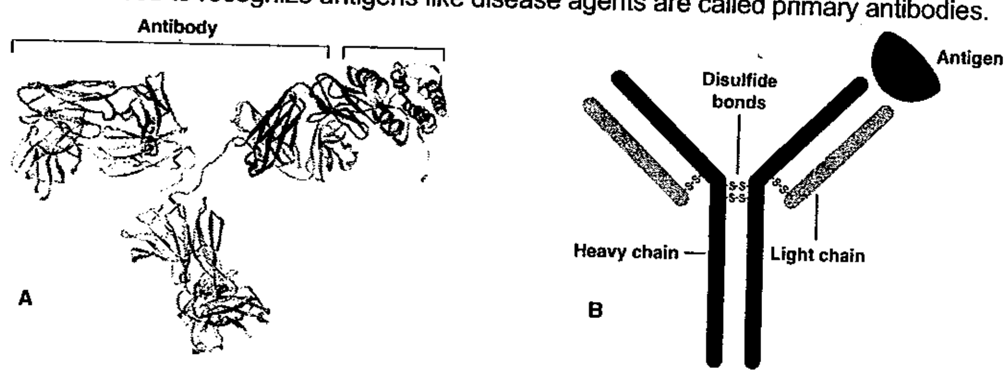
Positive Negative

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? (see p. 88, this handout)

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

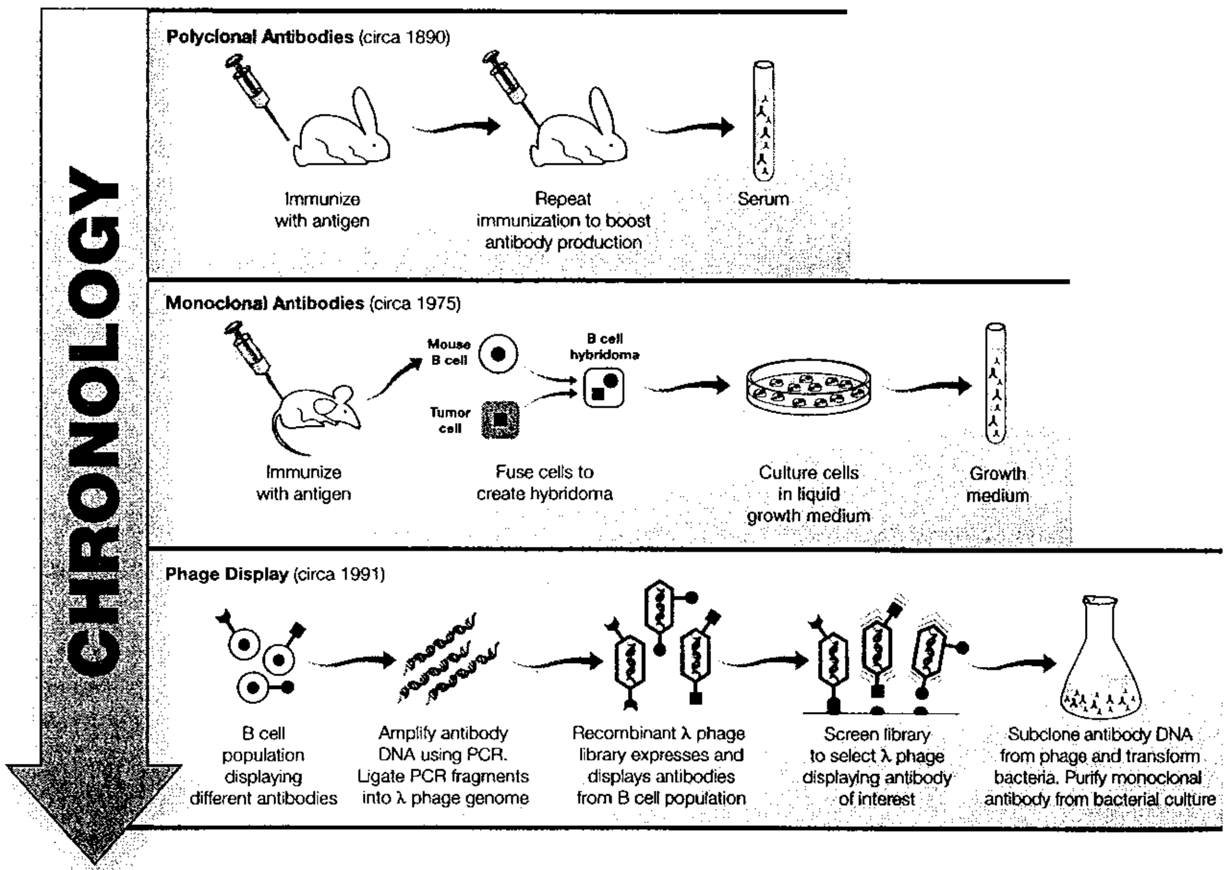
For any immunoassay to be valid, it must include both positive and negative controls, i.e., samples that will give known results. Controls are always run side by side with experimental samples. If you do not run a positive control and the experiment gives negative results, how can you be sure the results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

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.

Immunology **Lab #4: Practical application of Adaptive Immunity**
Biol 465 **Home pregnancy tests/antigen-antibody reactions**

*You should have received and read an introduction to the immunology behind the home pregnancy test. Please refer to that document as you perform today's tests.

You will be expected to explain the results of your test using the one of the urine samples as well as the results from your classmates' tests, so make sure you observe all brands and samples tested today.

We will use 3 different brands of pregnancy tests, which use slightly different methods of detecting hCG from urine. Each box contains 2 tests. I will assign each group a test to be sure each of the three are used and samples to be sure we see both positive and negative results with each test.

Protocol

1. Obtain one of the pregnancy tests and read the directions for use from beginning to end.
2. Using one of the 3 samples provided, (marked 1,2,3) use the test as directed, except for dipping the absorbent end into the tube for the suggested length of time, rather than holding it in a stream of urine.
3. Allow the test to run for the recommended time and decide whether your sample came from a pregnant woman or not.
4. Share your results with other classmates and come to a consensus on the samples.

Understanding the results. (Dr. Super will reveal the origin of each sample).

1. Why are these tests called lateral flow tests? What is the purpose of having the urine slowly progress down the length of the strip? Why is the viewing area sealed?
2. Why do the indicator areas look so uniform? (straight lines/plus signs.)
3. Describe several possible immunological explanations for the results of your test and that of your classmates. Be as specific as possible.
4. What is the purpose of the control window/line?
5. Describe how a control might have worked in your specific test. Propose a situation in which the control indicated you should not trust the results of the test.

6. Clearly the tests used today require very specific antibodies. Spend some time discussing the nature of antibodies and the technology used for generating antibodies of choice for applications such as those done today. (See handout, follow up with more research into the procedure).

Write-up:

Write a 1-2 page summary of how antibodies are produced for medical diagnostic purposes. Define *monoclonal* and *polyclonal* in your explanation.

Identify at least one reference which describes the use of monoclonal or polyclonal antibodies in research. Cite your reference (s). Use references from journals, not just on-line postings of information. Include in your reference: Title of the article, authors of the article, Journal in which the article was published, volume, date and page numbers of the article.