Received 2.11.06 | Revisions Received 3.1.06 | Accepted 3.2.06

Principles of Immunochemical Techniques Used in Clinical Laboratories

Marja E. Koivunen, Richard L. Krogsrud

(Antibodies Incorporated, Davis, CA)

DOI: 10.1309/MV9RM1FDLWAUWQ3F

Abstract

Immunochemistry offers simple, rapid, robust yet sensitive, and easily automated methods for routine analyses in clinical laboratories. Immunoassays are based on highly specific binding between an antigen and an antibody. An epitope (immunodeterminant region) on the antigen surface is recognized by the antibody's binding site. The type of antibody and its affinity and avidity for the antigen determines assay sensitivity and specificity. Depending on the assay format, immunoassays can be qualitative or quantitative. They can be used for the detection of antibodies or antigens specific for bacterial, viral, and parasitic diseases as well as for the diagnosis of autoimmune diseases. Immunoassays can measure low levels of disease biomarkers and therapeutic or illicit drugs in patient's blood, serum, plasma, urine, or saliva. Immunostaining is an example of an immunochemical technique, which combined with fluorescent labels allows direct visualization of target cells and cell structures.

Immunochemistry offers simple, rapid, robust yet sensitive, and in most cases, easily automated methods applicable to routine analyses in clinical laboratories. Immunochemical methods do not usually require extensive and destructive sample preparation or expensive instrumentation. In fact, most methods are based on simple photo-, fluoro-, or luminometric detection. Immunochemical methods have rapidly replaced chromatographic techniques in clinical diagnostics, offering fast detection of antibodies associated with specific diseases, disease biomarkers, hormones, and pharmaceuticals. The assays most often used in clinical immunochemistry involve either quantitative or qualitative formats using enzyme-linked immunosorbent assays (ELISAs), immunochromatography in the form of lateral-flow devices like dip-sticks and test strips or Western Blot assays used to interpret data from protein analysis with gel electrophoresis. Similarly, immunohistochemistry, one of the main diagnostics tools in today's clinical laboratories, is also based on the principles of antigen-antibody binding.

Antigen-Antibody Binding

All immunochemical methods are based on a highly specific and sensitive reaction between an antigen and an antibody. Antigen is a substance that induces the production of antibodies [ie, proteins from the class of immunoglobulins (MW about 150 kDa) that are produced in the immune system of any vertebrate or human as a result of a defense reaction (immunity) to this foreign substance]. Antibodies are a large family of glycoproteins that share key structural and functional properties. Functionally, they can be characterized by their ability to bind both to antigens and specialized cells or proteins of the immune system. Structurally, antibodies are often visualized as Y-shaped molecules, each containing 4 polypeptides—2 identical polypeptide units called heavy chains and another 2 called light chains (**Figure 1**). Immunoglobulins are divided into 5 classes: IgG, IgM, IgA, IgE, and IgD based on the number of Y-like units and the type of heavy-chain polypeptide (γ, μ, α, ε, and δ) they contain. The most abundant serum antibody is usually IgG, which contains 3 protein domains, 2 of which (Fab fragments) are identical and form the arms of the Y. Each Fab region contains a site that can bind to an antigen. The third domain (complement-binding Fc fragment) forms the base of the Y, and is important in immune system function and regulation.

The region of an antigen that interacts with an antibody is called an epitope or an immunodeterminant region. The binding of an antibody to the antigen is dependent on reversible, noncovalent interactions, and the complex is in equilibrium with the free components. The binding pocket of an antibody can accommodate from 6 to 10 amino acids. Small changes in the antigen structure (such as a single amino acid) can affect the strength of the antibody-antigen interaction. The measure of the strength of the binding is called **affinity**, and it is usually expressed in terms of the concentration of an antibody-antigen complex measured at equilibrium. It typically ranges from micro (10^{-6}) to pico (10^{-12}) molar. High-affinity antibodies can bind

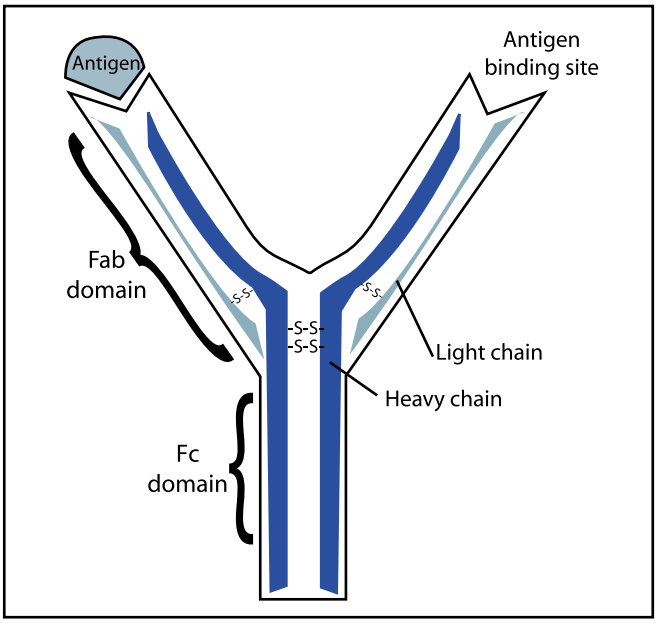

Figure 1_Schematic of antibody structure.

more antigen in a shorter period of time than low-affinity ones, and they form more stable complexes. Hence, high-affinity antibodies are usually preferred in immunochemical techniques.[1]

Avidity is another parameter used to characterize the antibody-antigen binding reaction. It is the measure of overall stability of the complex, determined by the antibody's affinity for the epitope, the number of binding sites per antibody molecule and the geometric arrangement of the interacting components. Avidity describes all factors involved in the binding reaction, and it determines the success of all immunochemical techniques. Antibodies are usually highly specific for the antigen. However, some antibodies show **cross-reactivities** to similar epitopes on other molecules. This makes the immunochemical method less specific but at the same time more applicable to situations where the target is a class of structurally related molecules.

Assay specificity and sensitivity are determined by the quality of antibodies used in the method. Polyclonal antibodies are isolated from the serum of immunized animals, usually rabbits, and the serum is a combination of numerous antibodies with different specificities and affinities. Monoclonal antibodies are more homogenous in terms of specificity and affinity because of the production method involving immunization of mice followed by hybridoma technologies.[2,3]

Precipitin Test

The precipitin test is an example of a clinical test based on antigen-antibody reaction. Antibodies are able to precipitate antigens through multivalent binding, in which 2 Fab fragments in a single antibody can simultaneously bind to 2 antigens. A matrix of antigen:antibody complexes in a solution will then lead to a formation of a visible precipitate. In the precipitin test, a soluble antigen and antibody diffuse toward each other, and a visible precipitate forms when the 2 solutes meet at an optimal concentration. The double immunodiffusion, or Ouchterlony gel diffusion, takes place in an agarose gel, with adjacent wells filled with an unknown soluble antigen and the known antibody solution. If antigen specific for the known antibody is present, the 2 components produce a visible precipitin band in the gel.[3]

In counterimmunoelectrophoresis (CIE), electrical current is applied to speed up the migration of soluble antigens and antibodies. In basic pH, most microbial antigens have a net negative charge migrating towards the positively charged electrode. On the other hand, antibody molecules are only weakly negatively charged or neutral under alkaline conditions and hence, they do not migrate in the electrical field but are carried toward the cathode by the buffer ions—a phenomenon called electroendoosmosis. When target antigen and antibody are present, they will meet and form a visible precipitin band. Compared to passive diffusion, the electrophoretically-enhanced system is much faster, requiring less than an hour for completion.[4] In the 1970s, CIE was widely used in clinical laboratories, but it has now been replaced by faster and more sensitive immunochemical tests for antigen detection.

Complement Fixation

In public health laboratories, complement fixation (CF) tests are used for typing of viral isolates. In brief, the presence of a specific antibody in a patient's serum is detected by using the antigen, complement, and red blood cells (RBCs). If antibody is present, it will bind to the specific viral antigen. Addition of a complement that binds to the antigen-antibody complex forms a system, which allows RBCs to settle out of the serum as a pellet. If no antigen-specific antibodies are present, no complex is formed, and the complement will lyse the RBCs when they are added.[3]

Hemagglutination

Hemagglutination inhibition (HI) assay is an indirect or passive agglutination assay where the antigen is bound to an inert substance and then mixed with patient serum containing the possible antibody. Serially diluted serum samples are placed in a microtiter plate to which the viral antigen and the RBCs are added. The last serial dilution that yields total inhibition of agglutination is the serum titer for the patient. Hemagglutination inhibition assays are quite tedious and labor-intensive because they require fresh erythrocytes and antigen dilutions each time the test is performed. At the moment, 1 of 3 immunochemical treponemal tests used for the identification of syphilis is based on a qualitative hemagglutination test. In this assay, anti-*T. pallidum* antibodies (MHA-TP) are detected using sheep red blood cells (SRBC) as a carrier for the antigen. For the test, the patient serum is serially diluted and added to both *T. pallidum*-sensitized and unsensitized SPBCs, and the results are reported as titers for the hemagglutinization reaction.[4]

Particle Agglutination

Agglutination reactions are similar to precipitin reactions, but they involve antigens or antibodies bound to the surface of carriers such as polystyrene beads. The visible precipitation (clumping) of antibody-antigen complexes with the beads occurs only when the antigen molecules contain multiple epitopes leading to crosslinking and a visible agglutinization reaction (**Image 1**). Attachment of antibodies to carrier particles increases the assay sensitivity and as a result, particle immunoassays are 3 orders of magnitude more sensitive than the standard agglutination tests.

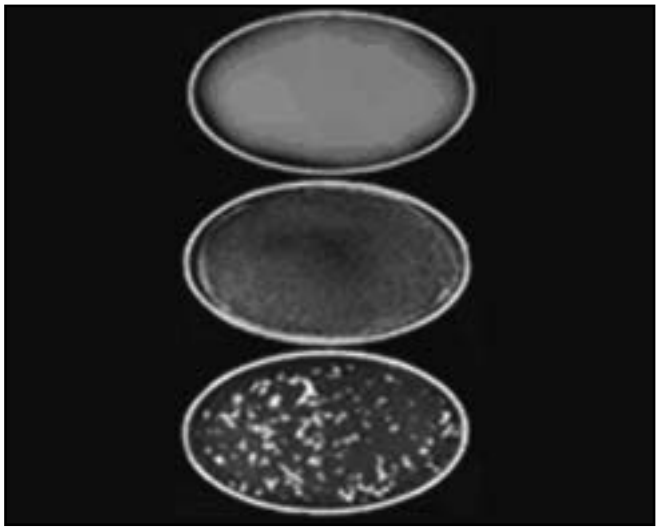

Image 1_Agglutinization test showing negative (milky) and positive (speckled) reactions.

Latex and Staphylococcal Agglutination

Latex agglutination utilizes latex beads mixed with a liquid specimen on a glass slide. After a short mixing, the reaction can be read with a naked-eye. Latex agglutination tests are prone to false-positive results due to nonspecific agglutination reactions. Hence, it is important to include proper positive and negative controls in the test. Sample pretreatment methods, like centrifugation, boiling, or filtration can be helpful in elimination or minimization of nonspecific agglutinations. Some specimens, like urine, can be concentrated by centrifugation or membrane filtration to increase the test sensitivity. Latex agglutination test kits are commercially available. The tests are easy to perform and sensitive. However, they might not be the fastest method of choice, and when the Strept A test kits based on latex agglutination were first introduced to the market, they often required an initial extraction of the group A carbohydrate antigen from the cell wall of the organism. The sensitivity and specificity of current group A streptococci (GAS) tests for throat swab specimens are high enough to justify their use for adult patients, but for the pediatric population, a simultaneous throat culture is still recommended to rule out false-negative results. Even though latex agglutination tests are rapidly being replaced by faster methods like lateral-flow test strips, latex agglutination tests are still routinely used for culture confirmation and serotyping in many clinical laboratories.

In Staphylococcal coagglutination tests, intact formalin-killed *Staphylococcus aureus* cells are used for visualization instead of latex beads.[3] The cell walls of these organisms contains protein A, which binds to the Fc portion of the IgG antibody, leaving the Fab portion available to react with specific antigens. Coagglutination is more susceptible for nonspecific reactions, and sample preparation is even more important than when using latex particles. Coagglutination is highly specific, though, and it is often used to confirm the identification of bacterial colonies in culture plates, but it is often not the best choice for rapid antigen detection in a clinical specimen.

Immunoturbidimetric Assays

Microparticle immunoturbidimetric assays can be used for quantitative measurement of drugs or biomarkers in body fluids like serum, plasma, or urine. The assays are based on an agglutination reaction induced by the antigen-antibody binding. For drug monitoring purposes, the assay often includes a competition between drug in the sample and drug coated onto a microparticle. In the absence of free drug, the drug-coated microparticle is rapidly agglutinated in the presence of antibody. When a light is directed to the sample mixture, the absorbance change measured photometrically is proportional to the rate of agglutination of the microparticles. If the sample contains free drug, the agglutination reaction is partially inhibited, slowing down the rate of absorbance change. A concentration-dependent classic agglutination curve can be obtained, with maximum rate of agglutination with the lowest drug concentration, and the lowest agglutination rate at the highest drug concentration.[5] Biomarkers, such as serum ferritin, can be measured using competition between protein in the sample and the antigen for the specific antibody coated onto a microparticle.

Light-Scattering Immunoassays

Nephelometric assays used in clinical laboratories utilize the fact that antigen-antibody complexes in solution scatter light at various angles to the direction of the incident light. A nephelometer uses a high-intensity light source that passes through a reaction vessel containing the immunoreactants, and a photodetector collects the light scattering signal. The immunoprecipitation curve is the key concept for understanding the light-scattering immunoassays.[4] (**Figure 2**). The reaction requires at least a bivalent immunoglobulin and an antigen with at least 2 epitopes. The curve contains 3 main areas: the antibody excess zone is the first one occurring when there is excess antibody available, little or no cross-linking of antigen, and only minimal precipitation. As more antigen is added, the reaction moves to the equivalence zone at which every antibody is attached to 2 antigens; maximal lattice formation occurs with maximal precipitation of the Ag-Ab complexes. As more antigen is added, the reaction moves to the third zone, antigen excess. All free antibody has been used, and no precipitation occurs due to the lack of antibody. As a rule of thumb, all nephelometric assays have to operate in the first zone (antibody excess) up to the zone of equivalence. In these 2 zones, there is a stoichiometric relationship between the number of complexes formed and the concentration of antigen. The 2 basic types of nephelometers measure the scattered light at different points of the immunoprecipitation curve: rate or kinetic nephelometers utilize the initial portion of the curve, while endpoint nephelometers utilize the equilibrium phase of the reaction.

A wide variety of proteins like immunoglobulins, albumin, antithrombin III, fibrinogen, rheumatoid factor, and myoglobin can be measured in human biofluids using light-scattering assays. Basic endpoint nephelometric assays can be made more sensitive by coupling antigens to small particles. These, particle-enhanced nephelometric assays, can be used for the measurement of high-sensitivity C-reactive protein (CRP) used as a biomarker for cardiovascular risk. Recent studies have indicated that elevated levels of CRP are not just an indicator of inflammation but can also be associated with a higher risk of future cardiac events.[6,7] The new, high-sensitivity CRP (hs-CRP) immunochemical tests can assess cardiovascular risk in the apparently healthy population, especially when combined with an index of lipid status. In the past, CRP was only used as an acute-phase protein with cutoff values between 0.5 and 1 mg/dL. Conventional nephelometric and turbidimetric tests have a working range of 0.2 to 0.4 mg/dL while the latest commercial tests based on particle-enhanced nephelometry can measure concentrations as low as 0.02 mg/dL.

Nephelometric assays should always be run with both positive and negative controls as well as a multilevel control material recommended by instrument manufacturers. The standard curve for rate nephelometers has a linear portion, which can be

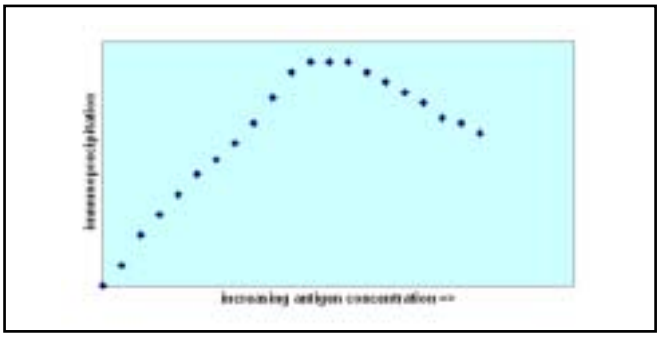

Figure 2_Immunoprecipitation curve: precipitation is observed when an increasing amount of antigen is added to a fixed concentration of antibody.

generated with a single calibrator. For endpoint nephelometers, at least 5 calibrators are necessary. Special attention is required when measuring patient samples with interfering substances causing either excess background or decreased scatter. Another potential problem is the presence of IgM rheumatoid factor (RE), which may bind to the anti-human antibody used in the assay.

Enzyme Immunoassays

The first immunoassay applications in the clinical laboratories often involved detection with radioactive compounds. These radioimmunoassays (RIAs) have now been replaced by enzyme immunoassays (EIA), which offer easy and nonhazardous detection of antigens or antibodies in clinical samples. In EIA, enzyme molecules are conjugated to secondary (detection) antibodies, which bind to the primary antigen-antibody complex. When the appropriate substrate is added, the enzyme catalyzes the production of a colored end-product, which can be visualized and quantitated. The enzymes most often used are alkaline phosphatase (AP) or horseradish peroxidase (HRP).[8]

Most commercially available EIA systems require separation of specific antigens from nonspecific complexes. Such systems are called solid-phase immunosorbent assays (SPIA) or enzyme-linked immunosorbent assays (ELISA). Separation is achieved through binding the antigen or capture antibody on a solid support such as polystyrene microtiter plate, latex bead, or magnetic bead. The solid matrix allows for separation through repeated washings to minimize nonspecific binding.

For a capture (sandwich) ELISA, a clinical sample is first added to a well coated with an antigen-specific antibody. If the antigen of interest is present, it will bind to the antibody. Unbound sample is removed by washing, and a secondary antibody specific for the antigen is added.[9] In a direct method, an enzyme is conjugated to this secondary antibody. In an indirect method, the second antibody is not conjugated, and hence, a third, conjugated antibody against the Fc-portion of the second antibody is added to give the color reaction after the addition of substrate. The amount of color is directly proportional to the amount of antigen present in the original sample. This assay set-up utilizing 2 antigen-specific antibodies is called a sandwich assay—and they can be either direct or indirect depending on which antibody is conjugated to the enzyme label.

A requirement for a successful sandwich assay is an antigen that is large enough to contain different epitopes for at least 2 different antibodies. In cases where the analyte is a small molecule, like a toxin or a pesticide, a competitive immunoassay can be used.[10] In a competitive ELISA format, a structural mimic of the antigen is coated on the walls of the microtiter plate wells. This mimic is usually an antigen conjugated to a protein through a specific 'handle.' The sample together with the antigen-specific antibody is then incubated in the wells, at which stage, the antigen in the sample is competing with the surface antigen for the limited amount of antibody in solution. After washing, a conjugated secondary antibody specific for the first one is added, and after substrate addition a color is formed. The intensity of the color is now reversely proportional to the amount of antigen in the solution—the more antigen in the sample, the less antibody is bound to the surface, and the lighter the color.

Enzyme immunoassays are rapid and sensitive tools for the detection of disease-specific antigens or antibodies in the patient's serum.[11,12] One of the common targets in clinical laboratories is hepatitis B (HBV). There are numerous serological markers for hepatitis B including hepatitis B surface antigen (HbsAg), anti-HbsAg antibody, hepatitis B e-antigen (HbeAg), IgM antibody to the HB core antigen (HBc), and they all have distinct serological time profiles making it possible for the immunologists to determine the stage and time course of the disease in each patient. Commercially available HIV EIAs utilize indirect antibody capture and sandwich methodologies. The latest development of these tests targets the detection of viral infection even before the antibody against HIV-1 is detectable. For example, the viral p24 antigen can be used to detect active viral replication in blood. Flow-through and dot plot membrane HIV EIAs are now commercially available and are used in many laboratories where a rapid turnaround time is critical.

An indirect sandwich assay is also used for the diagnosis of giardiasis, an intestinal infection caused by a parasitic protozoan (single-celled organism) called *Giardia lamblia*. These protozoans are found in the intestines of many animals, including dogs and humans. This microscopic parasite clings to the surface of the intestine, or floats free in the mucous lining the intestine causing malabsorption of nutrients. For detection of *Giardia* in stool samples, immunoassays have proven more reliable than direct microscopic examination. There are several diagnostic kits available, including GiardEIA—an ELISA test detecting *Giardia lamblia* antigens in infected fecal samples in about 30-minute analysis time (**Figure 3**). In addition, FDA-approved lateral flow devices can be used for simultaneous detection of *G. lamblia*, *Cryptosporidium parvum*, and *Entamoeba histolytica* in stool samples.

A variation of enzyme labeling involves coupling of a reactive molecule such as biotin to the primary antibody. Signal is then detected when an enzyme-labeled streptavidin is added to the system. The avidin-biotin interaction tends to multiply the signal and hence, increase the assay sensitivity. Another type of avidin-biotin system is currently used in a variety of clinical assays where increased sensitivity and shorter incubation times are required. In these modified assays, biotinylated capture antibodies are bound on the solid support through avidin (or streptavidin) (**Figure 4**), which provides the amplification needed for the detection of analytes in low concentrations.[13]

EIA in Lateral Flow Systems

A well-known application of enzyme immunoassay is immunochromatography in which a capture antibody is immobilized onto a surface of a porous membrane (nitrocellulose, nylon, Teflon), and a sample passes along the membrane. This modification utilizes a disposable plastic cassette with the

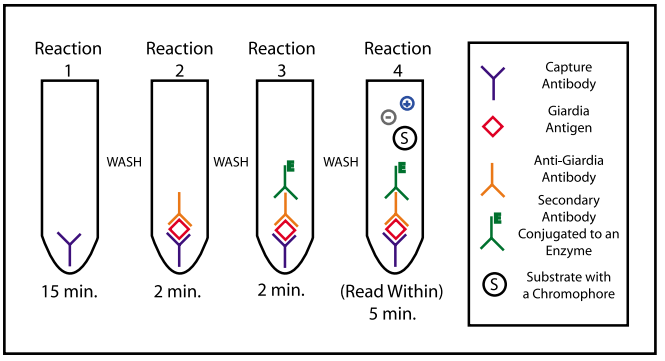

Figure 3_A schematic of the GiardiEIA capture (sandwich) assay used to detect *Giardia lamblia* antigens in fecal samples. The test takes less than 30 minutes to complete.

membrane attached to a chamber into which the clinical sample is added. An absorbent material placed below the membrane pulls the liquid reagents through the along the membrane allowing separation of nonreacted components from the membrane bound antibody-antigen complex.

The first major target analyte for in vitro diagnostic test kits utilizing the principles of immunochromatography was (human) chorionic gonadotropin (hCG) for the detection of pregnancy. For these lateral flow assays, the ligand (antibody) specific for the analyte is immobilized to the membrane (**Figure 5**). The detector reagent, typically coupled to a latex or colloidal metal particle, is deposited into a conjugate pad. When a sample (urine, plasma, whole blood) is added to the conjugate pad, the detector reagent is solubilized and begins to move with the sample flow front up the membrane strip. Analyte in the sample is bound by the antibody which is coupled to the detector reagent. As the sample passes over the zone to which the capture reagent has been immobilized, the analyte detector reagent complex is trapped, and a color develops in proportion to the analyte present in the sample.

Lateral-flow systems based on the principles of immunochromatography are quite popular because of the shorter analysis time compared to ELISAs. They can easily be performed as simple rapid qualitative tests for a single analytes—both in clinical laboratories and at home. The set-up usually contains both a negative and positive control for easy result interpretation and quality control. Membrane-bound EIA (lateral flow) tests are commonly used for rapid detection of hormones (hCG, LH), viruses (hepatitis B and C), bacteria (*Streptococcus*, *Chlamydia, Helicobacter pylori*), bacterial toxins, parasites (malaria), therapeutic and illicit drugs, as well as biomarkers such as troponin (cardiovascular disease) or prostate specific antigen (PSA, prostate cancer).

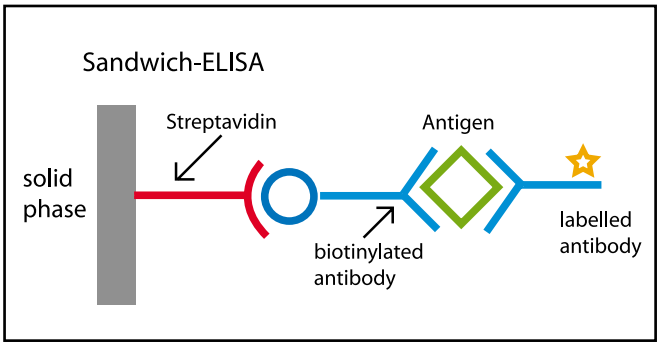

Figure 4_Biotinylated antibodies can be bound to the solid support via streptavidin. The 4 biotin-binding sites on 1 streptavidin molecule facilite the increase in assay sensitivity through amplification.

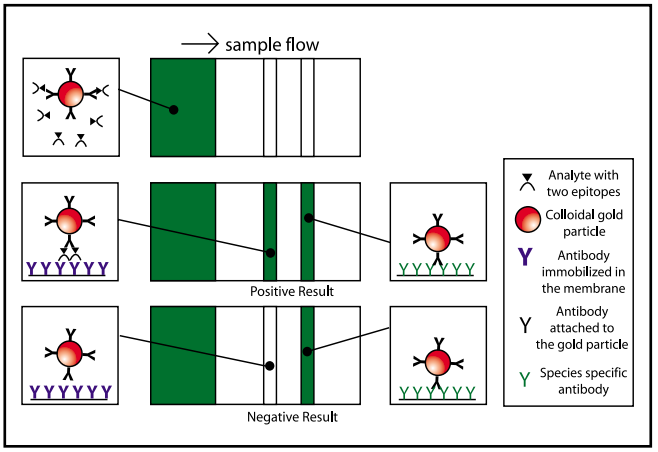

Figure 5_A schematic of a lateral flow capture (sandwich) assay used for detection of analytes (like hormones) with multiple epitopes.

Immunofluorescence Assays

In immunofluorescence (IFA), specific monoclonal or polyclonal antibodies are conjugated to fluorescent dyes (fluorochromes), which can be visualized using a fluorescence microscope, fluorometer, fluorescence scanner, or flow cytometer. Fluorophores are excited by light at a specific wavelength, and they release the extra energy by emitting light at another, longer wavelength. In a direct fluorescent antibody test (DFA) used for histochemistry, the antigen-specific labeled antibody is applied to a fixed specimen on a microscope slide, incubated, washed, and visualized under a fluorescence microscope. When a secondary, species-specific antibody is labeled with a fluorophore instead of the primary, the method is called indirect fluorescent antibody (IFA) test (**Figure 6**). Fluorescence microscopes or plate-readers used for detection contain filters specific for each emission wavelength. Conventional fluorophores like fluorescein isothiocyanate (FITC) emit light at the 550 nm wavelength. However, dyes emitting at longer wavelengths (red) are more desirable because most biological specimen emit green light after exposure to UV light (autofluorescence).

In clinical laboratories, fluorescent antibody tests are currently used for detection of bacterial, viral, and fungal infections as well as for bioimaging of tissue samples. A number of respiratory viruses can be directly detected in nasopharyngeal samples using direct fluorescent antibody (DFA) test. This technique is commonly used for respiratory syncytial virus (RSV), influenza A and B, and adenovirus. Through the development of immunochemical tools, rapid diagnosis of these infections is now possible in the point-of-care (POC) facilities. Fluorescence immunochemical tests are also successfully used for the diagnosis of autoimmune diseases such as lupus.

A systemic autoimmune disease, lupus (also called systemic lupus erythematosus or SLE), is a disorder of the immune system, which normally functions to protect the body against invading infections and cancers. In lupus, the immune system is over-active and produces increased amounts of abnormal antibodies that attack the patient's own tissues. Lupus is difficult to diagnose and often misdiagnosed unless there are characteristic symptoms such as the butterfly-shaped rash over the cheeks. There is no single definitive blood test for lupus, but the screening test most often used for diagnosis is the antinuclear antibody (ANA) test. An ANA test is based on human epithelial cells (Hep-2) cultured on a microscope slide, and detection of a reaction between antinuclear antibodies in the patient serum (diluted 1:40) and the antigen present in the cell nucleus. Detection involves microscopic examination of the slide after a fluorescent secondary antibody is added to the complex. If the nuclei fluoresce more brightly than the negative control with a discernible pattern, the reaction is considered positive. High titers (>1:160) usually indicate the presence of an autoimmune disease. Besides the concentration of antibodies, patterns of antibodies can be identified that indicate certain diseases. The ANAs present different "patterns" depending on the staining of the cell nucleus in the laboratory:

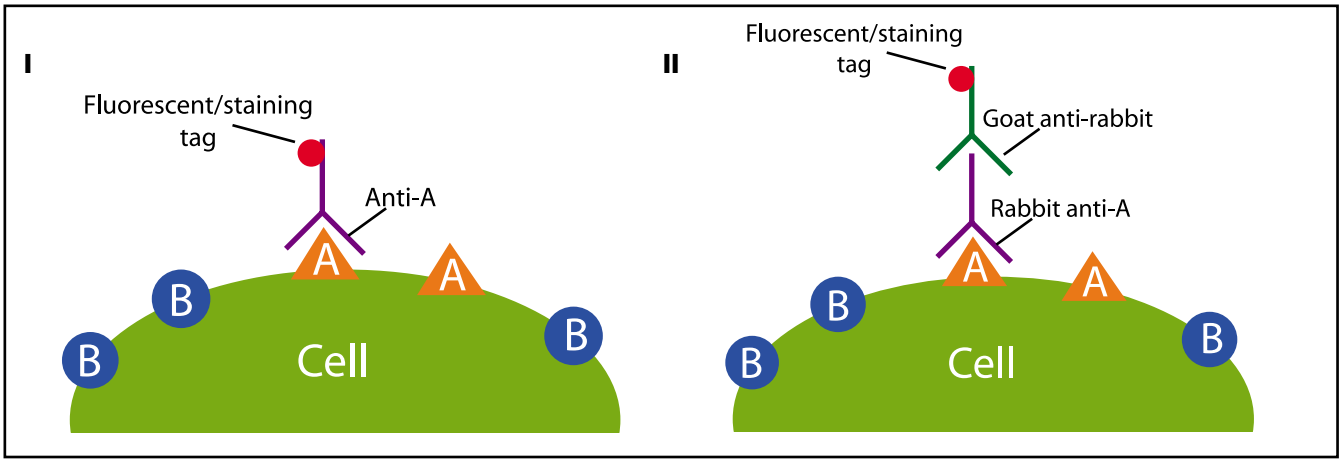

Figure 6_Direct fluorescent antibody test (**I**) uses antibodies that recognize specific antigens on the cell surface. In the indirect test format (**II**), the detection is based on a secondary (anti-species) antibody conjugated to a fluorescent label.

homogeneous or diffuse; speckled; nucleolar; and peripheral or rim (**Image 2**). While these patterns are not specific for any one illness, certain illnesses can more frequently be associated with one pattern or another.

Fluorescence Polarization Immunoassay

Fluorescence polarization immunoassay (FPIA) is based on the capacity of a fluorescein-molecule to emit plane-polarized light upon excitation. In FPIA, a fluorescein-labeled compound (drug) competes with unlabeled compound for a specific antibody-binding site. When a serum sample of interest is added into a mixture containing labeled drug and the specific anti-drug antibody, and the mixture is excited with plane-polarized light (490 nm), the fluorescein molecules emit plane-polarized light at a longer wavelength (520 nm). Small, free drug-fluorescein rotates faster leading to less emission whereas larger complexes of antibody-drug-fluorescein rotate slower producing more emission. The compound of interest (free drug) in serum competes for antibody with the fluorescein-bound drug. The more drug in the sample, the less fluorescein-labeled drug bound to antibody resulting in lower emission of plane-polarized light. The FPIAs are currently widely utilized in therapeutic drug-monitoring schemes in hospitals and point-of-care facilities. The more recent advent of microplate readers equipped with polarizing optics has led to the adoption of fluorescence polarization as a readout mode for high-throughput screening.

Enzyme Multiplied Immunoassay—EMIT

Similar to FPIAs, enzyme multiplied immunoassays (EMIT) have a wide application in therapeutic and illicit drug monitoring. In this type of assay, a sample of interest with the analyte (drug) is added to a fixed quantity of enzyme-bound drug, and the anti-drug antibody. After the addition of substrate, absorbance measurements are taken at time intervals to determine the speed of the enzyme reaction. The more free drug in the sample, the faster the enzyme reaction because only the unbound enzyme-drug complexes are capable of binding the substrate (**Figure 7**). The method can be used for whole blood, serum, or urine. Enzyme multiplied immunoassays can be fully automated with a fast throughput of clinical samples especially in laboratories specializing in monitoring therapeutic drugs like cyclosporin in transplant recipients.[14,15]

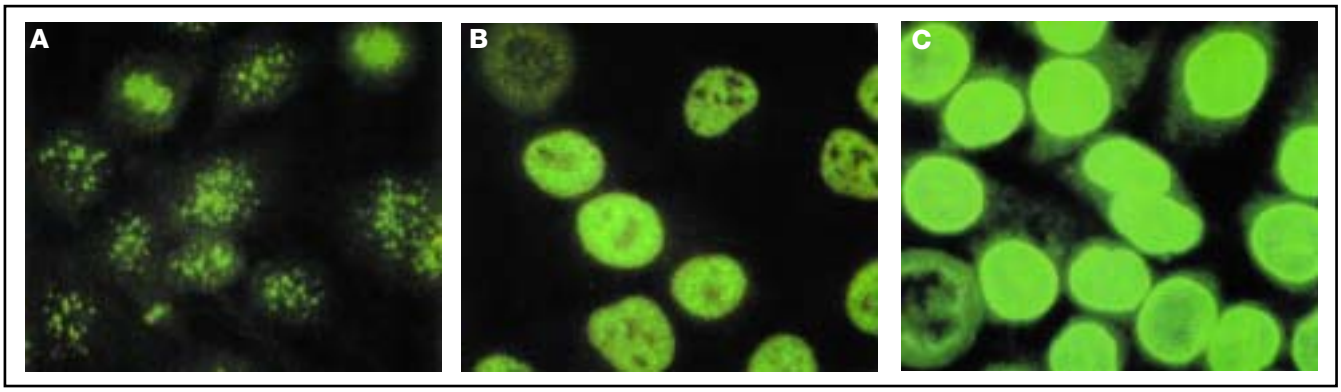

Image 2_Photos representing different types of staining in the antinuclear antibody (ANA) test kit (Antibodies Incorporated, Davis, CA) using human epidermal cells (Hep-2) fixed on a glass slide. The different staining patterns: (**A**) discrete speckled, (**B**) fine speckled, and (**C**) peripheral can be associated with different forms of autoimmune diseases. Photos are courtesy of Gerald M. Penn, MD, PhD, Chief of Pathology, Childrens Hospital, Columbus, OH.

Immunostaining

Immunostaining is a term that applies to any use of an antibody coupled to an enzyme or dye to detect a specific protein or antigen in a sample. The most common applications are gel electrophoresis (immunoblotting, Western Blot) or within tissue slices in immunohistochemical staining. For example, the standard confirmatory test for an anti-HIV reactive screening test result is a **Western Blot** assay for which separation of viral proteins by molecular weight is done by electrophoresis. The gel is then blotted on a membrane allowing the transfer of viral proteins onto the membrane. The membrane is then incubated with patient serum. A HIV specific antibody binds to viral proteins on the membrane and can be detected using an enzyme-labeled secondary antibody or a biotin-avidin detection system.

Fluorescent markers can be applied to a wide selection of tests including **histochemistry** and **tissue microarray** for detection of specific proteins like cancer markers in cells or tissues and for cell visualization in flow cytometer measurements. Some immunostaining agents can be applied in a single stage, where the primary antibody is directly linked to a coloring agent, but in most cases the primary antibody is targeted by a labeled "secondary" antibody giving an amplified signal as multiple secondary antibodies will bind to a primary antibody (**Image 3**).

The advantages of using immunofluorescence include rapid visualization of specific tissue, cell culture, body fluids, and swap specimen. However, high background fluorescence might be a problem when green-emitting dyes are used. In most cases, assay sensitivity can be improved by using fluorescent dyes, and the problem of photobleaching (fading over time and after multiple excitations) is diminished in new types of fluorophores (quantum dots, long-lifetime nanoparticles).

Chemiluminescence Assays

The chemiluminescence (CL) assay is very popular and widely used in many different assay formats. Chemiluminescence refers to the emission of light that occurs when a substrate decays from an excited state to a ground state. While fluoresence reactions utilize incident radiation for energy, chemiluminescence energy is generated from a chemical reaction, most often an oxidation reaction. Because of an extremely low background of chemiluminescence in biological samples, CL assays are most sensitive immunoassays with detection limits as low as attomole (10^{-18}) or zeptomole (10^{-21}) level.[16] The results are recorded with a luminometer, which can read either tubes or microtiter plates or the light signal can be recorded onto photographic film.

Chemiluminescence can be applied either as a direct label or a chemiluminescent compound can be used as a substrate for an enzyme-labeled immunoreactant. Most common chemiluminescent compounds are acridinium esters and derivatives of isoluminol.[17] Acridinium compounds can be conjugated to either antibody or antigen. A chemical reaction producing light is induced by addition of either sodium hydroxide or hydrogen peroxide. Assays utilizing isoluminol are even more sensitive than the one with acridinium—the reaction is developed by adding a mixture of hydrogen peroxide and a catalyst into the sample with isoluminol label.

Optical Immunoassays

Optical immunoassay (OIA) is based on the interaction of antigen-antibody complex on inert surfaces. Specific binding of antibody increases the thickness of the reactants on the surface, and changes the color of the light reflected from the surface. Optical immunoassays are used in many laboratories to detect group A *Streptococcus* (GAS) in children as well as respiratory viruses.

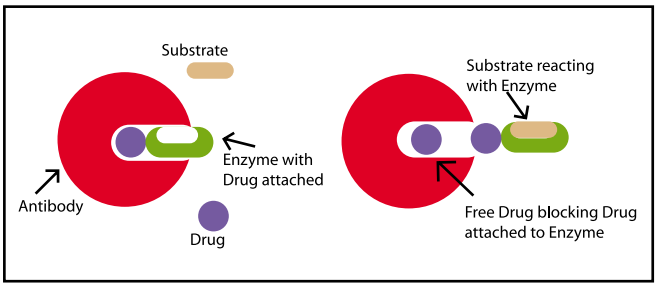

Figure 7_Enzyme multiplied immunoassay (EMIT) for the measurement of free drug in the sample involving a fixed quantity of enzyme-bound drug, and an anti-drug antibody. After substrate addition, the development of color is measured at 15 and 45 seconds, and the change in absorbance is converted into a concentration reading using a standard curve based on known amounts of free drug in solution.

Analytical Problems

As described above, the antigen-antibody binding is highly specific, allowing for specific detection of analytes with a particular epitope. However, some antibodies bind to non-target compounds that have structural similarities with the target analyte—in other words, they have cross-reactivity against these related compounds. Cross-reactivity makes assays less specific but at the same time more robust, and suitable for detection of different classes of compounds, like pyrethroid metabolites in human urine samples.[18] Nonspecific binding of primary or secondary antibodies onto solid support can lead to false positive readings. Usually, nonspecific binding is eliminated using blocking agents

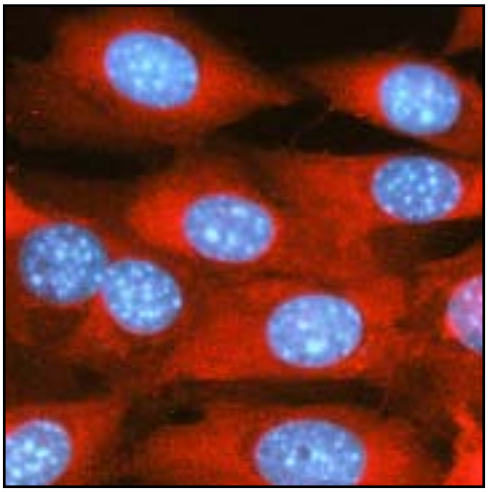

Image 3_NIH 3T3 Fibroblasts labeled indirectly for: (1) actin (red) using rabbit anti-pan actin IgG and goat anti-rabbit IgG coupled to Fort Orange Evitags (Evident Technologies) and (2) the kinetochore (blue/green) using human anti-kinetochore IgG and goat anti-human IgG coupled to FITC (Antibodies Incorporated) with a DAPI counterstain (purple). The image was acquired on a Leica TCS laser scanning confocal microscope at 60x with an oil-immersion objective. Photo is courtesy of Antibodies Incorporated, Davis, CA.

like bovine serum albumin (BSA) or non-fat dry milk, which bind to potential non-specific binding sites without interfering with the assay itself. One of the problems with the analysis of biofluids (blood, urine, saliva) is the interference caused by the structurally similar compounds in the sample matrix. Sometimes this matrix interference is so strong that the sample has to be cleaned up prior to analysis. Common clean-up techniques that eliminate structurally similar, interefering substances include liquid-liquid or solid phase extraction (SPE).

Precision and Accuracy

Precision is a measure of the reproducibility of sample data from measurement to measurement and is affected by both the consistency of the test and the analyst's technique. Precision is assessed by conducting several analyses of a sample or a control sample and calculating the relative standard deviation of the sample results. As with all bioassays, immunoassays have inherently more variability between replicate measurements than strictly chemical chromatographic methods.[8,19] For example, a coefficient of variation of 15% between replicate measurements in an LC-MS analysis would be an indication of a problem, whereas it is considered acceptable for ELISAs. Accuracy is a measure of how close the result of an analysis comes to the "true" concentration in a sample. Control samples are used to assess the accuracy of the immunoassay method and the application being used. Such samples are solutions of known concentration, often supplied by the manufacturer. They are to be analyzed with each set of calibration standards before the samples are analyzed. The control sample will have an acceptance range that approximates the known concentration. If the method is to be considered accurate, the concentration obtained by the user for the control sample must fall into that range.

It should be kept in mind that precision and accuracy are measures applicable only to quantitative immunoassay data. It is impossible to measure the precision or accuracy of qualitative or semi-quantitative data reported as either: (1) greater or less than a given value, or (2) within a range of pre-established values. Performance evaluation samples can be used to check the accuracy of the quantitative method. Performance evaluation samples are solutions of known concentration of target analytes, and they should be purchased from a different vendor than the control samples. When results from immunochemical analysis are used for diagnostic purposes, they should always be validated against another, gold-standard method to eliminate the possibility of both false positive and false negative results.[8]

Future Development of Immunochemical Tests

There are many advantages to using immunochemical methods in clinical laboratories. Specificity, sensitivity, speed, portability, ease of use, low cost, and the range of analytes that can be measured are the main reasons for the increased use of these techniques in laboratories and point-of-care facilities. The future for clinical immunochemistry looks very promising. We have come a long way from the most simple antibody-based tests like the PPD skin test for tuberculosis to fully automated ELISAs with fluorescent markers for quantification of small molecules in patient serum to a pico or femto mole level. Future development will undoubtedly include more micro-scale instrumentation with ultrasensitive detection,[20] faster turnaround time, and increased throughput in clinical laboratories. Rapid development in the area of quantum dots and other fluorescent nanoparticles[21] will produce more applications in which multiple analytes can be detected simultaneously.[22,23] Advances in the fields of multiplex immunoassays and sensitive cellular imaging will eventually benefit routine clinical laboratory analysis as well. LM

1. Devey ME, Steward MW. The role of antibody affinity in the performance of solid phase assays. In: Kemeny DM, Challacombe SJ, eds. *ELISA and other solid phase immunoassays. Theoretical and practical aspects.* Chichester, UK: John Wiley & Sons, 1988:135-154.
2. Goding JW. *Monoclonal antibodies: principles and practice*. London: Academic Press; 1983:276.
3. Harlow E, Lane D. *Antibodies - a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1988:726.
4. Mahon CR, Tice D. *Clinical Laboratory Immunology*. Upper Saddle River, NJ: Pearson Education, Inc.; 2006:325.
5. Carpenter AB. Antibody-based methods. In: Rose NR, Hamilton RG, Detrick B, eds. *Manual of Clinical Laboratory Immunology*. Washington, DC: ASM Press; 2002:6-25.
6. Ridker PM, Morrow DA. C-reactive protein, inflammation, and coronary risk. *Cardiol Clin.* 2003;21:315-325.
7. Ridker PM. High-sensitivity C-reactive protein, inflammation, and cardiovascular risk: from concept to clinical practice to clinical benefit. *Am Heart J.* 2004;148:S19-S26.
8. Crowther JR. *The ELISA guidebook*. Totowa, NJ: Humana Press, 2001:421.
9. Butler JE. The immunochemistry of sandwich ELISAs: principles and applications for the quantitative determination of immunoglobulins. In: Kemeny DM, Challacombe SJ, eds. *ELISA and other solid phase immunoassays. Theoretical and practical aspects.* Chichester, UK: John Wiley & Sons, 1988:155-180.
10. Koivunen ME, Gee SJ, Park E-K, et al. Application of an enzyme-linked immunosorbent assay for the analysis of paraquat in human-exposure samples. *Arch Environ Contam Toxicol.* 2005;48:184-190.
11. Chantler SM, Clayton A.-L. The use of ELISA for rapid viral diagnosis: viral antigen detection in clinical specimens. In: Kemeny DM, Challacombe SJ, eds. *ELISA and other solid phase immunoassays. Theoretical and practical aspects.* Chichester, UK: John Wiley & Sons, 1988:279-302.
12. Duncan RJS. The use of ELISA for rapid viral diagnosis: antibody detection. In: Kemeny DM, Challacombe SJ, eds. *ELISA and other solid phase immunoassays. Theoretical and practical aspects.* Chichester, UK: John Wiley & Sons, 1988:303-318.
13. Schetters H. Avidin and streptavidin in clinical diagnostics. *Biomolec Eng.* 1999;16:73-78.
14. Dessars B, Cotton F, Thiry P, et al. Comparison of automated ACMIA and EMIT immunoassays for whole blood cyclosporin monitoring. *Clin Lab.* 2003;49:135-140.
15. Schutz E, Svinarov D, Shipkova M, et al. Cyclosporin whole blood immunoassays (AxSYM, CEDIA, and EMIT): A critical overview of performance characteristics and comparison with HPLC. *Clin Chem.* 1998;44:2158-2164.
16. Weeks I, Woodhead JS. Chemiluminescence immunoassay. In: Kemeny DM, Challacombe SJ, eds. *ELISA and other solid phase immunoassays. Theoretical and practical aspects.* Chichester, UK: John Wiley & Sons, 1988:265-278.
17. Kricka LJ. Clinical applications of chemiluminescence. *Anal Chim Acta.* 2003;500:279-286.
18. Shan G, Huang H, Stoutamire DW, et al.. A sensitive class specific immunoassay for the detection of pyrethroid metabolites in human urine. *Chem Res Toxicol.* 2004;17:218-225.
19. Fare TL, Sandberg RG, Herzog DP. Considerations in immunoassay calibration. In: Van Emon JM, Gerlach CL, Johnson JC, eds. *Environmental immunochemical methods. Perspectives and applications.* Washington, DC: American Chemical Society, 1996:240-253.
20. Christodoulides N, Mohanty S, Miller C, et al. Application of micro-chip assay system for the measurement of C-reactive protein in human saliva. *Lab Chip.* 2005;5:261-269.
21. Medintz IL, Uyeda HT, Goldman ER, et al. Quantum dot bioconjugates for imaging, labelling and sensing. *Nature Mat.* 2005;4:435-446.
22. Kartalov EP, Zhong JF, Scherer A, et al. Anderson WF. High-throughput multi-antigen microfluidic fluorescence immunoassays. *BioTechniques.* 2006;40:85-90.
23. Rao RS, Albala, JS, Lane SL, et al. Developing rapid, point-of-care, multiplexed detection for use in lateral flow devices. SPIE Symposium, Smart Medical and Biomedical Sensor Technology III, Nov. 2005; 6007:227-233.