IMMUNOASSAY

1. Introduction

Immunoassay is a method that identifies and quantifies unknown analytes using antibody–antigen reactions. Techniques are based in immunochemistry, analytical chemistry, and biochemistry, with a history of development paralleling advances in microbiology and immunology (see also IMMUNOTHERAPEUTIC AGENTS).

Immunoassay has developed primarily as a subdiscipline of clinical diagnostics. From the first agglutination assays through radioimmunoassay (RIA) and other immunoassay formats, the focus of each advancement was a specific clinical need, such as the identification of infectious agents in biological tissues and fluids. The development and commercialization of RIAs in the 1960s and 1970s placed immunoassay as a leading technology in clinical diagnostics, eventually evolving into a variety of immunoassay formats which, in 2000, captured >30% of the *in vitro* clinical diagnostic market (1).

The success of immunoassay in clinical diagnostics, and the generic nature of immunoassay technology has resulted in the application of the method in other areas. By the late 1980s, commercial immunoassay products and systems were available for detection and diagnosis in environmental, food, and chemical processing applications. In the 1990s, immunoassays were adapted for high throughput screening used in new drug discovery. Whereas the application of immunoassays in these areas is small in relation to clinical diagnostic immunoassays, nonclinical applications of immunoassays have become commonplace for nonclinical applications (see AUTOMATED INSTRUMENTATION, CLINICAL CHEMISTRY).

2. Historical Perspective

The modern discipline of immunochemistry began in the late 1800s when Ehrlich and others discovered specific serum agents, now known as antibodies, that neutralized infectious agents (2). Based on the discoveries of specific precipitation of plague bacilli with serum from animals previously exposed to the bacillus and specific agglutination/clumping of bacterial cells (3), one of the first diagnostic immunoassays, an agglutination test for typhoid, was developed. By the 1930s, precipitating antibodies were being used routinely to quantify bacteria. For example, Heidelberger and Kendall (4) developed the typhoid precipitation test into a quantitative diagnostic method for typhoid, able to detect <0.1 μg of bacterial antigen.

Immunodiffusion and immunoprecipitation, developed in the 1940s as a means to identify and semiquantitate specific proteins, were the direct precursors to the development in 1953 of immunoelectrophoresis, a method used in many clinical laboratories (5). Single- and double-gel immunodiffusion and immunoelectrophoresis were, in effect, the first standardized and routinely used immunoassay methods (see ELECTROPHORESIS).

Although useful, immunodiffusion and immunoelectrophoresis are tedious and time consuming, difficult to automate, and not easily applied to mass sample analysis. In the early 1960s, a method was introduced that revolutionized immunoassay and clinical diagnostics. Developed from the pioneering work of Berson

and Yallow (6), radioimmunoassay became a routine, automated, highly adaptable and cost-effective method of analyzing large numbers of clinical samples. Quickly commercialized, by the early 1970s hundreds of RIAs were available for not only a wide spectrum of clinical analytes, but also environmental, food, and chemical analytes.

The success of RIA led to the development of other immunoassay methods in the late 1960s and early 1970s. Whereas these methods used the basic format of RIAs, the quantifiable indicators were changed from radioactive isotopes (qv) to ones based on the production of color, fluorescence, or light. The first of the nonisotopic immunoassays to be developed and commercialized utilized enzymes as indicator agents and utilized technology based on the work of Avremeas for enzymatic labeling of antibodies and antigens (7). These assays became known as enzyme-linked immunoadsorbant (or immunosorbant) assays (ELISAs) or simply enzyme immunoassay (EIA), and have become the primary laboratory and commercially used immunoassay method, applicable to hundreds of analytes and available in kit or automated analyzer format. EIAs and other nonradioactive immunoassays have largely replaced RIAs. Further evolution of immunoassays includes the development of near real-time, homogenous immunoassays and use both in clinical laboratories and for point-of-care (POC) diagnosis, the adaptation of immunoassay formats to immunosensors, [biosensors (qv) based on antigen–antibody reactions], and the use of antibodies in microfluidic microarrays in diagnostic panels and for high throughput sceening in drug discovery.

3. The Antibody–Antigen Reaction

Immunoassays are based on the binding and complexing of an antigen to an antibody, and the use of some physical or chemical means to measure and quantify the antigen–antibody complex. The antibody–antigen reaction is a typical reversible bimolecular reaction having rate constants for the forward and backward reactions that are dependent on the concentration of the antigen (Ag) and antibody (Ab), affinity for the antigen as defined by the association constant of the antibody for its antigen, temperature, pH, and other environmental conditions. This reaction is represented by an equation common to reversible receptor–ligand assays:

$$\mathrm{Ab} + \mathrm{Ag} \rightleftarrows \mathrm{AbAg} \tag{1}$$

and the equilibrium constant for the reaction is determined by the mass action equation:

$$K = \frac{[\mathrm{AbAg}]}{[\mathrm{Ab}]\,[\mathrm{Ag}]} \tag{2}$$

where [Ab] is the concentration of antibody sites for antigen, and [Ag] is the concentration of free antigen. The association, or affinity, constant for the antibody–antigen reaction is then further defined as the equilibrium constant at half-saturation of the antibody with antigen. Because at half-saturation AbAg and

Ab are equal, these cancel in the above equation and the association constant, K_a, is equal to the reciprocal of the free antigen concentration:

$$K_a = \frac{1}{[\mathrm{Ag}]} \tag{3}$$

Thus, if the antibody has a high affinity for the antigen, it has a high association constant. Typical association constants range from 10^6 to 10^{10}L/mol, and as high as 10^{13}L/mol for some monoclonal antibodies.

The definition of an association constant for an antibody–antigen reaction can become more complex if the antibody–antigen reaction involves a multivalent antigen, as is the case when a polyclonal antiserum is used for detection of an antigen. This type of multivalent binding is termed avidity and is defined by the equation:

$$x\ \mathrm{Ab} + y\ \mathrm{Ag} \longrightarrow \mathrm{Ab}_x\mathrm{Ag}_y \tag{4}$$

Definition of the association (or avidity) constant for such multivalent antibody–antigen reactions must consider not only the heterogeneity of the antibodies and the antigen determinant site(s), but also an apparent additive effect of binding two antigen molecules to a single antibody. Such effects lead to a multiplying of the individual association constants and an apparent large increase in the total association–avidity constant. This multiplication of avidity through multivalent binding has been exploited to increase the sensitivity of many immunoassays. A more detailed discussion of antigen–antibody reaction kinetics may be found in the literature (8,9).

As the result of many studies on the antigen–antibody reaction, it is known that the primary antibody–antigen binding event (in solution) occurs in ~10 s, whereas formation of the final complex (final binding equilibrium) takes ~30 min. Antigen–antibody reactions on solid surfaces, such as in the case where the antigen or antibody is chemically immobilized onto a solid support, can take significantly longer, up to a few hours, to reach final binding equilibrium. This time dependence of antigen–antibody binding plays a central role in immunoassay development and the need to balance maximal antigen–antibody binding within a minimal amount of time.

Most antibody–antigen reactions are optimal at ~ pH 7 in a low salt medium between 0 and 40°C. The typical free energy changes associated with the antibody–antigen reaction are ca. −33 to −42 kJ/mol (−8 to −10 kcal/mol). This is about the same amount of energy change associated with the nonspecific binding of drugs to a plasma protein such as serum albumin. The free energy varies depending on the nature of the antigen, being the lowest for large antigens, such as proteins (qv), and highest for antigens such as small peptides and low molecular weight drugs. Free energy changes are directly related to antibody affinity for the antigen. The free energy of the antigen–antibody binding event and antibody affinity for its antigen are thus also important factors to be considered during the development of an immunoassay.

4. Basic Technology

The principal approach to immunoassay is illustrated in Figure 1, which shows a basic sandwich immunoassay. In this type of assay, an antibody to the analyte to be measured is immobilized onto a solid surface, such as a bead or a plastic (microtiter) plate. The test sample suspected of containing the analyte is mixed with the antibody beads or placed in the plastic plate, resulting in the formation of the antibody–analyte complex. A second antibody, which carries an indicator reagent, is then added to the mixture. This indicator may be a radioisotope, for RIA; an enzyme, for EIA; or a fluorophore, for fluorescence immunoassay (FIA). The antibody-indicator binds to the first antibody–analyte complex, free second antibody-indicator is washed away, and the two-antibody–analyte complex is quantified using a method compatible with the indicator reagent, such as quantifying radioactivity or enzyme-mediated color formation (see AUTOMATED INSTRUMENTATION, CLINICAL CHEMISTRY).

In fact, most RIAs and many nonisotopic immunoassays use a competitive binding format (see Fig. 2). In this approach, the analyte in the sample to be measured competes with a known amount of added analyte that has been labeled with an indicator that binds to the immobilized antibody. After reaction, the free analyte–analyte-indicator solution is washed away from the solid phase. The analyte-indicator on the solid phase or remaining in the wash solution is then used to quantify the amount of analyte present in the sample as measured against a control assay using only an analyte-indicator. This is done by quantifying the analyte-indicator using the method appropriate for the assay, eg, enzyme activity, fluorescence, radioactivity, etc.

There are many variations on these two basic approaches for immunoassays (Figs. 1 and 2). For example, antigen instead of antibody may be immobilized onto a solid surface to allow antibody detection in samples. A displacement rather than a competitive reaction may be used, ie, the analyte displacing analyte-indicator bound to the antibody. Additionally, supports can be designed to allow better separation and quantification. For example, the capture antibody (or antigen) may be immobilized onto magnetic beads. After the immunoreaction, the antibody–analyte complex on the beads can be separated rapidly using a magnetic field.

4.1. Immunoassay Design. The basic reagent and design requirements of an immunoassay are antibody, antigen, conjugates of either or both, and a means for separating bound and unbound reagents. Both antibodies and antigens may be used per se in an assay or as indicator conjugates. Conjugating involves the chemical linkage of the antibody or antigen to another molecule such as a radioactive isotope; an enzyme, usually peroxidase, alkaline phosphatase, or glucose oxidase [9001-37-0]; a fluorophore, usually fluorescein [2321-07-5] or rhodamine B [81-88-9]; or a chemiluminescent molecule, such as luminol [521-31-3]. Conjugation reactions are discussed in detail elsewhere (7,10–12). In summary, the antibody or antigen is linked to the labeling molecule using a variety of chemical methods including glutaraldehyde cross-linking; carbodiimide, epichlorohydrin, and *N*-hydroxysuccinimide coupling; mixed anhydride formation and coupling; and periodate cleavage followed by reductive alkylation. In most

cases, the conjugation reactions occur spontaneously in a buffered solution and the products–reactants can be easily separated by chromatography (qv) or ultrafiltration (qv).

Noncovalent methods for conjugating antibodies and antigens are also used in many immunoassays. For example, one well-documented approach labels the antigen and/or antibody using either biotin [58-85-5] or strepavidin, and then uses the conjugates in a variety of competitive and displacement-type immunoassays. The advantage in the use of such conjugates is the extremely high affinity constant of the avidin–biotin complex, estimated at $\sim 10^{14}$/L/mol (13).

Once the appropriate reagents are prepared for an immunoassay, a laboratory assay is developed to serve both as a means of assessing the performance of the individual assay components and as a prototype for the final assay. The prototype is then used for reagent optimization and development of the final assay methodology. In the case of commercial immunoassays for clinical applications, development from an initial laboratory prototype of the assay, which includes development of antibodies and reagents, through final market release may take from 6 to 36 months depending on whether the new assay is an extension of existing, in-place technology, or represents an entirely new assay format and/or application (1). Time to market also depends on regulatory approval of the new immunoassay by the U.S. FDA. If the new assay has been preceded by another, approved assay that measures the same analyte, then the new assay can be approved under a 510 K application by showing equivalent performance to the preceding assay. If there is no precedent assay, the new assay will require a PMA (Pre-Market Approval) application supported by extensive clinal trials to prove its performance. The difference between 510 K and PMA approval can be a delay of 6 months to several years before the assay reaches the market.

5. Immunoassay Classification

There are many possible means for quantification of the antigen–antibody reaction. Immunoassays may be classified according to the technology used for detection and quantification of the analyte being detected.

5.1. Turbidimetric Agglutination Immunoassays. Agglutination–precipitation immunoassays were among the first practical applications of the antigen–antibody reaction in diagnostic tests. These assays are not as widely used today as EIA and FIA, because they are either not quantitative enough or lack the sensitivity limits of RIA, EIA, and FIA.

Classic agglutination assays utilized the formation and visual determination of antibody–antigen precipitates resulting from the formation of insoluble complexes between antibodies and high molecular weight antigens, or between antibodies and cell wall or surface antigens of microbes or animal cells. These simple precipitation reactions also form the basis for immunoelectrophoresis, which is the quantification of antigenic determinants of a cell after electrophoretic separation in a gel by the addition of specific antibody and precipitation in the gel.

Immunoprecipitation is also the basis for immunodiffusion methods such as Ouchterlony double immunodiffusion (14). The most frequently used method for

this procedure is to apply various antigen (or antibody) solutions to wells punched out of an agar base that surrounds a central well containing antibody (or antigen). The antigen–antibodies diffuse through the gel and when an antibody–antigen reaction occurs, a distinct precipitin line results. Variations of well locations and contents can result in the identification and semiquantification of an antigen (or antibody).

Latex particles, in most cases actually made of monomeric polystyrene, coated with a specific antibody or antigen are the basis for a number of commercial agglutination assays (see LATEX TECHNOLOGY). Particle reaction with the target antigen or antibody in a sample results in antigen–antibody binding and, if enough target analyte is present, the clumping or agglutination of the particles as the antigens or antibodies bridge two or more particles to form a visible precipitate.

Latex agglutination immunoassays are easily formatted into simple kits that can provide yes/no and semiquantitative estimates of antigen (or antibody) in a sample. The first such assay was developed in 1957 for rheumatoid factor (15) and assays are on the market for the determination of many species of bacteria, fungi, Mycoplasma, parasites, Rickettsia, and viruses, as well as for the determination of autoimmune disease, hormones (qv), drugs (see PHARMACEUTICALS), and blood proteins (16). Latex agglutination is also the basis of many home pregnancy tests.

An example of a latex bead-based diagnostic for Group A Streptococcal antigen (17) manufactured and sold for *in vitro* use in physician's offices and clinical laboratories is shown in Figure 3. In fact, the assay is based on immunochromatography, a combination of immunoassay and chromatography. The chromatography is used for the separation of bound and free antibody and/or antigen. Initially, a specific carbohydrate containing antigen is extracted from bacteria collected on a throat swab sample from the patient. The extract is then added to the beginning of a paper strip in a plastic housing about the size of a tape cassette. There the antigen (if present) reacts with antibody to the Streptococcal antigen that has been immobilized onto a blue-colored latex bead. The solution then migrates on the strip, carrying the latex bead–antibody–antigen complex with it until it reaches a location where a second antibody to the Streptococcal antigen has been chemically immobilized. If the antigen is present, a sandwich of immobilized antibody–antigen–antibody-bead forms and a blue line, indicative of a positive result, forms. The assay takes $\sim$5 min and has a reported sensitivity of $>$96%, ie, $<$4% false positives or negatives, and a specificity of $>$97% tested against 40 specific bacteria including 16 Streptococcal species (18). The sensitivity of the test appears to be $\sim 10^8$–10^9 organisms.

Latex agglutination tests can also be quantitated using a particle counter, spectrophotometer, or nephelometer. These detectors can overcome the sensitivity problems associated with visual determination of the agglutination reaction, increasing the sensitivity for a protein, eg, from the ppm or μg/mL to the ppb or ng/mL range. Such assays and support equipment are offered by a number of commercial companies for blood factors and proteins, eg, rheumatoid factor, α-fetoprotein, plasminogen [9001-91-6], prothrombin [9001-26-7], and fibrinogen [9001-32-5]; and drugs, eg, gentamicin [1403-66-3], phenobarbital [50-06-6], and theophylline [58-55-9].

5.2. Radioimmunoassay. RIA caused a revolution in clinical diagnostics, providing a rapid, ultrasensitive method for the detection of nearly any agent to which an antibody could be developed. Sensitivity limits in the parts per trillion (pptr) or pg/mL are commonplace and RIAs are available for nearly every class of clinically relevant analyte including drugs, hormones, immunoglobulins, immune complexes, blood factors, microbes, viruses, and tumor antigens.

Most modern RIAs utilize a competitive assay format (Fig. 2) in which radiolabled antigen, Ag*, competes with unlabeled antigen, Ag, in a sample for binding to the antibody, Ab. The free antigens are then separated from the antigen–antibody complexes, and the amount of radioactivity in the complex, or that remaining free in the wash, is quantified by radioactivity counting and related to antigen concentration. This reaction may be represented by

$$\mathrm{Ab}_y + \mathrm{Ag}^* + \mathrm{Ag}_x \longrightarrow [\mathrm{Ab} - \mathrm{Ag}]_x + [\mathrm{Ab} - \mathrm{Ag}^*]_{y-x} + \mathrm{Ag}^* \qquad (5)$$

and assumes that [Ag*]>[Ab]>[Ag];. In this case, the antibody–antigen complexes could be captured for counting after removal of the free Ag* from the reaction by ion exchange or gel permeation chromatography (gpc); adsorption of the complex onto charcoal, plastic, or a filter; or by addition of another antibody to result in immunoprecipitation.

The primary limitation to RIA is its need for development, purification, and standardization of radioactive reagent(s). Moreover, methods must be in place for the safe use and disposal of the assay reagents. One of the most commonly used radioisotopes for RIAs is iodine-125 [14158-31-7], ^{125}I. This isotope has a half-life of 60 days, requiring frequent resynthesis and purification of assay reagent(s). Many proteins labeled with ^{125}I also undergo significant autodegradation in as little as 2 weeks owing to the incorporated radioactive iodine.

In spite of these drawbacks, RIA remains a principal immunoassay method and it is expected to continue to be used extensively in many clinical and research laboratories for the foreseeable future.

5.3. Enzyme Immunoassay. In EIA, antibody (or antigen) is labeled with (or conjugated to) an enzyme, and this reagent is used to complex and quantify the target antigen (or antibody) in a sample. Conjugation may utilize a variety of chemical methods.

The primary use of EIA when it was first developed was for histological labeling and localization of specific cell macromolecules. For example, enzymes labeled with peroxidase were used to locate specific cellular compartments and structures for microscopic examination. The flexibility of EIA was recognized quickly and it was adapted for use as a laboratory assay.

The specific enzyme to be used in an EIA is determined according to a number of parameters including enzyme activity and stability (before, during, and after conjugation), cost and availability of the enzyme substrate, and the desired end point of the EIA, such as color. Most EIAs utilize a colored end point that can be readily determined both visually and spectrophotometrically. Table 1 lists a number of enzymes that have been used in immunoassays and their substrates.

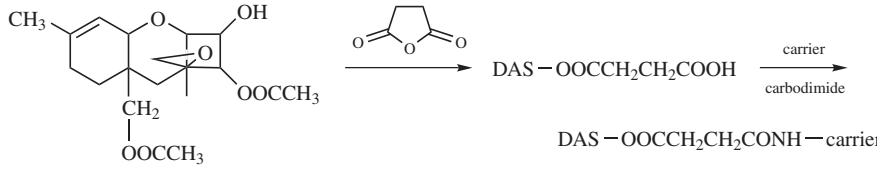
EIAs can be used per se or with a spectrophotometer. Traditionally, EIAs have been developed in 96-well microtiter plates that provide the immobilization

support for the assay, the reaction vessel, and, when linked to a spectrophotometer-based reader, a rapid means to detect and quantify the color resulting from interaction of a substrate with the antibody–antigen–enzyme complex. Automated immunoassay analyzers targeted primarily for use in the clinical laboratory have taken automation one step further, utilizing robotics to carry out all reagent additions, washings, and final quantification including report preparation.

In addition to instrument-dependent EIAs, a number of EIA kits have been developed for remote lab or field use that depend either on visual determination of color or portable spectrophotometers–readers for quantifying the color response. Whereas such field assays are moderately complex (multistep) and require technical training, they are providing rapid means for determination of a variety of analytes, from microbes in food processing (qv) to environmental pollutants. These include commercial assays for herbicides (qv), pesticides, aflatoxins, polychlorinated biphenyls (PCBs), dioxin, and various volatile organic compounds (solvents and chlorinated hydrocarbons), and microorganisms including Salmonella, Listeria, Clostridium, and Staphylococcus species. These kits are sensitive for their target analytes in the ppm to ppb range, produce results in 15–30 min, and cost from $1 to 10/sample (1). The same technology is being applied to plant and animal diagnostics, including EIA tests for plant viruses, animal diseases, antibiotics in meat and milk, and animal hormones.

There are several basic steps in developing an EIA. One example is the immunoassay developed for the determination of a trichothecene mycotoxin, diacetoxyscirpenol (DAS) (19), also known as anguidine, which is a scirpen-type sesquiterpenoid secondary metabolite of the parasitic plant fungus *Fusarium sambucinum*. DAS appears to cause vomiting when fungus-infected wheat and corn is ingested. DAS has also been shown to have anticancer activity. DAS is an example of a low molecular weight, environmental analyte, and as such, presents different challenges to EIA development as compared to EIAs for high molecular weight analytes.

As a low molecular weight compound, DAS is not significantly immunogenic, ie, it is a hapten and thus requires conjugation to a suitable antigenic carrier in order to elicit a successful antibody response in animals. DAS treated with succinic anhydride results in the DAS–hemisuccinate (DAS–HMS) shown.



DAS

The available free carboxyl groups of the DAS–HMS can be linked via a peptide bond to available primary amine groups onto highly antigenic carriers using a carbodiimide (19). The carriers used in this case were bovine serum albumin (BSA) and poly-L-lysine (molecular weight 150,000 to 300,000). The resulting conjugates may be purified using Sephadex G-25 gpc. Typical DAS/carrier ratios

were found to be ~25:1 for BSA, and 100–350:1 for poly-L-lysine as determined by high pressure-gpc analysis.

Polyclonal antibodies to the DAS conjugates were produced by inoculating mice with conjugate equivalent to 10–30 μg of DAS in Freund's complete adjuvant, ip, five times over a 4-week period. Ascites fluid was collected at 6 and 10 weeks and processed for antibody. DAS antibody (αDAS) was purified from the ascites fluid, ie, cellular fluid containing antibody that accumulates in the peritoneal cavity of animals inoculated with an immunogenic compound, using the standard purification scheme shown in Figure 4. As is common for polyclonal antibodies raised to a conjugated antigen, further purification was necessary to separate αDAS antibodies from antibodies to the carrier BSA or poly-L-lysine. Using affinity chromatography, in which the original carrier, BSA or poly-L-lysine, is immobilized on Sepharose 4B support material, the antibodies for carrier were adsorbed by the column; antibodies having primarily DAS activity rapidly moved through the column, resulting in αDAS preparations which had a specificity ratio of DAS to carrier of >100:1.

The αDAS antibodies were used as the basis to develop a number of sandwich and competitive EIAs for DAS. In the simplest application, αDAS was conjugated to alkaline phosphatase (AP) using glutaraldehyde as the conjugating agent. After purification by gpc, the αDAS–AP was characterized for DAS specificity using a standard microtiter format, ie, varying amounts of DAS were bound to the microtiter wells and then a constant amount of αDAS–AP was added to all wells. After reacting for 30–90 min, free αDAS–AP was washed out of the wells and *p*-nitrophenyl phosphate, an indicator reagent, was added. The production of color in the wells after 15 min was then quantified using a standard microtiter plate reader. Using this approach, one preparation of αDAS–AP was calculated as having a $K_a = 1.9 \times 10^6$ L/mol for DAS, as shown in Figure 5.

This same experimental approach can be used to determine the applicability of the αDAS–AP to a competitive assay for DAS. As shown in Figure 6, increasing amounts of free DAS were used to define the 50% inhibition level (ID_{50}) of DAS for binding of two αDAS–AP conjugates to immobilized DAS. This approach was also used to determine the sensitivity of an EIA, as well as the specificity of the assay, as shown in Table 2. Increasing amounts of trichothecene mycotoxins closely related to DAS were added to microtiter plate wells containing a constant amount of prereacted DAS–αDAS–AP. After 30 min, excess toxin and any free toxin–αDAS–AP were washed out, and substrate was added. Quantification of the color produced was directly related to the ability of the added toxin to displace αDAS–AP from the immobilized DAS, which is an indication that the αDAS also has an avidity for that toxin. As shown in Table 2, free DAS, as expected, is its own best displacing agent, whereas only DAS–HMS showed any appreciable displacing capability. This can be expected because the hemisuccinate linker is also immunogenic and leads to the production of antibodies specific for the linker in the polyclonal antibody population. All the other toxins had at least 100×less the avidity for the antibody, illustrating the specificity of the αDAS for DAS.

Based on such characterization studies, both sandwich (Fig. 1) and competitive (Fig. 2) EIAs were developed for DAS having a lower detection limit for DAS of 10–20 ppb, ie, 10–20 ng/mL (19).

There are many variations to this basic type of EIA. These include variations aimed at enhancing or increasing the sensitivity and specificity of an assay. For example, an EIA may utilize enzyme amplification to increase the speed and sensitivity of an immunoassay. In this approach, the enzyme label in the EIA produces a substance that triggers a second enzyme-based system that can generate large quantities of color in a very short time. Thus the product of the enzymatic activity of the antigen–antibody–enzyme complex does not need to be detected; rather it can serve as a catalyst to begin the second reaction. The second enzyme system can be present in relatively large quantities, facilitating rapid color formation, because the second enzyme is silent and noninteractive with the assay until the first reaction product turns it on. For example, a standard microtiter plate-based EIA for thyroid stimulating hormone (TSH), using alkaline phosphatase as the enzyme, can be amplified by adding nicotine adenine dinucleotide phosphate ($NADP^+$) and a second enzyme system such as alcohol dehydrogenase and lipoamide dehydrogenase, which requires NAD^+, produced by alkaline phosphatase-dephosphorylation of $NADP^+$, for the production of the colored formazan dye (20).

The enzyme mercuripapain, HgP, is being utilized as an enzyme amplification system for immunoassay (19). HgP is papain (EC 3.4.22.2) which is allosterically inhibited by mercury. Upon removal of the mercury by, eg, a mercaptan, the protease is activated. Displacement EIAs are being developed for the detection of viruses where the virus or a viral antigen is labeled with a mercaptan such as 3-mercaptopropionic acid and bound to immobilized antibody to the virus. Upon addition of a sample containing the virus, the mercaptan-labeled antigen is displaced and is able to sequester mercury away from the HgP present in the reaction mixture. This then activates the enzyme which acts on a suitable substrate such as *N*-α-benzoyl-L-arginine ethyl ester (BAEE), rapidly producing hydrogen ions. The hydrogen ions can be detected directly by use of an electrode or can be linked to an indicator. The system acts as an efficient means to amplify signal output, with as much as a 10-fold increase in assay sensitivity.

Another approach to enzyme activation allows the EIA to be carried out in a homogenous format in which no washing steps are required. Termed prosthetic group label immunoassay (PGLIA), the method involves the activation of apoglucose oxidase (apo-GOD) by its cofactor, flavin adenine dinucleotide (FAD). Essentially, FAD is covalently liked to antigen and the labeled antigen competes with sample antigen for antibody. FAD-antigen not bound by antibody can combine with apo-GOD, activate the enzyme, and a color reaction results. The advantage to this approach is that no separation steps are required for the assay. This approach has been used for the determination of drugs such as theophylline (21). A similar system for the detection of gentamicin utilized NAD^+-labeled drug linked to a NAD^+ peroxidase/glucose-6-phosphate dehydrogenase enzyme system (22).

5.4. Fluorescence Immunoassay. Basic FIA follows the same formats and approaches as EIA. The difference lies in the indicator: A fluorophore is used instead of an enzyme. This allows direct quantification of the indicator–antibody–antigen complex, or free indicator–reagent, without the need for a substrate.

FIA was originally developed as a histological technique to localize specific cellular sites using the specificity of an immunological reaction (23). The resulting fluorescent antibodies can be detected in animal tissues at levels as low as 1μg/mL of body fluid. Fluorophore-labeled antibodies have also been used widely for flow cytometry applications using fluorescein antibodies to cell surface markers to detect and quantify specific cells (24).

The most used FIA reagents conjugate a fluorophore such as fluorescein–isothiocyanate (FITC) or rhodamine–isothiocyanate to antibody (or antigen) free amino groups. Examples of other commonly used fluorophores for FIA and their spectral characteristics are presented in Table 3. FIA assays are available in sandwich and competitive formats similar to EIAs. Unlike EIA kits that can be used directly with visual color determination, FIAs require a fluorometer, and thus are primarily laboratory based.

The high sensitivity achievable with FIA has led to the development of a number of FIA assay formats that attempt to both increase assay sensitivity while decreasing background interference, a primary drawback of FIA. For example, time-resolved fluorimetry (25) is used in one commercial automated FIA system to decrease background fluorescence interference and, as a result, increase sensitivity. The antibody–antigen complex formed in this system includes a second antibody conjugated to europium. When an organic enhancer such as naphthoyltrifluoroacetone is added to the antibody–antigen–antibody–europium complex, a europium-enhancer chelation complex results. Lanthanide chelates have fluorescent lifetimes up to 10 times longer than other fluorophores, such as those occurring naturally and representing fluorescence background (see LANTHANIDES). Therefore measurements of fluorescent signals from the complex can be delayed until most or all of the background fluorescence has decayed, resulting in time-resolved fluorimetry.

Another variation of FIA is based on fluorescence polarization (FP). Based on the random rotation and orientation of fluorescent molecules in plane-polarized light, FP uses the rotation time and fluorescence lifetime for the molecules to determine the amount of filtered light transmitted to a detector (26). These characteristics can be exploited for immunoassay because the binding of a fluorescent (labeled) molecule to an antibody leads to the ordering of the complex with a concurrent and proportional increase in the transmittance of light through the filters. For example, the binding of two antibody molecules to a fluorescein-labeled protein A molecule can result in a complex having a molecular weight approximately eight times that of the original fluorescein-protein A complex (Fig. 7).

Immunoglobulin G (IgG) can be detected in <1 min by FP using either a commercial fluorimeter or a portable field detector using a one-tube, homogenous format (Fig. 7**b**). Only one reagent needs to be prepared for this assay, αIgG-fluorescein. Fluorescein conjugates with antibodies are easily prepared by reaction of the antibody with fluorescein isothiocyanate at pH 9 for 3 h at room temperature followed by gpc purification. The resulting conjugate reacts directly with the sample containing IgG. Using this assay, ppm to ppb (μg to ng/mL) amounts of IgG could be detected directly in blood serum in <1 minute. This same approach has been used for the direct measurement of viruses in <1 min (19).

FP is also the basis for a very successful line of automated immunoanalyzers for the clinical measurement of application drugs and other analytes in a competitive mode (28). For example, to measure theophylline, drug antibody, theophylline–fluorescein conjugate, and the theophylline sample are combined. The labeled and unlabeled drug then compete for antibody. The amount of theophylline in the sample is then determined by the amount of polarized light transmitted by the solution. The higher the amount of drug in the sample, the less drug-fluorescent conjugate is bound to the antibody, and the less light is transmitted to the photometer-based detection system. As in all competitive binding assays, the amount of drug in a sample is inversely proportional to signal output, in this case transmitted light.

Other examples of FIA techniques include a fluorescence protection immunoassay, where a competition reaction is used between antibody, antigen–fluorophore, antibody to the fluorophore (antifluor), and free antigen such that the free antigen causes antifluor to bind to the antigen–fluor and fluorescent quenching occurs (29); and a homogenous FIA using a nonfluorescent drug-fluor conjugate as the substrate for an enzyme where free drug prevents antibody binding to the drug-fluor resulting in enzymatic degradation of the drug–fluor complex and fluorescence (30).

5.5. Chemiluminescent Immunoassay. Chemiluminescence is the emission of visible light resulting from a chemical reaction. The majority of such reactions are oxidations, using oxygen or peroxides, and among the first chemicals studied for chemiluminescence were luminol (5-amino-2,3-dihydro-1,4-phthalazinedione [521-31-3]) and its derivatives. Luminol or isoluminol can be directly linked to antibodies and used in a system with peroxidase to detect specific antigens. One of the first applications of this approach was for the detection of biotin (31).

In the most common method for chemiluminescent immunoassay (CLIA), after the immunological reaction and any necessary separation steps, the labeled compounds or complexes react with an oxidizer, eg, hydrogen peroxide, and an enzyme, eg, peroxidase, or a chelating agent such as hemin or metal ions to produce light. This light is then detected and quantified using a photon or liquid scintillation counter (see PHOTODETECTORS).

A number of companies have developed and commercialized CLIA immunoassay systems. One system utilizes a competitive, heterogenous CLIA containing acridinium ester-labeled antigens or antibodies and micrometer-sized paramagnetic particles coated with antigens or antibodies (32). For an assay to measure an analyte such as ferritin [9007-73-2] or human chorionic gonadotrophin [9002-61-3] (hCG), the analyte in the sample competes with acridinium-labeled analyte for binding to the antibody on the magnetic beads. The beads are then magnetically separated, from the unbound antigens, and a chemiluminescent reaction occurs with the addition of hydrogen peroxide and sodium hydroxide. Photon emissions at 430 nm are then measured and compared to a standard curve to determine the sample concentration of unknown analyte.

Another commercial CLIA system detects blood endotoxin as an indicator of gram-negative sepsis is a homogenous assay that utilizes neutrohils in the blood sample to amplify the luminescent signal (33). The blood sample is mixed with MAb to endotoxin. The resulting antibody–endotoxin complex activates surface

receptors on neutophils, that then release oxidants that oxidize luminol to produce light that is detected by a luninometer. The more endotoxin present, the higher the light output.

Bioluminescence can also be used as the basis for immunoassay. For example, bacterial luciferase has been used in a coimmobilized system to detect and quantify progesterone using a competitive immunoassay format (34), and other luciferase-based immunoassays have been used to quantify insulin, digoxin, biotin, and other clinically important analytes (35).

5.6. Examples of Other Immunoassay Methods. A number of other immunoassay technologies have been developed, but are not widely commercialized. These include liposome-mediated immunoassays, in which liposomes labeled with a fluorophore are used as the basis for detection (36); metalloimmunoassays, in which antigens or antibodies are labeled with a metal ion and then quantified after the immunoreaction using atomic absorption spectrophotometry or fluorometry (37); and a variety of magnetic bead-based immunoassays in which the application of a magnetic field aids separation of antigen–antibody complexes from free antigen during the assays (38). These are only a few of the many variations possible on the basic antigen–antibody binding reaction.

5.7. Comparison of Methodologies. *Heterogenous and Homogenous Immunoassays.* The DAS EIA is an example of a heterogenous immunoassay, ie, a multistep assay requiring the sequential addition of reagents with washing steps between reagent additions. In a typical protocol for the sandwich assay (see Fig. 1) and using an enzyme-based indicator, the sample containing the analyte is added to the immobilized antibody and permitted to react between 15 min and several hours, depending on the affinity of the antibody for the analyte. Repeated washings rinse away excess sample, and the second antibody linked to an enzyme is added. After another incubation (lasting 15 min–2 h) excess (nonbound) antibody–enzyme is washed away. A substrate is then added to the resulting two-antibody–analyte–enzyme complex, the action of the complexed enzyme on the substrate causes a color change, and that color is then quantified with a spectrophotometer. This whole process takes from 2 to 6 h to complete. In competitive immunoassay (see Fig. 2) a similar series of reagent additions and washing steps would be required.

Most immunoassay kits and many commercial immunoassay analyzers are based on heterogenous EIA or FIA. These include an immunoassay system that uses FIA linked to radial partition chromatography of the antibody–antigen complex (39); a system that uses antibody-coated tubes for enzyme immunoassay of a variety of hormones and drugs (40); and a system that uses either a sandwich or competitive FIA format to measure a variety of analytes (41).

During the 1980s, a number of homogenous immunoassays were developed and commercialized. Homogenous immunoassays occur in one vessel, requiring no separation of components prior to quantification. Examples of homogenous immunoassays include a series of kits for stand-alone and automated immunoassays based on an enzyme activated assay using glucose-6-phosphate [56-73-5] (42), two very successful automated immunoanalyzers based on fluorescence polarization (FP) immunoassays and having a total menu of assays for >100 analytes (43), and the FP technology developed and described earlier (19,27).

The advantages of homogenous immunoassays are simple formats and rapid data output producing user-friendly and cost-effective products. Technical challenges to consider, however, are the necessity to remove or minimize background interference from the reagents and nonspecific binding reactions.

Monoclonal vs Polyclonal Antibodies. A continuing question facing the developer of an immunoassay is whether to use monoclonal (MAb) or polyclonal (PAb) antibodies in the assay. Polyclonal antibodies are the natural mixture of antibodies resulting from the immune response to an antigen. A family of antibodies results, each binding specifically to a different antigenic determinant (or part of a determinant) on the same antigen. Subsets of polyclonal antibodies can also exist in which all the antibodies are specific for one antigenic site (epitope), but having varying avidities for that site.

Whereas such diversity in the immune response may have evolved as a protective means to the host animal, PAbs present problems to the immunoassay developer looking for high antibody specificity and low total protein. For example, in most crude antisera, >90% of the antibodies present have no or very low avidity for the antigen. This means that extensive purification must be used to isolate the specific antibodies. Routine purification measures cannot, however, separate the resulting specific antibodies further according to the specific determinant they bind to on the antigen. As a result, it is difficult to use the antibodies from the same preparation in an assay requiring two antibodies owing to differences in avidities between the antibodies and cross-reactivity problems.

In 1975, the first successful production of MAbs was reported (44). By fusing normal antibody-producing cells with a B-cell tumor (myeloma), hybridoma cell lines resulted that produced antibodies having a specificity to only one determinant on an antigen; ie, all the antibodies produced from the cell line are identical. These studies resulted in a standard approach to MAb production. In this approach, the hybridoma cells are produced in large quantities in culture and screened to select specific clones producing the desired MAb using an appropriate assay. The selected clones are then expanded in culture (or in animals), the cells are collected, and the MAbs are extracted and purified.

The singularity of MAbs and the ease of mass production appeared to be the answer to rapid development of highly specific immunoassays. Companies were formed to produce MAbs and incorporate them into assays. In fact, such assays have been developed and have proved very successful for infectious diseases, hormones, and other clinical analytes.

Whereas MABs appear to be the choice for use in immunoassays, a majority of immunoassay developers and suppliers use polyclonal antibodies. The primary reason for this choice lies in the investment of time and costs required to fuse, clone, and screen thousands of hybridomas to discover those producing MAbs having the high avidity required for an assay. In most cases, PAb preparations with avidities equal to or better than the great majority of MAbs produced from a fusion can be produced and purified in less time with far less investment. In addition, MAb-producing hybridoma cells can be extremely unstable, losing antibody production capabilities or simply dying out in a few passages (generations). Whereas PAb sources, such as animals and cell culture, are also susceptible to loss or change, these are more easily replicated.

The question of whether to use MAbs or PAbs in an assay is a matter of assay requirements (specificity and sensitivity) and economics and cannot be answered on technical merit alone.

6. Immunoassay–DNA Probe Hybrid Assays

Nucleic acid [deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)] probes utilize labeled, ie, radioactive, enzymatic, or fluorescent, fragments of DNA or RNA (the probe) to detect complimentary DNA or RNA sequences in a sample. Because the probe is tailored for one specific nucleic acid, these assays are highly specific and very sensitive (45).

As the result of high specificity and sensitivity, nucleic acid probes are in direct competition with immunoassay for the analytes of some types of clinical analytes, such as infectious disease testing. Assays are being developed, however, that combine both probe and immunoassay technology. In such hybrid probe–immunoassays, the immunoassay portion detects and amplifies the specific binding of the probe to a nucleic acid. Either the probe per se or probe labeled with a specific compound is detected by the antibody, which in turn is labeled with an enzyme or fluorophore that serves as the basis for detection.

Assays using the technology have been developed for a number of bacteria, viruses, and human chromosome segments (46). For example, a method has been developed for the detection of hepatitis B virus DNA in serum using a sulfonated DNA probe. The DNA probe is then detected using (mouse) antibody to sulfonated cytosine and anti-mouse IgG conjugated to alkaline phosphatase. The resulting complex (DNA-sulfonated probe-α, sulfonated probe-α,α-sulfonated probe–alkaline phosphatase) is then incubated with an alkaline phosphate substrate to produce color (47).

7. Immuno(bio)sensors

Immunoassay technology is also being applied in the development of antibody-based biosensors (qv), or immunosensors (27). A biosensor is an electronic detection device containing a biological molecule, such as an antibody, enzyme, or receptor, as its basic detection element. The biological molecule is immobilized onto a transducer that detects the immobilized biomolecule analyte interaction and sends a signal to the electronic portion of the biosensor for amplification and report output (Fig. 8). The ideal biosensor employs a homogenous (one-step, no-prep) format, real-time detection (results in <1 min) in a cost-effective, portable, user-friendly design.

Immunosensors have been designed that use both direct and indirect immunoassay technology to detect specific analytes within a minute or less in a variety of matrices (see Fig. 9). Indirect immunosensors may employ EIA, FIA, or CLIA principles whereby enzyme-, fluorophore- or chemiluminescent-labeled analyte competes with the target (nonlabeled) analyte for binding sites on the immobilized antibody. Unbound (free) labeled analyte is then quantitated using an electrochemical, optical, or electromechanical transducer and compared to the amount of target analyte in the sample.

Direct immunosensors measure the actual interaction between the immobilized antibody or antigen and the target analyte, which may be an antigen or antibody, respectively. Measurements may be based on the perturbation of an electrical field by the antibody–antigen binding event; changes in light scattering fluorescence or chemiluminescence on an optical fiber; or changes in the weight of an antibody–antigen complex as compared to the weight of antibody alone on a piezoelectric crystal.

A number of immunosensors have been developed that use an interdigitated electrode, capacitance transducer to measure direct antigen–antibody interactions (Fig. 10**a**) (48). The antibody (or antigen) is immobilized in a protein-based film covering one side of the chip, whereas a film containing no antibody (which serves as the background control) coats the other set of electrodes on the chip. The chip fits into a portable electronics module that supplies a low ($\sim$1 V) potential across the chip and that measures changes in impedance across the two sets of electrodes on the chip. After addition of sample, the impedance of the control film is subtracted from that of the antibody (or antigen) containing film to result in the measurement of specific binding. The output resulting is directly proportional to the amount of target analyte in the sample. Sample is added directly to the chip and the result read in $\sim$20 s; thus this is a direct, homogenous immunosensor.

Figure 10**b** illustrates the detection of human IgG (hIgG) by an immunosensor. The bioactive film on the chip contained αhIgG entrapped with glutaraldehyde in a bovine serum albumin film. The chip was challenged with increasing concentrations of hIgG. Saline washings were used between challenges. As shown, the change in impedance is directly proportional to hIgG concentration in the ppb (ng/mL) to ppm (μg/mL) range.

Immunosensors have become principal players in chemical, diagnostic, and environmental analyses. Due to the practical detection limits of immunosensors (low ppb or ng/mL to mid-pptr or pg/mL) and their portability, immunosensors are being used as rapid screening devices in noncentralized clinical laboratories, in intensive care facilities, as bedside monitors, in physicians' offices, and in environmental and industrial settings (49–52). Industrial applications for immunosensors include use as the basis for automated on-line or flow-injection analysis systems to analyze and control pharmaceutical, food, and chemical processing lines (53) and as drug screening assays in new drug discovery. Immunosensors are not expected to replace laboratory-based immunoassays, but to open up new applications for immunoassay-based technology.

8. Microarrays

Microarrays are detection devices that extend the basic priciples and technology of biosensors, allowing the detection and identification of multiple analytes in the same sample simultaneously (54). Linked to microfluidic technology, microarrays can be automated to carry out thousands of individual assays in hours. This capability has been especially important in the development of microarrays for use in drug discovery. The first microarrays were developed using oligonuleotides for genomic and expression profiling (55).

Antibody-based microarrays are now being developed for the detection multiple analytes in a single sample for clinical, environmental, food, and military applications (56) and promise to simplify the analysis of multiple analytes in a single sample. For example, Figure 11 illustrates a microarray being developed for the simultaneous detection of a number of biological toxins and infectious agents in an environmental or suspected biological warfare agent sample (57).

In this case, the array has been designed to test for 12 agents using specific antibodies to each. Each antibody is present on the array at five concentrations to allow for a graded response to its specific analyte. After reacting with sample, the array is developed by first reacting with a solution containing fluorescent second antibodies to each analyte, washing, and detecting fluorescence in the spots. Detection can also use enzymes to produce color if an analyte is present, or chemiluminescence. Another variation of the array utilizes conductivity for instantaneous, homogenous detection of analytes similar to biosensors.

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Table 1. **Examples of Enzymes Used in EIA**

Enzyme	Enzyme classification number	Substrate	Wavelength of detection, nm
aldolase	4.1.2.13	fructosediphosphate	600
alkaline phosphatase	3.1.3.1	*p*-nitrophenyl-phosphate	405
acetylcholinesterase	3.1.1.7	5,5′-dithiobis(2-nitrobenzoic acid)	412
amylase	3.2.1.1	starch–iodine	440–500
cholesterol oxidase	1.1.3.6	cholesterol–amino-antipyrene	500
creatine phosphokinase	2.7.3.2	α-napthol–creatine phosphate–diacetyl	520
elastase	3.4.21.36	elastin–congo red	490
glucose oxidase	1.1.3.4	thiozolyl blue–phenazine methosulfate	540
β-glucuronidase	3.2.1.31	phenophthalein glucuronide	550
peroxidase	1.11.1.7	*o*-phenylenediamine, *o*-dianisidine, 5-aminosalicycliate	492 460 474
urease	3.5.1.5	bromcresol purple	588

Table 2. **Inhibition of DAS–αDAS Binding by Various Mycotoxins**

	ID_{50}[a]	
Mycotoxin	μg	10^{-6} M
DAS diacetoxyscirpenol	0.21	5.7
DAS–HMS hemisuccinate	16.3	350
T2 toxin	>23	>500
HT2 toxin	>21	>500
verrucarol	>13	>500
verrucarin A	>25	>500
zearaleone	>16	>500

[a] The concentration required to displace 50% of αDAS–AP bound to immobilized DAS.

Table 3. **Commonly Used Fluorophores in FIA**

Fluorophore	CAS Registry number	Excitation wavelength[a]	Emission wavelength
allophycocyanine		620	660–665
7-amino-4-methylcoumarin-3-acetic acid		347	456
bis-benzimide (Hoechst 33258)	[*2349-45-4*]	340–360	470–480
fluorescein-isothiocuprate FITC	[*3326-32-7*]	495	525
indocarbocyanine (Cy3)		550	570
indodicarbocyanine (Cy5)		650	680
lissamine rhodamine B-sulfonyl hydrazine		556	580
lucifer yellow CH	[*67769-47-5*]	428	540
phycocyanine	[*11010-15-2*]	620	655
phycoerythrin		495	578
rhodamine isothiocyanate	[*36877-69-7*]	540–550	570
Texas red	[*82354-19-6*]	596	620

[a] Excitation and emission wavelengths are for the unconjugated fluorophore. Wavelengths for conjugates may vary according to the composition of the conjugate.

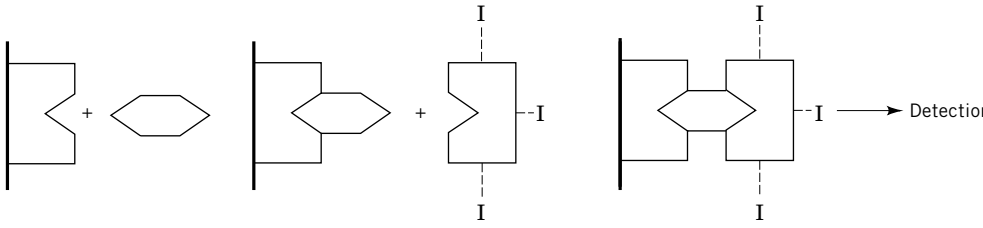

Fig. 1. A principal approach to immunoassay, the sandwich immunoassay, where the thick line represents the solid matrix, (⏍) the antibody, (⬭) the antigen, and I an indicator molecule such as an enzyme, fluorophore, or radioisotope.

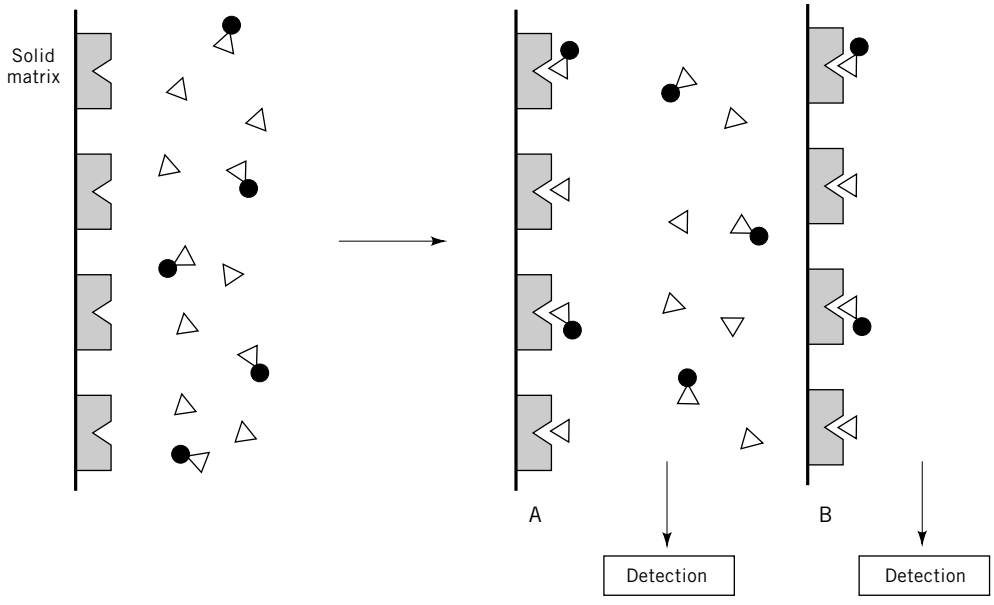

Fig. 2. The basic approach for a competitive immunoassay. The analyte (△), and analyte-indicator (◬) compete for sites on the antibody (⏍) which may be immobilized or in solution. Quantitation may utilize the remaining (free) indicator in solution A, or the indicator bound to the antibody, B.

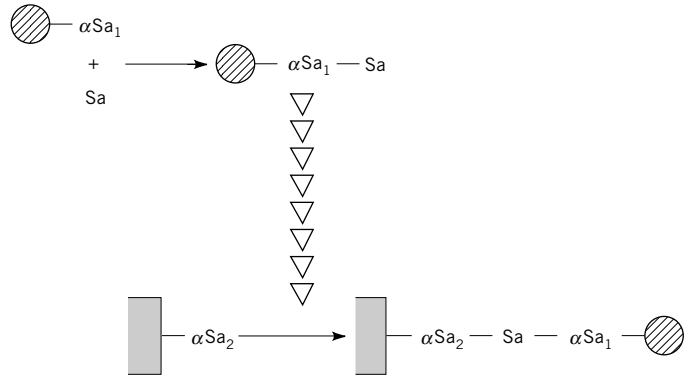

Fig. 3. Example of an immunochromatographic assay for a Streptococcal antigen (Sa) using antibody to the antigen (αSa_1) linked to a blue-colored latex bead (▨). The downward pointing triangles represent chromatographic migration. Formation of a sandwich linking the antigen between the latex bead and a second, immobilized antibody (▮ αSa_2) results in an immobilized colored complex giving a positive visual test (18).

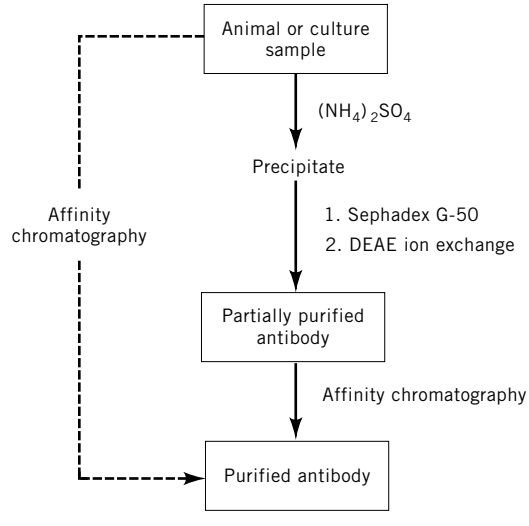

Fig. 4. Example of antibody purification from animal or culture sources. In some cases, affinity chromatography may be used directly with the source material, bypassing the precipitation and other column chromatography steps.

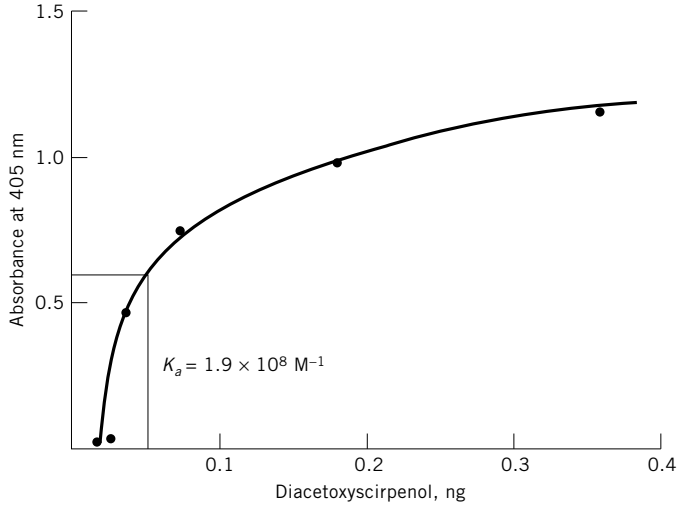

Fig. 5. Determination of the K_a for the binding of DAS to its polyclonal antibody raised in mice. A fixed amount of immobilized antibody in a microtiter plate is reacted with increasing amounts of DAS and the amount of DAS bound at each concentration is determined using an EIA based on alkaline phosphatase (AP) (19).

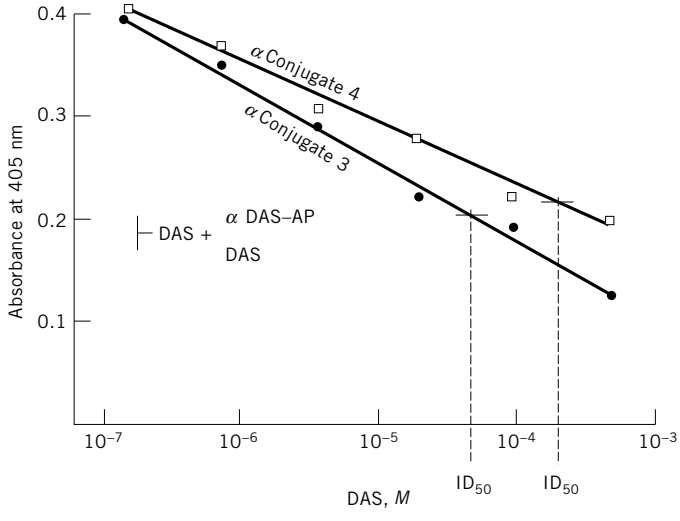

Fig. 6. Determination of the amount of free DAS required to cause 50% binding inhibition, ID_{50}, of αDAS–AP to immobilized DAS, as a means to determine the specificity of an antibody for its target analyte (19).

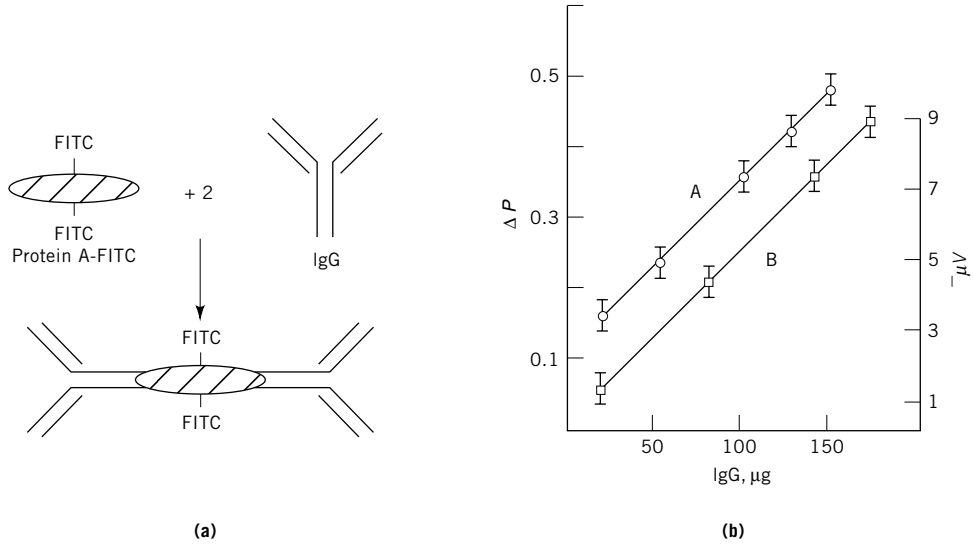

Fig. 7. Fluorescence polarization (FP). (**a**) The formation of the large FITC–protein A–IgG complex that leads to a net increase in plane polarized light transmitted from the solution. Molecular weights of the protein A-FITC, IgG, and complex are ~43,000, 150,000, and 343,000, respectively. (**b**) Detection of IgG by fluorescence polarization immunoassay using A, a laboratory fluorimeter where (○) represents ΔP = change in polarization, and B, a portable detection unit where (□) is $-\mu V$ = change in voltage (27). The field detector proved to be more sensitive than the fluorimeter.

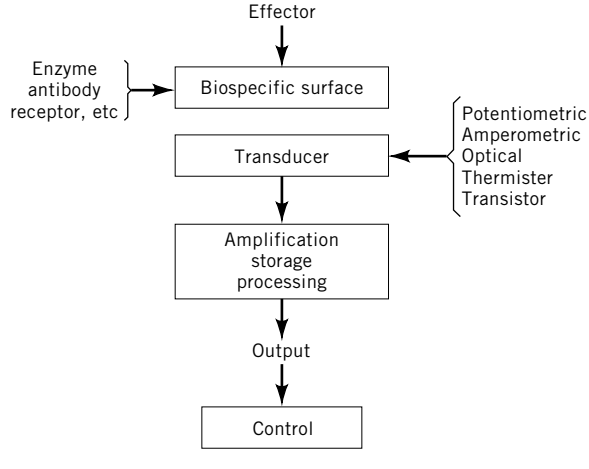

Fig. 8. Basic components of a biosensor. In the case of an immunosensor, the antibody (or antigen) would be immobilized onto the transducer.

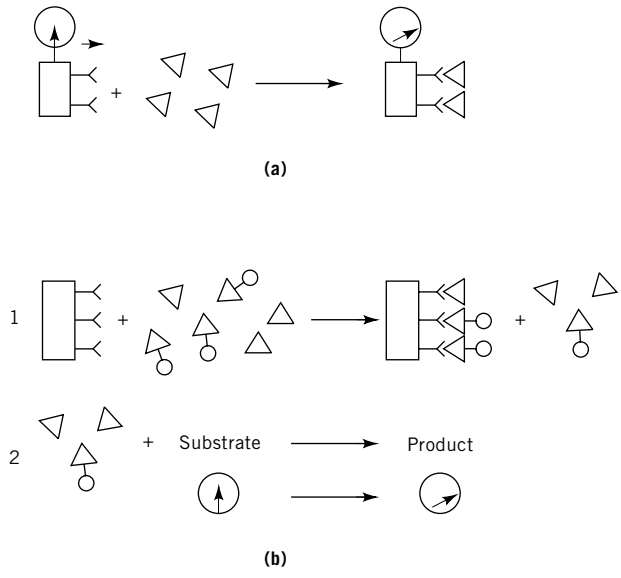

Fig. 9. Immunosensor approaches where △ is the analyte, ⟁ is the labeled analyte, and ⊣ is the antibody. (**a**) Direct immunosensors, where the actual antigen–antibody interaction is measured; (**b**) indirect immunosensors **1** and **2**, which utilize formats similar to competitive and displacement immunoassays.

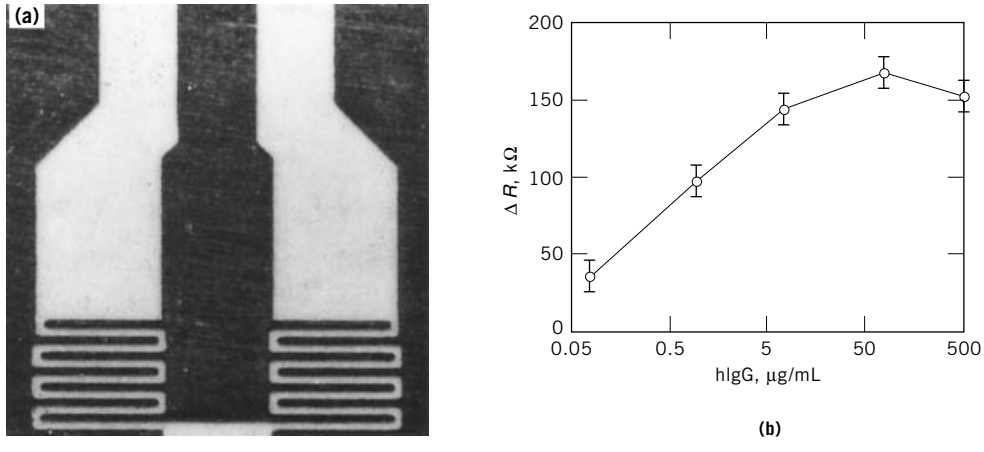

Fig. 10. (**a**) An interdigitated electrode biochip. One set of electrodes is coated with membrane containing antibody, the other set with membrane alone. The nonantibody containing membrane–electrode serves as a control to compensate for nonspecific binding and background. (**b**) Response of biochip containing immobilized antibody to human IgG (αhIgG) to increasing amounts of hIgG in saline. The electronics unit reports specific antigen–antibody binding corrected for background and nonspecific binding as a change in impedance across the interdigitated electrodes (48).

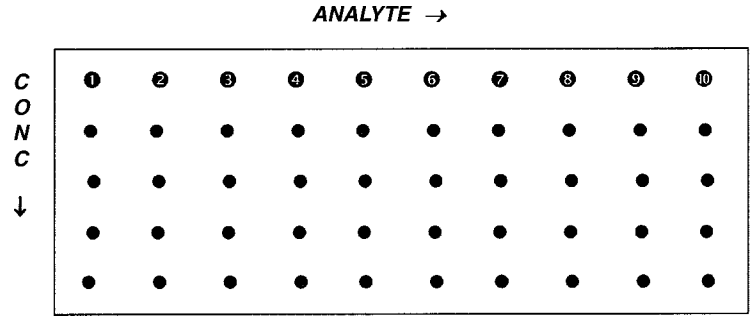

Fig. 11. Antibody-based microarray for the simltaneous detection and qualitative concentration of 10 potential biological agents in a single sample. Five microdots of increasing amounts of immobilized capture antibody for each agent indicates approximate concentration of the agent. Agents detected: column 1, *Eschericia coli* 0157:H; 2, *Salmonella tyhimurium*; 3, gram-negative bacterial endotoxin; 4, hepatitis B surface antigen; 5, viral Influenza A/B; 6, *Clostridium botulinum* type A toxin; 7 *Vibrio cholerae* exotoxin; 8, concanavalin A (lectin); 9, α-bungarotoxin; 10, acetylcholinesterase inhibitors.