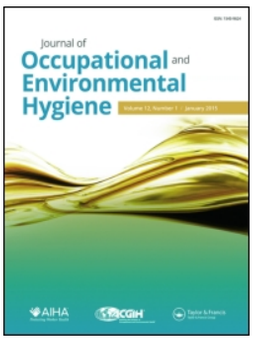

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Analytical Performance Criteria

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Analytical Performance Criteria
The Use of Immunochemical and Biosensor Methods for Occupational and Environmental Monitoring. Part I: Introduction to Immunoassays

BACKGROUND

Immunoassays, especially enzyme immunoassays (EIAs) and enzyme-linked immunosorbent assays (ELISA), are commonly used analytical techniques for clinical diagnostic measurements, drug screening, and measurements for evaluating exposure to environmental agents.[1–16] ELISAs have been performed directly on diluted biological matrices[1–3] (blood, urine, saliva, breast milk, etc.), extracts from filters used for air sampling,[4] soil extracts,[5] food extracts,[6,7] and myriad other varied matrices.

The first ELISA, used to quantitatively measure immunoglobulin G (IgG), was described in 1971.[8] EIAs display accuracy comparable to that of traditional lab-based analytical methods, such as gas chromatography (GC). Their low cost, speed, and portability have made them attractive to environmental chemists in a variety of fields.[9]

Immunoassays are based on the formation and detection of immune complexes between antigens (Ag) and antibodies (Ab). Antigens are principally macromolecules (e.g., proteins, polysaccharides, nucleic acids) that can act as complete immunogens able to stimulate an immune response. Other substances too small to act as immunogens on their own (drugs, pesticides, etc) must be coupled to a macromolecular carrier molecule (usually a protein) to become immunogenic and elicit an immune response. These small molecules are called haptens. Many environmental agents (such as pesticides or their metabolites) are haptens. *In vivo*, haptens may form adducts with constitutive proteins, becoming complete immunogens.

Experimentally, the selection of the protein carrier used to form the hapten-protein conjugated immunogen is important. Keyhole limpet hemocyanin (KLH), a protein from the shelled keyhole limpet, a marine gastropod, is often used as a carrier protein, since it is highly immunogenic and vertebrate exposure is unlikely (rabbits are commonly immunized to produce antibodies).[10] The number of haptens bound to the carrier, the chemistry of the conjugation reactions, and other factors will impact the final affinity and avidity of the resultant antibodies. The purity of the hapten is also important, as conjugation of closely related structures to the carrier may result in the formation of nonspecific antibodies.

Spacer molecules (e.g., reaction of morphine with succinic anhydride, forming a morphine-hemisuccinate-protein conjugate)[11] are often used in the preparation of haptens for conjugation in efforts to increase the specificity of the antibodies for the hapten portion of the conjugate. The optimal spacer molecule has a chain length of about four to six atoms.[12] The ability of an antibody (Ab) molecule to bind an antigen (Ag) or a hapten specifically is controlled by structural and chemical interactions between the ligand and the Ab at the combining site.[13] The Ag–Ab interaction is reversible and does not involve the formation of covalent

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bonds.[13] This interaction is controlled by the law of mass action:

$$Ag + Ab = AgAb \quad \textbf{(1)}$$

with

$$K = \frac{AgAb}{[Ag][Ab]} mol^{-1} \quad \textbf{(2)}$$

where K is the affinity constant and AgAb = the antigen-antibody complex.

High affinity constants, resulting from stronger Ag/Ab interactions, lead to lower limits of detection (LODs) in immunoassays. The mammalian immune system has the capacity to produce five distinct classes of Abs. Immunoglobulins are Y-shaped proteins consisting of two identical heavy chains (50–60 kDa) and light chains (~25 kDa). Both the heavy and light chains have a variable region (V_H and V_L, respectively) whose sequence varies between antibodies. The variable region is the portion where antigen binding occurs. The remainder of both chains is referred to as the constant region (C_H and C_L), since it has minimal variation in its amino acid sequence. Portions of the constant region are where the antibody binds to cells.

In mammals, there are five types (isotypes) of antibodies, IgA, IgD, IgE, IgG, and IgM. IgG (Figure 1) is the preponderant antibody class in most mammals and, as such, is the major antibody used in the development of EIAs. IgM accounts for ~5–10% of total serum immunoglobulins and is the first immunoglobulin class produced in response to antigen. Isotype switching, however, occurs to form other antibody classes. IgA constitutes 10–15% of total serum immunoglobulins but is the predominant immunoglobulin class in external secretions (e.g., saliva, tears, breast milk, digestive tract). IgE is present in serum at very low concentrations (~0.3 μg/mL) and is important in immediate hypersensitivity reactions, such as hives and asthma. IgD is present in serum at ~30 μg/mL and is a major membrane-bound immunoglobulin. No biological effector function has been identified for IgD.[14]

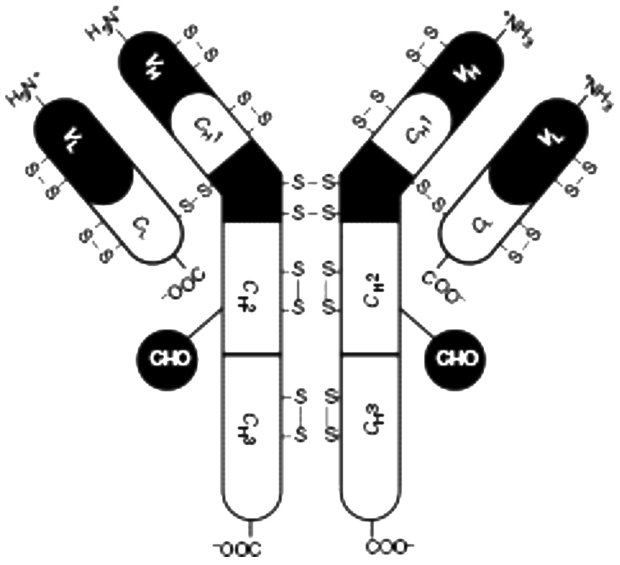

FIGURE 1. Structure of immunoglobulin G. IgG consists of two identical heavy chains (50–60 kDa) and light chains (~25 kDa). Both the heavy and light chains have variable regions (V_H and V_L) whose sequences vary between antibodies. The variable top region is the portion where antigen binding occurs. The remainder of both chains is referred to as the constant region (C_H and C_L).

The antibodies used in an ELISA can be monoclonal or polyclonal. Monoclonal antibodies are produced by fusing tumor cells with cells that produce antibody (hybridomas). Hybridoma cells produce antibody to essentially one epitope (an epitope is the smallest fragment of an antigen to which an immune response can be directed; antigens can have numerous epitopes), hence the name "monoclonal." Monoclonal antibodies provide a continuous and unlimited supply of a standardized reagent with defined specificity and assay characteristics.[15]

Polyclonal antibodies are usually prepared by injecting animals (usually rabbits) with antigen and adjuvant (a mixture that stimulates the immune response) and collecting serum from the animals.[1] Polyclonal antibodies may be further purified and isolated, yielding essentially monospecific polyclonal antibodies.[16] Polyclonal Abs, as the name implies, are a mixture of immunoglobulins directed against specific epitopes present in an antigen. The Ab response to each epitope is the result of clonal expansion of specific epitope directed B-lymphocytes.

Radioimmunoassays (RIA) use radiolabeled (e.g., ^{125}I) reagents to detect the reaction between antigens and antibodies, and the presence of antigen-antibody reactions are measured using a gamma counter.[17] Most radioimmunoassays have been replaced by ELISAs. In ELISAs, the solid support binding of a reactant (usually a microtiter plate, although other solid supports such as magnetic particles, microspheres, and coated tubes have been used) allows for separation of bound vs. unbound reactants by simple washing. The detector system in ELISAs is usually an enzyme (e.g., horseradish peroxidase, alkaline phosphatase) bound to a reactant (an antibody or analyte). Common chromogens (enzyme substrates) used in ELISAs include p-nitrophenyl phosphate and 2,2′-azino-di-(3-ethylbenz-thiazoline) sulfonic acid, o-phenylenediamine, and tetramethylbenzidine.

IMMUNOASSAY FORMATS

ELISAs can be performed in many different formats (direct, indirect, capture, competitive, etc.). In the following descriptions, generic overviews of ELISA formats are given. Many differing variations of these generic formats have been utilized to detect numerous analytes that would be too exhaustive to cover in this column. In a direct ELISA (Figure 2A), which is the most basic ELISA format, an analyte (hapten, antibody, or antigen) is attached to a solid support. Antibody, specific for the analyte and containing a reporter system (usually an enzyme), is incubated with the captured analyte. After washing, a chromogen (enzyme substrate) is added and allowed

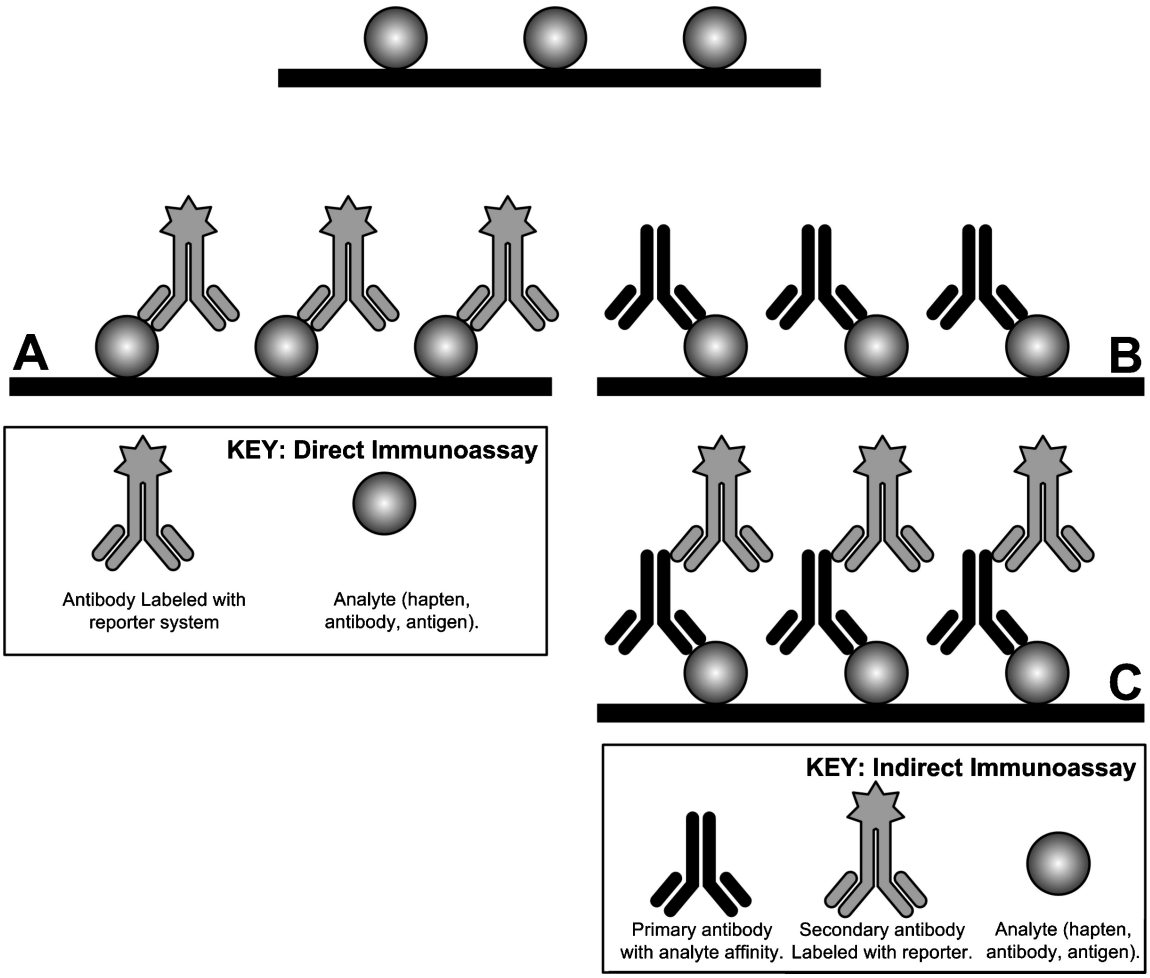

FIGURE 2. Direct and indirect immunoassay. In a direct assay (A), analyte (hapten, antibody, antigen) is bound to a solid support (i.e., bead or microplate). Reporter-labeled antibody is introduced to the immobilized analyte, forming an antigen-antibody complex. After washing, the concentration of analyte is measured by radiometric, colorimetric, or fluorometric detection of the reporter system. In an indirect format (B,C), a primary antibody specific for the analyte is introduced to the solid support bound analyte. After washing, a secondary labeled antibody, specific for the primary antibody, is added to the system. The concentration of analyte is then measured by radiometric, colorimetric, or fluorometric monitoring.

to react, forming a colored product that can be measured by spectrophotometry.

In an indirect ELISA (Figures 2B,C), analyte (hapten, antibody, antigen) is again attached to a solid support. A primary antibody, specific for the analyte, is incubated in the system and the excess removed by washing. A secondary labeled antibody, specific for the primary antibody, is added to the system and incubated. After washing, chromogen is added and the color measured in a spectrophotometer (usually a specialized microplate reader that can read the optical density of many microplate wells quickly). The amount of color produced is proportional to the amount of secondary antibody that was bound.

ELISAs may also be designed in capture formats (Figure 3). In an antigen capture (sometimes called a sandwich) ELISA, antigen is captured by antigen-specific antibody that has been attached to the solid support (Figure 3A). After washing, another labeled antibody, specific for another epitope on the antigen, is added (Figure 3B). After incubation and washing, chromogen is added and the resultant color measured in a spectrophotometer. ELISAs may also be designed as antibody capture ELISAs, which are performed in a similar fashion to antigen capture ELISAs, except the analyte of interest is an antibody.

Another format is the competitive ELISA. In a competitive ELISA (Figure 3D), the analyte (either antibody or antigen) competes with labeled analyte for binding. With higher concentrations of analyte, less of the labeled analyte is bound, yielding reduced signal. In a modification of this format (blocking ELISA), unlabeled analyte is added prior to the addition of labeled analyte.

In most ELISAs, antigens/antibodies are coated onto plastic microwell plates by electrostatic attraction and van der Waals forces. Antigen or antibody are diluted in coating buffers to

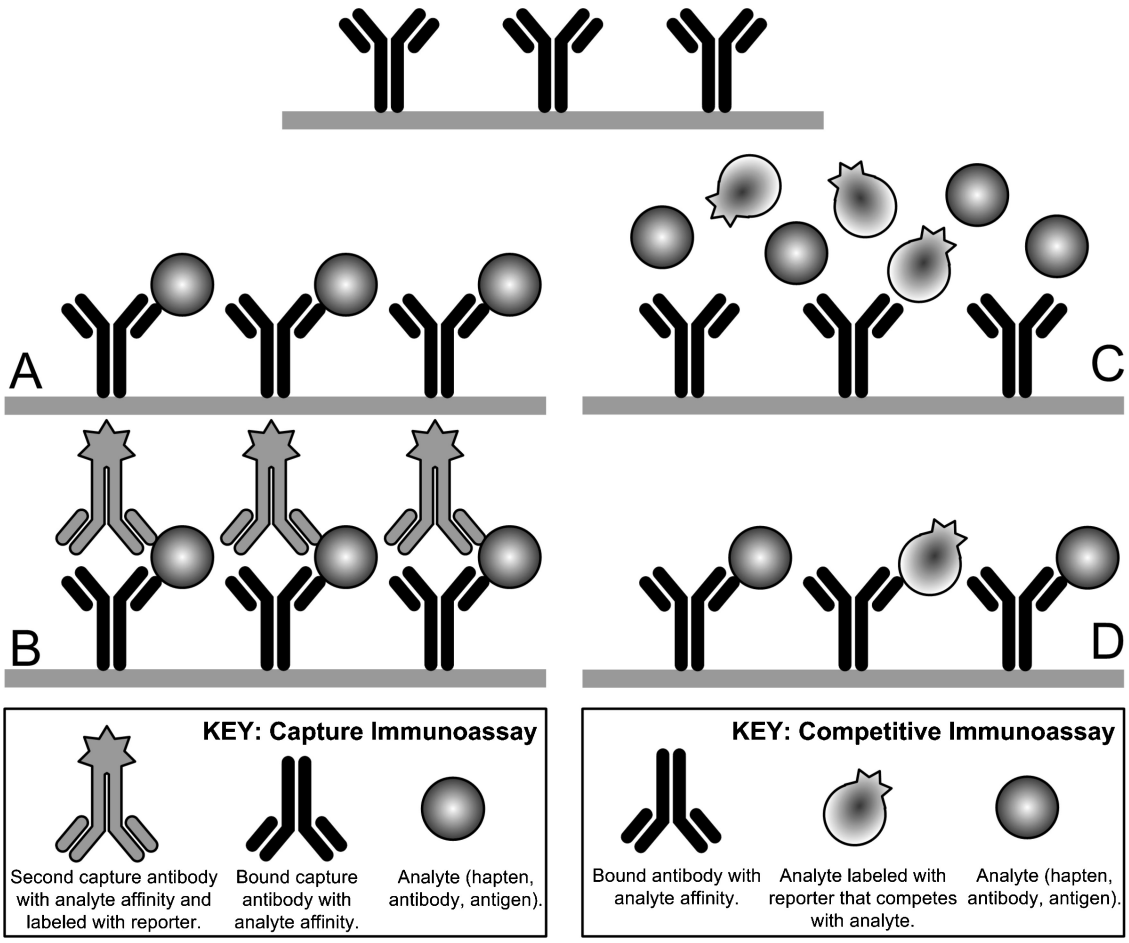

FIGURE 3. Capture and competitive immunoassay. In an antigen capture (sometimes called sandwich) assay, the antibody which is specific for the analyte is bound to a solid support. Added analyte is bound by the first specific antibody (A). After washing, another labeled antibody, specific for another epitope on the antigen, is added (B). Concentration of analyte is then measured by radiometric, colorimetric, or fluorometric monitoring. In a competitive assay, analyte and a reporter-labeled analyte are allowed to compete for binding sites with the immobilized antibodies (C) and bind in relation to their relative concentrations (D). The concentration of analyte is measured by radiometric, colorimetric, or fluorometric detection of the reporter system. With higher concentrations of analyte, less of the labeled analyte is bound, yielding reduced signal.

assist in immobilizing them to the microplate. Commonly used coating solutions include sodium carbonate, Tris-HCl, and phosphate buffered saline solutions. To minimize nonspecific binding to the microtiter plates, solutions of proteins are used to "block" unbound sites. Commonly used blocking agents are bovine serum albumin, nonfat dry milk, and casein.

Another immunoassay format is the lateral flow immunochromatographic assay (LFIA or LFA). A common example of a LFIA is the home pregnancy test, which detects the presence of human chorionic gonadotropin in urine. The authors have reported on an LFIA for the detection of antiprotective antigen (PA) IgG from vaccination with the U.S. anthrax vaccine.[18] The device consists of a plastic support to which a nitrocellulose membrane is mounted. Purified anthrax recombinant PA (rPA) is striped in the "test line" position, while a biotinylated-bovine serum albumin (BSA) conjugate is striped at the "control line" position. Gold particles (40 nm), individually conjugated to PA and streptavidin, are prepared and mixed. The PA and streptavidin colloidal gold conjugate mixture is dispensed onto a conjugate pad. The conjugate pad is then affixed to the test strip by overlapping the nitrocellulose membrane at its proximal end; the addition of a sample pad completes the assembly by overlapping onto the conjugate pad (see Figure 4). Thirty μL of either serum, or with the addition of a blood separation sample pad, whole blood are added to the sample port of the device, followed by chase buffer.

If present in the sample, the PA-conjugated nanoparticles bind to anti-PA IgG and the chase buffer carries them down the nitrocellulose membrane along with the streptavidin-gold nanoparticles. On reaching the rPA at the test line, the beads are immobilized, as is the case with the streptavidin-gold nanoparticles when they interact with the biotin at the control line. The device is inspected for reactions at 15 min. For a positive test, two distinct red lines are observable at the "C" line and the test ("T" line) identified on the device. The nanoparticle gold

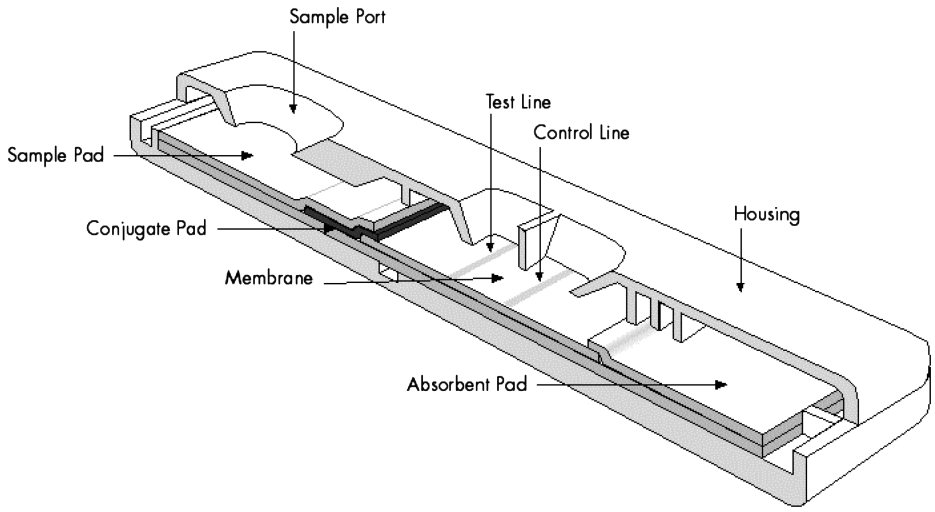

FIGURE 4. Diagram of lateral flow immunochromatographic assay cartridge

becomes a distinct red color as a result of localized surface plasmon resonance. Negative tests yield distinct red lines at the control area (C line) on the device with no evidence of red lines at the test (T line). A schematic of the mechanism of the assay is shown in Figure 5.

FLUORESCENCE COVALENT MICROBEAD IMMUNOASSAYS

Classically, body burdens of pesticides are analyzed using chemical/instrumental analysis (CIM) or enzyme immunoassays

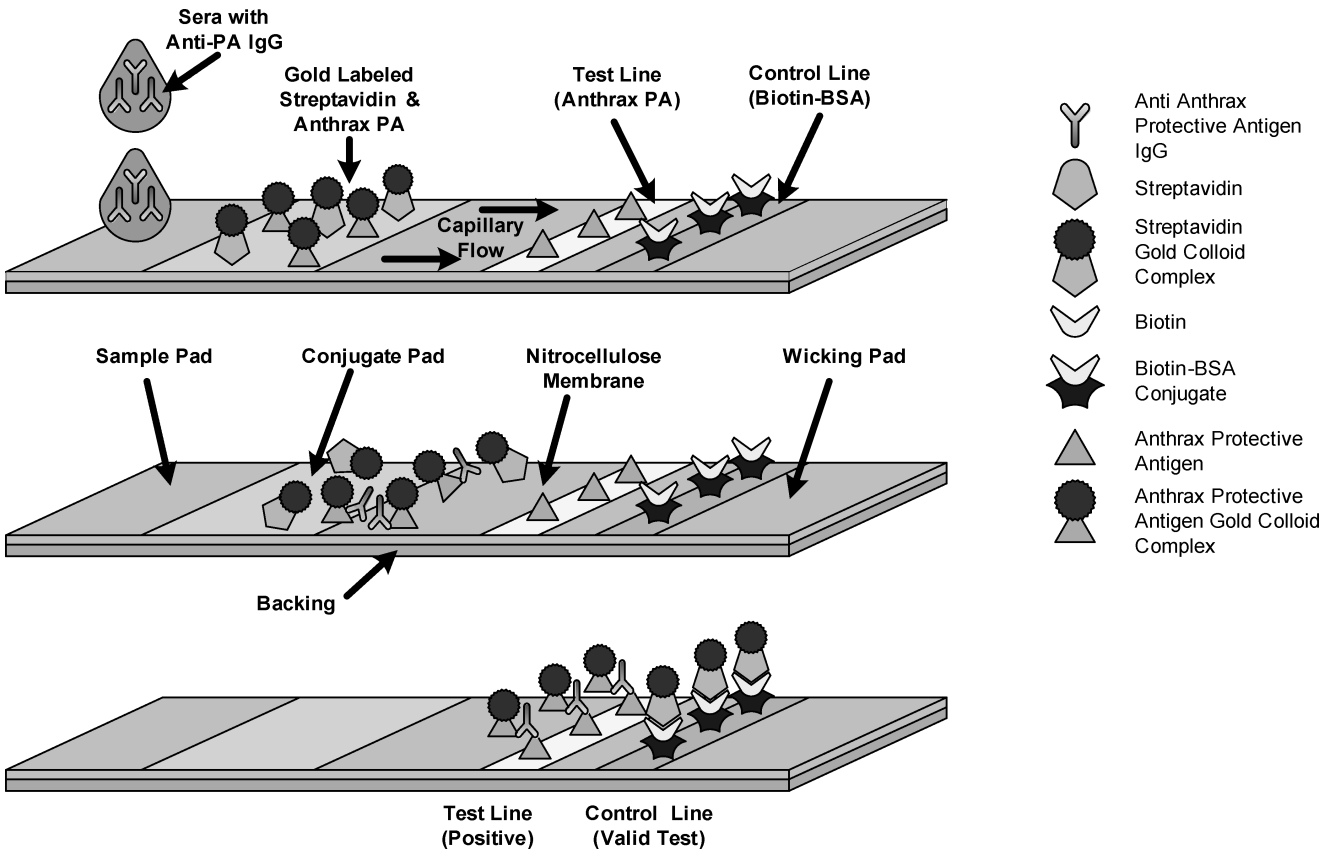

FIGURE 5. Diagram of the mechanism of the anti-anthrax PA-IgG LFIA

(EIAs). Both of these technologies can usually be used by CIM to quantitate one analyte or closely related groups of analytes per assay. In addition, CIM assays usually require numerous cleanup and extraction steps before the sample can be introduced into the instrumentation. For example, the NIOSH analytical method for triazine herbicides and their metabolites in urine,[19] which employs gas chromatography with a mass selective detector, has 39 steps from sample preparation to calculations. To perform multiple analyses for multiple unrelated pesticides by CIM could entail a prodigious amount of effort. Alternatively, multiple analytes can be measured simultaneously using a multiplexed fluorescence covalent microbead immunoassay (FCMIA).

In one example of this method,[20] three distinct spectrally addressable microspheres were coupled with three pesticide conjugates (glyphosate-ovalbumin, atrazine-bovine serum albumin and metolaclor mercapturate-keyhole limpet hemocyanin) using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS). The primary antibodies were anti-atrazine, anti-glyphosate and anti-metolachlor mercapturate prepared by immunizing rabbits (Figure 6).

To prepare standard curves, mixtures of atrazine, glyphosate, and metolachlor mercapturate were mixed with the conjugated microspheres, and a mixture of primary antibodies added. After a period of incubation, biotin labeled anti-rabbit IgG was added and allowed to incubate. After washing, streptavidin R-phycoetythrin was added, and after incubation and washing, the bead mixture was analyzed in a commercial liquid suspension array instrument. This type of assay essentially consists of numerous competitive immunoassays being performed simultaneously using microspheres as solid supports. As the concentration of analyte increases, the reporter signal decreases.

The multiplexed suspension contains a mixture of spectrally distinguishable 5.6 μm diameter microspheres (composed of polystyrene, divinylbenzene, and methacrylic acid), internally dyed with proprietary red and infrared-emitting fluorochromes. By adjusting the concentrations of each fluorochrome, spectrally addressable microsphere sets are obtained. A 635-nm classification laser excites and classifies the microspheres based on embedded red and infrared fluorochromes. A 532-nm reporter laser excites the green fluorescent molecules bound to the surface of the microspheres.

Using this method, concentrations of numerous analytes can be measured simultaneously by isolating detector responses (green fluorescence) from numerous spectrally addressable microspheres simultaneously (multiplexing).[21] When the microsphere sets are mixed, they can be analyzed with a standard benchtop flow cytometer or a commercially available dedicated liquid suspension array instrument. The three major components of the system are a benchtop flow cytometer, microspheres, and computer hardware and software. Microsphere size, determined by 90° light scatter, is used to eliminate microsphere aggregates from the analysis. The microspheres in a liquid suspension array technique can conjugate with receptors,[22] oligonucleotides,[23] proteins[20,24] and antibodies,[25] such that studies of numerous biological interactions and assays can be performed.[21,24,26–28] Both ELISA and FCMIA are analytically sensitive techniques, having limits of detection for some analytes <1.0 pg/mL.[29]

The reporter signal from ELISAs and FCMIAs can be amplified by various methods designed to increase assay sensitivity. One example of this, rolling circle DNA amplification (RCA), can yield 100-fold greater sensitivity.[30] This is done by covalently attaching an oligonucleotide primer to the reporter Ab in the presence of circular DNA, DNA polymerase, and nucleotides. Amplification results in a long DNA molecule containing hundreds of copies of the circular DNA sequence that remain attached to the Ab. The amplified product is labeled *in situ* by hybridization with numerous fluor-labeled oligonucleotides,[31] which are then measured in a liquid suspension array instrument. Another amplification method, surface enhanced raman scattering (SERS), also has the potential to enhance sensitivity and limits of detection manyfold.[32]

FCMIAs are considerably faster than EIAs or CIMs, having three 30-min incubations and a $\sim$20 sec read time for each well. In FCMIA, since the time for analyses is essentially independent of the number of analytes measured, incredibly high throughput can be realized. Multiplexed analyses for 25 analytes[27] have been described simultaneously, which equates to measurement of $\sim$2,500 individual analyses in under 80 min. Multiplate (3) runs using an autosampler with this assay yield measurements of $\sim$7500 analytes in $\sim$240 min.

Multiplexed methods also have potentially reduced errors compared with sequential and/or contemporaneous (running numerous ELISAs at the same time) measurements. Unless the samples are stored as many independently frozen aliquots, or other contingencies are used to ensure sample integrity, sample degradation is likely in repeated freeze-thaw cycles. Errors in the estimates of the concentrations of individual analytes are likely, as the individual methods most probably have their own unique inter- and intra-assay coefficients of variation and recoveries. Propagation of these errors in each individual analysis could lead to potentially large combined errors, especially for the relative ratios of concentrations of individual analytes.

Multiple analytes can be measured using CIM after liquid-liquid extraction, liquid-solid extraction or the use of solid-phase microextraction fibers, etc., with multistep and time consuming extractions and sample cleanup procedures sometimes necessary before samples can be introduced to the analytical instrumentation.[33–35] Diversity in the chemical properties of mixtures has been shown to negatively impact recoveries when measuring multiple analytes by CIM.[33] The final outcome of these efforts can be methods that are either sensitive and imprecise or precise and insensitive.[33–35]

Benefits of EIAs over CIM generally include enhanced speed, throughput, sensitivity, lower instrumentation and reagent cost, less technician training, linearity of response over wide dynamic ranges, and the ability to multiplex. A drawback inherent in any immunoassay is the cross-reactivity of reagent

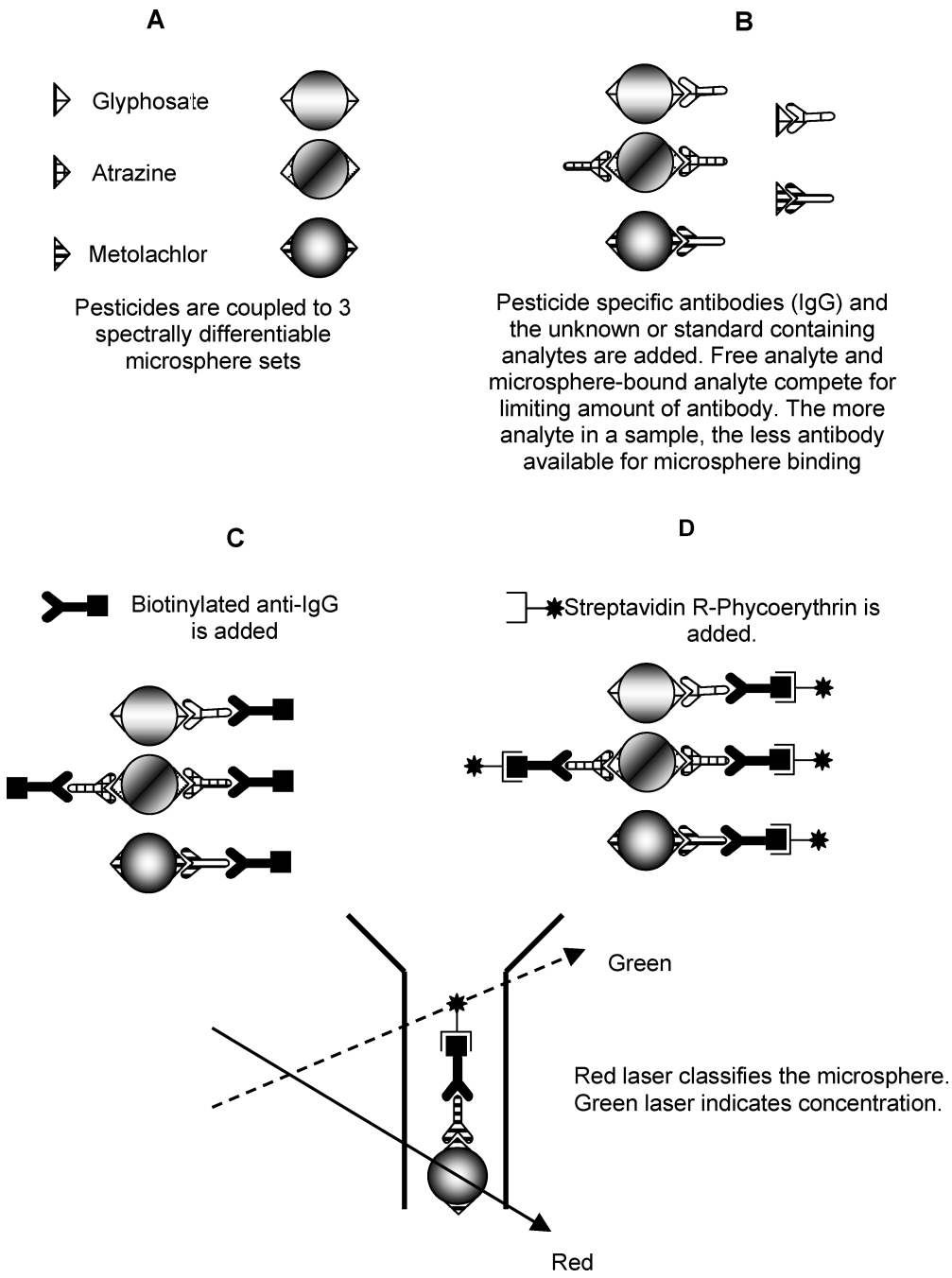

FIGURE 6. Diagram of a competitive multiplexed fluorescence covalent microbead immunoassay (FCMIA).

antibodies. This can be somewhat minimized by the use of monoclonal antibodies, but remains a significant analytical challenge.[36]

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