27 chapter

Immunoassays

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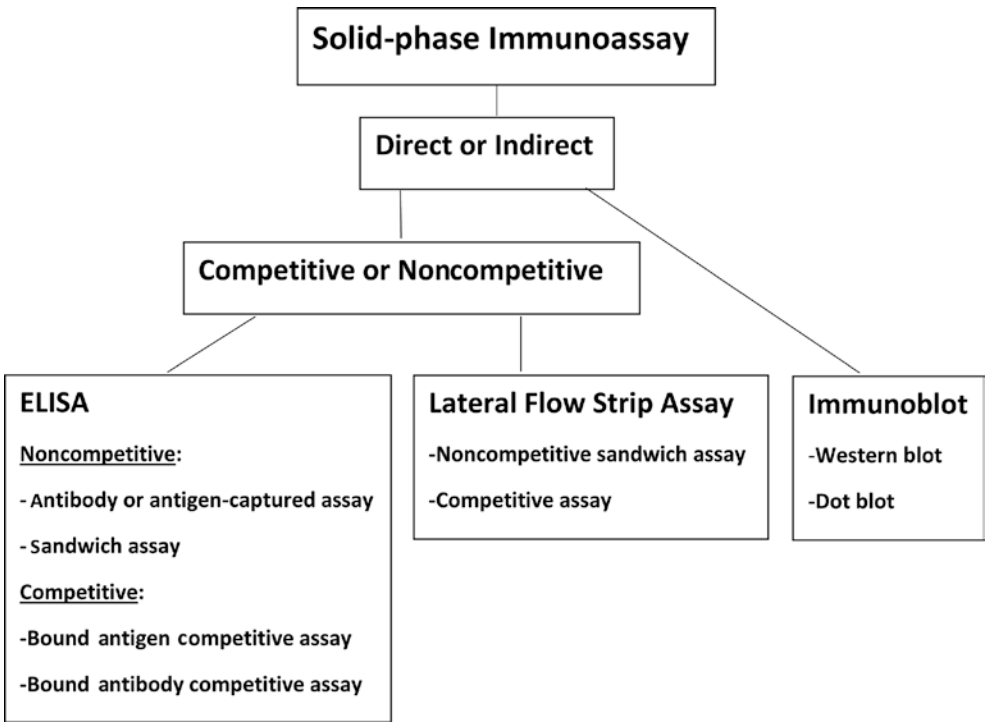
27.1 INTRODUCTION

Immunochemistry is a relatively new science that has developed rapidly in the last few decades. One of the most useful analytical developments associated with this new science is immunoassay. Originally immunoassays were developed in medical settings to facilitate the study of immunology, particularly the antibody-antigen interaction. Immunoassays now are finding widespread applications outside the clinical field because they are appropriate for a wide range of analytes ranging from microorganisms to proteins to small organic molecules. In the food analysis area, immunoassays are widely used for chemical contaminants analysis, identification of bacteria and viruses, and detection of proteins in food and agricultural products. Protein detection is important for determination of allergens and meat species content, seafood species identification, and detection of genetically modified plant tissues. While immunoassays of all formats are too numerous to cover completely in this chapter, there are several assay formats that have become standard for food analysis because of their specificity, sensitivity, and simplicity (Fig. 27.1).

27.1.1 Definitions

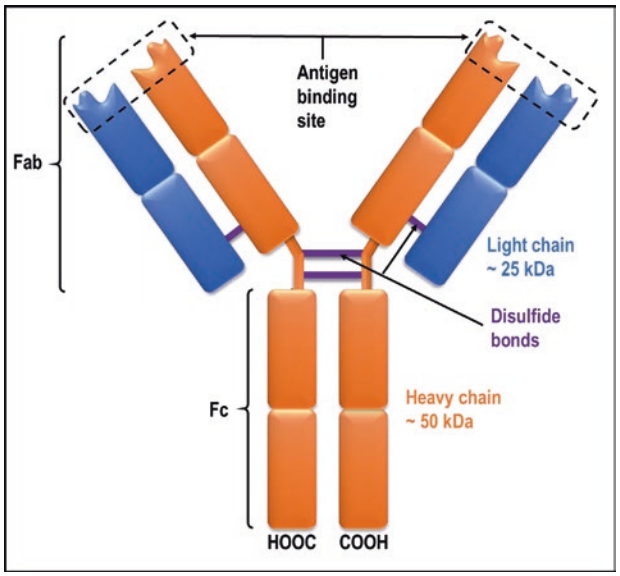
Immunoassays are analytical techniques based on the specific and high-affinity binding of antibodies with particular target antigens. To fully understand immunoassays, some of these terms need to be defined. The two essential elements of any immunoassay are antigens and antibodies. In an immunoassay, antigens and antibodies are used either as target molecules or capture molecules. In other words, a particular antigen can be used to capture its specific antibody, or a specific antibody can be used to trap the target antigen in a sample. An **antigen** is any molecule that induces the formation of antibodies and can bind to these antibodies. **Antibodies** are **immunoglobulin** (Ig) **proteins** produced by animals in response to an antigen. These antibody proteins are secreted by the activated B cells in immune system and bind the particular antigen responsible for their induction. Generally a molecule must be greater than 5000 Da, abbreviated as Da (unit of molecular mass), to be perceived as an antigen by a mammalian immune system. Almost all proteins are large molecules and have the ability to induce antibody formation in the body of humans and animals. However, many of the molecules analyzed in food are not as large as proteins but are small molecules such as



27.1 figure Commonly used immunoassays for food analysis

toxins, or antibiotics and pesticides. When animals are injected with small molecules, they do not develop antibodies against these molecules. To induce specific antibodies to recognize and bind the small target molecule, the solution is to covalently link the small molecule, or some appropriate derivative of the small molecule, to a larger carrier molecule. The small molecule that must be linked to a large carrier protein before it can be used as an immunogen to induce antibodies is called a **hapten**. The carrier protein-linked hapten is called a **conjugate antigen**. Haptens react specifically with the appropriate antibodies but are not immunogenic. The desired molecules used as carriers are proteins that are soluble, unrelated to proteins that may be found in the assay sample, and foreign to the host animal to properly stimulate an immune response. Typical carrier molecules include albumin proteins from a different species, such as bovine serum albumin and hemocyanins that are obtained from crustaceans. Of course, when a conjugate antigen is used for immunization of an animal, its immune system is stimulated to produce antibodies that bind not only the externally attached hapten but also the exposed exterior of the covalently linked carrier protein.

There are five major classes of antibodies, IgA, IgE, IgG, IgM, IgD, according to their heavy chain structure. Animal blood contains trace amounts of IgA and IgD. IgA is mainly found in mucous secretions and plays an important role in mucosal immunity. The unique function of IgD is still unclear. IgM is a very large molecule and can be regarded as a precursor of IgG. IgE is only associated with allergic response in humans and animals. Among these five classes of antibodies, IgG has the highest concentration in blood and is the most important class used in food immunoassay. Since the antibody and antigen are central to any immunoassay, it is useful to better understand the basic structure of the antibody and how it binds the antigen. Figure 27.2 is an idealized diagram of an antibody IgG. The IgG is a Y-shaped molecule made up of four polypeptide chains that are linked by inter- and intra-disulfide bonds. Two of the polypeptide chains are identical and roughly twice as large as the other two identical polypeptide chains. Because of their relative sizes, the former pair is known as **heavy chains** and the latter pair as **light chains**. Overall, an IgG antibody is a very large protein of approximately 150000 Da. Antigen is bound by two identical binding sites made up of the end portions (N terminals) of a heavy and light chain at the top of the Y. These two fragments capable of binding with antigen are called **Fab** (**fragment antigen binding**). The third fragment with no antigen-binding capability is called **Fc** (**fragment crystallizable**), because it can be crystallized. Different antibodies produced by different B cells can have many variations in amino acid sequences near the binding sites for both the heavy and light chains.

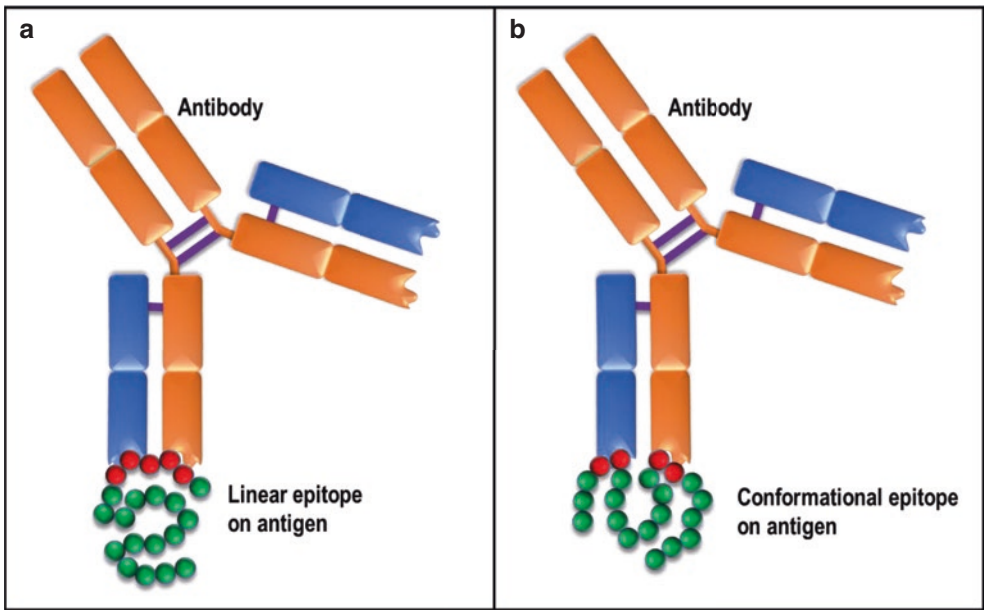


27.2 figure Antibody (IgG) structure

This leads to a tremendous diversity of binding sites for different antibodies. For example, a mouse has 10^7–10^8 different antibodies (and at least this number of different B cells), each with a unique binding site. The rest of the antibody (away from the binding site) is quite consistent, and small variations in this region on the heavy chains result in different antibody classes.

27.1.2 Binding Between Antigen and Antibody

Antibodies can develop remarkably strong binding affinities for their antigens. These affinities are among the strongest noncovalent interactions known between molecules. The binding strength (affinity) between antibody and antigen is one of the most important factors that determines the sensitivity of an immunoassay. The antibody binds to the outside of the antigen molecule in a specific region. This specific region bound by a single antibody binding site is known as an **epitope**. Two types of epitopes on an antigen can be formed. A **linear epitope** is formed by a continuous sequence of amino acid residues, and a **conformational epitope** is formed by noncontinguous amino acid sequences that are folded into close proximity from neighboring or overlapping peptide chain on the surface of the antigen (Fig. 27.3). If this 3-dimensional conformation of the antigen is altered by some kind of the environmental conditions, such as heating or pH changes, the conformational epitope will be destroyed, which means that the antigen cannot bind to the antibody. Moreover, the binding of the antibody to the antigen does not involve covalent bonding, but the same interactions that are responsible for the tertiary



27.3 figure Linear epitope (**a**) and conformation-dependent epitope (**b**)

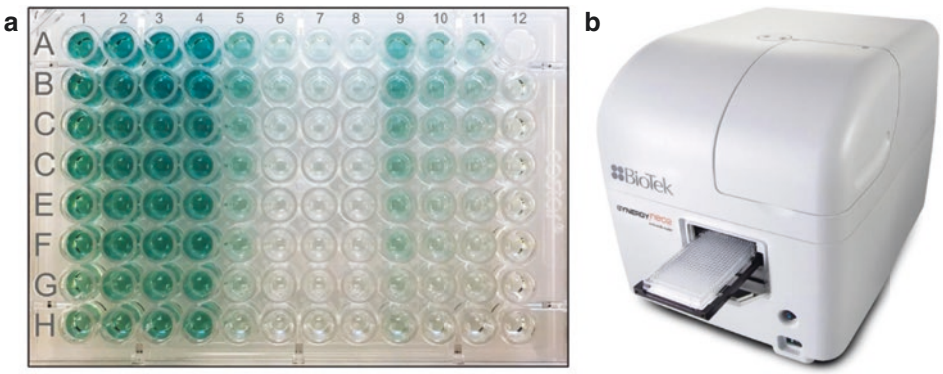
structure of proteins. These interactions include hydrogen bonds, electrostatic and hydrophobic interactions, and van der Waals forces. While the latter interactions, van der Waals, are the weakest, they often can be the most important because every atom can contribute to the antibody-antigen binding as long as the atoms are very close to each other (generally about 0.3–0.4 nm). This requirement for very close proximity is why antibody to antigen binding is considered something like a lock and key interaction, where the surfaces of the antibody binding site and the antigen epitope are mirror complements of each other.

27.1.3 Types of Antibodies

A major variable in an immunoassay is the type of antibody used. When serum antibody is used from any immunized animal, there are many different antibodies that bind different epitopes on the antigen. This collection of different antibodies is known as **polyclonal antibodies**. Scientists knew that individual B cells produced antibodies with only one binding site but were unable to culture B cells outside of the animal. However, in 1975, Köhler and Milstein [1] successfully fused cancer, or myeloma, cells with B cells. The new fused cells, or **hybridomas**, retained the properties of both of the parent cells. That is, they could be cultured, like cancer cells, and produced antibodies like the B cells. Hybridomas thus can be cloned and cultured individually to produce different antibodies with different epitopes. Antibodies produced with this procedure became known as **monoclonal antibodies**. Monoclonal antibodies produced from a single hybridoma are identical in every way and bind antigen with only one type of binding site, that is, a single epitope is bound; therefore, they can be used as standard reagents in immunoassays. Moreover, the hybridomas were "immortalized" by the procedure and with proper care could produce as much identical antibody as required. It did not take the scientific community long to appreciate the tremendous advantages of these monoclonal antibodies, so Köhler and Milstein were awarded the Nobel Prize for their work on this in 1984. While monoclonal antibodies are initially much more expensive to produce, there is the possibility for limitless identical antibody production, often from non-animal sources such as large-scale production of the hybridomas in cell growth chambers. These advantages outweigh the initial development costs for many immunoassay manufacturers. Detail procedures regarding the antibody production and characterization can be found in the book by Howard and Bethell [2].

27.2 THEORY OF IMMUNOASSAYS

Based on the specific antibody-antigen affinity, various types of immunoassays have been developed to either: (1) use antibody as the capture molecule to search the target antigen or (2) use the antigen as the capture molecule to trap the antibody in a complex sample. The basis of every immunoassay is the detection and measurement of the primary antibody-antigen reaction. In its simplest form, antibody capture of antigen can involve a simple precipitation and be detected visually. Since all antibodies have at least two identical binding sites, they can cross-link epitopes from two identical antigens. If other antigen epitopes are further cross-linked by different antibodies, a large, insoluble network can result which is seen as a precipitate. The immunoprecipitation techniques, including immunodiffusion and agglutination,



27.4 figure Image of a 96-well plastic microtiter plate (**a**) and plate reader (**b**) used for ELISA

formed the basis of early development of immunoassay techniques and have been used widely for protein and cell identification using antisera. However, these methods only work for antigens with multiple epitopes.

To measure the quantity of soluble antibody-bound antigen molecules in a solution, all immunoassays require two things. The first is that there must be some method to separate or differentiate free antigen from bound antigen. Secondly, these antibody-bound antigens must be quantifiable at low concentrations for maximum sensitivity. Detection at very low concentrations has required very active labels. One of the first successful immunoassay procedures was developed by Yalow and Berson [3] in 1960. This procedure used radioactive iodine, I^{131}, a "hot" radioisotope with a half-life of only 8 days, as a label to reveal the primary antibody-antigen complex. This radioactive label allowed for the second requirement of immunoassays: quantification at low concentrations. Yalow and Berson used paper chromato-electrophoresis to separate their antibody-bound antigen from free antigen, fulfilling the first requirement of an immunoassay. With all the variations in separation and detection techniques in the early stage of immunoassay development, however, the radioactive iodine labeling remained, and these assays became known as **radioimmunoassays** (RIA).

One of the techniques for the separation of unbound from bound molecules in immunoassays involves immobilizing protein on a hydrophobic solid surface. Proteins have large regions that contain hydrophobic amino acid groups that prefer not to be exposed to water. These nonpolar hydrophobic groups include hydrocarbons and aromatic groups that prefer to interact with similar groups, rather than a polar solvent such as water. In aqueous conditions these regions will bind to other hydrophobic surfaces excluding water. Surfaces commonly introduced in immunoassays to take advantage of this type of binding include charcoal, nitrocellulose, and plastic. Plastic surface in many forms is used commonly for immunoassays. Among the most popular are **microtiter plates** made of plastics such as polystyrene or polyvinyl. These microtiter plates typically are formatted to contain 96 individual wells, each with a maximal capacity of about 300 µl of liquid (Fig. 27.4a). To differentiate the wells, the vertical rows are labeled A to H and the columns numbered 1–12. It is important to realize that proteins bind to the bottom and sides of the wells in these plates randomly through hydrophobic interactions. Other forms of solid surface commonly used in immunoassays include plastic vials, magnetic beads, and nitrocellulose or polyvinylidene difluoride (PVDF) membranes or strips.

27.3 SOLID-PHASE IMMUNOASSAYS

27.3.1 Overview

Every immunoassay technique developed is based on the selection of an **amplification method** that will improve the sensitivity of assays. Labeling of the detecting molecule, either antigen or antibody, is necessary to locate or to quantify the target molecule in a solid-phase immunoassay that uses a solid surface for the separation of unbound from bound molecules. For example, having an easily detected label attached to the antibody allows the target antigen in a complex food sample matrix to be detected. A number of labeling compounds and materials have also been used, such as radioisotopes, fluorochromes, enzymes, biotin, and gold nanoparticles. These different label options become very useful for immunoassay development, or in the use of antibodies for detection in different systems such as examination of tissue under a microscope or proteins separated using electrophoretic techniques followed by an immunoassay.

While RIAs worked well in the clinical field, they were confined to specially equipped laboratories because of the dangers associated with the use of radioactive material. Immunoassays did not develop for more general use, including field use, until enzyme labels were developed. Any immunoassay that uses an enzyme label to reveal the primary antibody-antigen binding is called an **enzyme immunoassay**. Pioneers in this development were Engvall and Perlmann [4] who in 1971 developed a type of enzyme immunoassay that they called an **enzyme-linked immunosorbent assay**, or ELISA. ELISA involves the binding of a soluble antigen or antibody to a solid support (immunosorbent), typically in the form of a 96-well plastic microtiter plate. The bound and unbound molecules can be easily separated by a washing step of the plate. Microtiter plates of hydrophobic plastics are commonly used to immobilize proteins and to separate unbound molecules. Similar immunoassays using a solid support other than plastic microtiter plates also have been developed and are commonly used. Examples are the **dot blot** assay, **Western blot** assay, and more recently developed lateral flow immunochromatographic assay, which use nitrocellulose or polyvinylidene difluoride (PVDF) membranes. These developments have expanded the use of immunoassays to a wider range of applications.

27.3.2 ELISA

27.3.2.1 *Introduction*

Because enzyme immunoassay, especially ELISA, has become the most popular immunoassay for food analysis applications, the general principle and protocols of various formats of ELISA are illustrated in subsequent sections using ELISA as examples.

The enzyme label used in an ELISA converts a colorless substrate to a colored soluble product in the solution, thus generating a detectable signal for the assay. The amount of target protein antigen present in the sample extract is determined from the intensity of color developed in the immunoassay. The ideal enzyme for an enzyme immunoassay is one that is stable, easily linked to antibodies or antigens, and rapidly catalyzes a noticeable change with a simple substrate. With the many enzymes available, two enzymes, **horseradish peroxidase** and **alkaline phosphatase**, are by far the most commonly used in immunoassays. Other enzymes used include β-galactosidase, glucose oxidase, and glucose-6-phosphate dehydrogenase. The use of an enzyme to catalyze a chemical reaction that generates color signal contributes to the sensitivity of the assay because a single enzyme molecule present at the end of the test converts many substrate molecules to detectable colored product, thus amplifying the signal generated by the assay. However, it is more difficult to achieve a quantitative measure with enzyme immunoassays because the rate of the enzyme reaction involved is difficult to measure and the enzyme-labeled reagents are not homogenous. Minimal laboratory equipment is required to perform ELISA. The color generated from an assay can be visualized to determine the result in a qualitative assay or semi-quantitated spectrophotometrically. The type of spectrophotometer used to quantitatively monitor color development caused by the enzyme action is called a **microplate reader** (Fig. 27.4b). Automated **microplate washer** also is available although most assays can be washed manually.

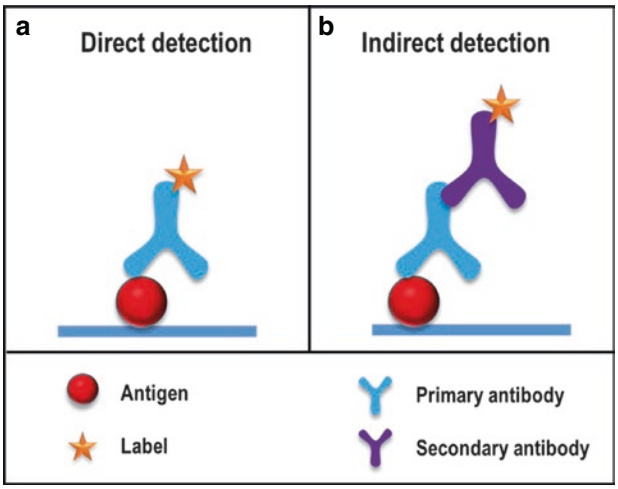
All ELISA protocols include the following five general steps:

1. *Coating* of antibody or antigen onto the wells of a microfiter plate (solid phase)
2. *Blocking* the remaining uncoated surface on the solid phase with a blocking buffer containing a nonspecific protein such as bovine serum albumin (this is to minimize the nonspecific reactions and also protect the adsorbed antigen or antibody from surface denaturation)
3. *Incubating* with different immunoassay reagents at a specified temperature and time
4. *Washing* the coated surface to separate free, unbound molecules from bound molecules
5. *Detecting* the color developed from the assay visually or spectrophotometrically

Specific procedures vary with the different variations of ELISA. It is important to include both positive and negative controls in an assay along with any analyzed food sample because materials in the food extract can vary widely and these other components can have an effect on the competition for the antibody binding site. This is to ensure that the immunoassay works well (positive control shows positive signal) and that there is no contamination or nonspecific reactions in the assay system (negative control shows negative signal).

27.3.2.2 *Direct Versus Indirect Detection*

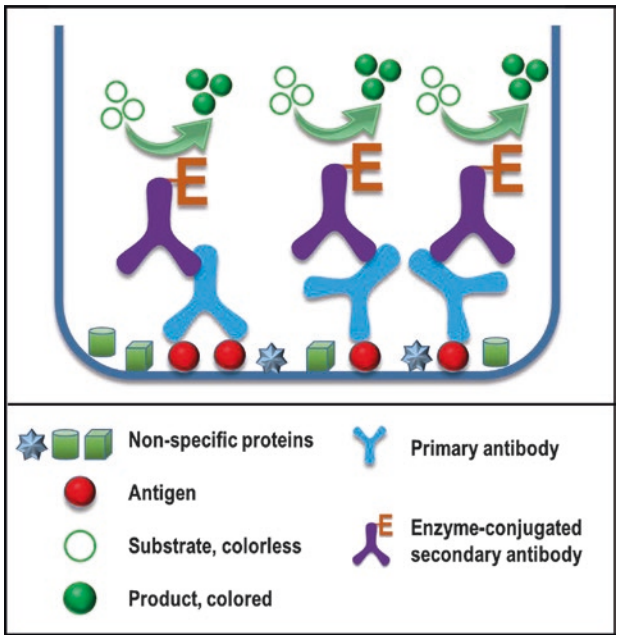
All immunoassay signals can be detected directly or indirectly. In the **direct detection method**, the detecting molecules are purified and linked to the label to directly measure the amount of the antibody-antigen complex (Fig. 27.5a). Therefore, more purified immunoreagents need to be used for the label conjugation procedure. In contrast, the **indirect detection method** uses a commercially available intermediate reagent to link the capture molecule. Most often a labeled **anti-species antibody** is used for an antibody-captured assay to indirectly measure the amount of antibody-antigen complex formed (Fig. 27.5b). Although an additional step is involved, indirect assays require less



27.5 figure Direct detection (**a**) and indirect (**b**) detection methods

immunoreagents and in many cases could be more sensitive because more labeling molecules can be linked to the detection antigen or antibody for enhanced signal production. While the direct detection method is essential when an accurate quantification is required for the assay, the indirect detection method is used in most solid-phase immunoassays.

A simple form of indirect antibody-captured ELISA is demonstrated in Fig. 27.6. The method is often used in the early stage of immunoassay development to detect primary antibodies in antisera or screen hybridoma supernatants for searching desired antibodies. **Primary antibody** refers to the antibody that binds the antigen. Theoretically, the antibody-captured ELISA can be made either as a direct ELISA or an indirect ELISA. However, because the target molecule is the primary antibody that appears in the biological fluid in a very low quantity, it usually is impossible to obtain enough quantity of the primary antibody to prepare the enzyme-conjugated labeling reagent for subsequent detection. Therefore, antibody-captured ELISA is almost always configured in an indirect assay format. The soluble antigen is adsorbed (coating) onto the surface of the microplate wells and incubated. After blocking, diluted samples of antisera or hybridoma supernatants are then added to the wells and incubated to allow the immobilized antigen to bind primary antibodies in the sample. After washing away any unbound molecules, those bound antibodies can be detected by adding an enzyme-linked secondary antispecies antibody, which can easily bind to the constant region of the primary antibody. After another incubation and washing steps, a solution containing substrate is added to generate color in the solution. The color is positively related to the amount of target antibody present in the sample.



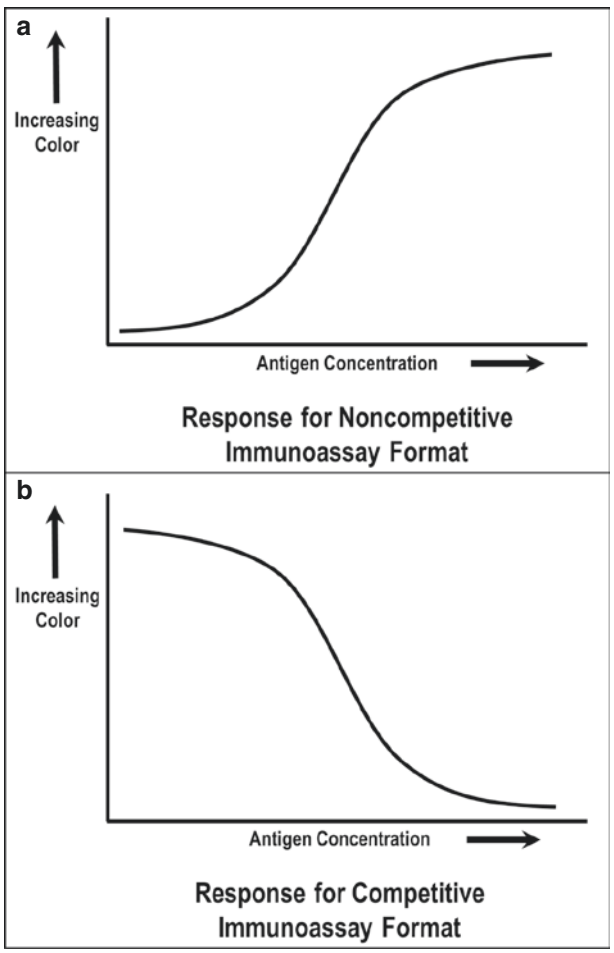
27.6 figure Indirect noncompetitive ELISA

The **secondary antibody** used in the indirect ELISA does not have the specificity to bind the antigen but only recognizes the primary antibody; thus it makes a link of the enzyme label to the bound immunomolecules without interfering with the primary antigen-antibody binding. Since antibodies are proteins, they can act as antigens in another animal species. For example, rabbit antibodies injected into a goat can stimulate the goat's immune system to produce goat antibodies that bind to the rabbit antibodies. In this way, goat anti-rabbit antibodies can be produced to bind any antibody produced in a rabbit. There are many advantages to these secondary anti-species antibodies. For example, when antispecies antibodies are used in the above antibody-captured ELISA format (Fig. 27.6), there is no need to label the primary antibody with an enzyme. The primary antibody does not need to be chemically modified; thus the loss of its activity is avoided. After excess material is washed away, goat anti-rabbit antibody labeled with an enzyme can be added to detect the presence of any primary antibody which is produced in rabbit antiserum and binds to the antigen coated on the microtiter plate. Although this procedure adds an additional step, there are many advantages: (1) antispecies antibodies of all types are commercially available from many manufacturers; (2) antispecies antibodies come with a variety of labels such as different enzymes, radioisotopes, or fluorescent compounds for different immunoassay systems; and (3) since the antibody is a very large protein, it has many sites for attachment of a labeled antispecies antibody. This multiplies the labels

per antibody, increasing the ability to detect the antibody and resulting in stronger signals with increased sensitivity in an immunoassay, do less primary antibody reagent is needed. Therefore, indirect methods are used in most detection applications.

27.3.2.3 *Noncompetitive Versus Competitive Immunoassay Variations*

Noncompetitive immunoassay is commonly employed to analyze large molecules such as proteins in a food sample, while **competitive immunoassay** is competitive in nature and is mainly used for small molecule analysis. Both competitive and noncompetitive immunoassays can be detected directly or indirectly. For simplicity, these variations are illustrated in the sections that follow using ELISA as examples. In general, the amount of color development for the noncompetitive ELISA is directly related to the amount of antigen present in the sample Fig. 27.7a. With any competitive ELISA format, there is an inverse relationship between the amount of color developed and the amount of antigen present in the sample (Fig. 27.7b).



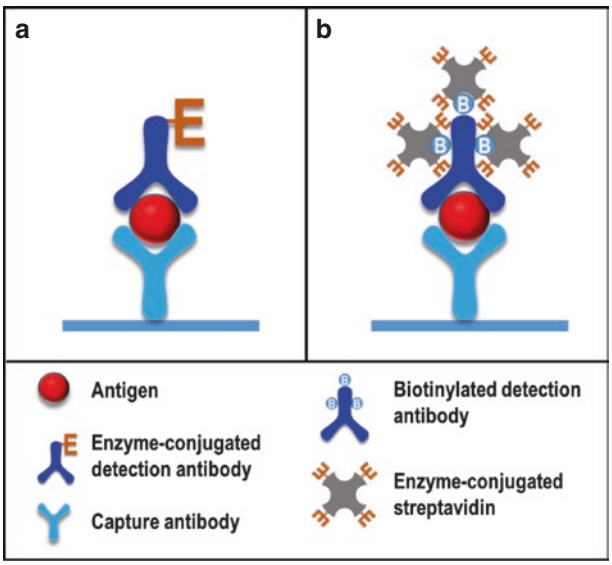
27.7 figure Relationship between color development and antigen concentration for noncompetitive (**a**) and competitive (**b**) immunoassay formats

27.3.2.3.1 *Noncompetitive ELISA*

Noncompetitive ELISA variations involve the revealing of the amount of primary antibody-antigen complex immobilized on the solid phase by the amount of enzyme linked to the detection antigen or antibody molecules to produce a colored product in the assay solution. Therefore, at the end of the assay, the color intensity is positively related to the amount of the target molecules. The absence of the target molecules produces no color, and the presence of high concentration of the target molecules produces strong color. This type of ELISA is used often to detect proteins in a food sample because protein molecules are large enough to link one or more antibodies or to an additional enzyme label on the surface of the protein.

One of the most popular formats for a noncompetitive enzyme immunoassay is the antibody **sandwich immunoassay**. A sandwich ELISA model using both direct and indirect detection method is demonstrated in Fig. 27.8a, b, respectively. The "meat" in the antibody sandwich is the target antigen. In food analysis this can involve identifying a protein adulterant such as undeclared pork meat in a beef product, a protein allergen such as peanut protein, or wheat protein in a product that would be a problem for people suffering from celiac disease.

Generally a primary antibody that binds to the antigen is first immobilized onto a hydrophobic solid phase such as plastic. Excess antibody is removed by washing with a washing solution or simply water



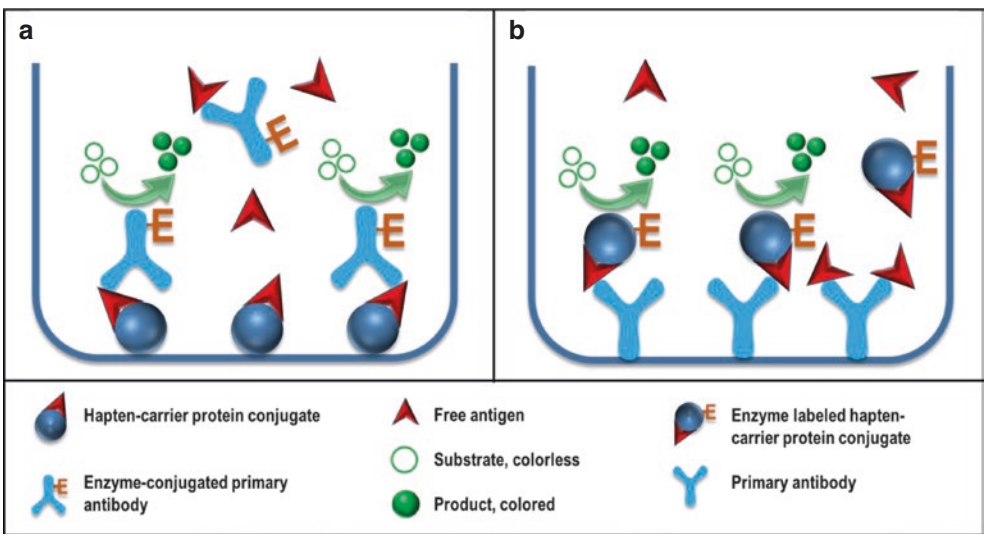
27.8 figure Direct (**a**) and indirect (**b**) noncompetitive sandwich ELISA

followed by a blocking step, and then the test is ready for analysis of a food extract. The immobilized antibody is called a **capture antibody**. The food extract being tested contains many compounds with or without the target antigen. However, the antibody was prepared by immunization of an animal with a specific, purified protein antigen, and only this protein antigen in the food extract solution will bind to the capture antibody. Now the antigen and the capture antibody are immobilized, and the remaining unbound molecules can be washed away. After the washing step, another primary antibody labeled with an enzyme (Fig. 27.8a) is introduced. This antibody, called the **detection antibody**, also recognizes the antigen, thus forming an antibody-antigen-antibody complex. Again excess detection antibody is washed away and then colorless enzyme substrate is added to develop a color if bound enzyme is present. Enzyme will only be present if the detection antibody has been immobilized by binding to the antigen. The greater the color development, the greater the amount of antigen present. That is, there is a direct proportionality between the amount of color seen in the final step and the amount of antigen present in the extracted food sample. To increase the sensitivity of a sandwich immunoassay, one can use more antibodies for capture of the antigen or link more enzyme molecules to the detection antibody through the use of intermediate reagents, such as biotin and labeled streptavidin. In the latter case, the detection antibody in the indirect sandwich assay (Fig. 27.7b) is purified and linked to biotin. Biotinylation of a primary antibody is relatively simple and rarely affects the antibody activity. Then an additional step of adding enzyme-labeled avidin or streptavidin to the reaction system is needed. Avidin/streptavidin easily and tightly binds to the biotin groups so that more enzymes are linked to the immobilized molecules (Fig. 27.8b). This indirect sandwich immunoassay format can be made remarkably specific and very sensitive since two antibodies must detect the antigen and more labels are involved in amplifying the reaction signal.

When polyclonal antibodies are used in the sandwich immunoassay, the polyclonal antibody solution is divided into two parts. One part is bound to plastic to become the capture antibody. The second portion of the polyclonal antibody solution is purified and conjugated to an enzyme or biotinylated and becomes the detection antibody. Monoclonal antibodies can also be used, but care must be exercised since a single type of monoclonal antibody cannot be used for both the capture and detection antibodies since only one unique epitope is recognized by any monoclonal antibody. In other words, the antigen must be large enough to bind two antibodies at the same time and therefore must use at least two distinct epitopes recognized by different monoclonal antibodies that recognize two distinct antigen epitopes.

27.3.2.3.2 Competitive Immunoassays

A problem in developing an immunoassay for detecting a small molecule is that a sandwich immunoassay format will not work since two different epitopes on the antigen are required for both antibodies to bind. A small molecule represents only one epitope or even only part of one epitope. The competitive immunoassay format (Fig. 27.9a, b) was, therefore, developed to solve this problem. The first requirement in a competitive ELISA involves immobilizing the small molecule, often as a hapten, or immobilizing the antibody. Subsequent procedures involve the competition between the free small antigen (from a sample) and



27.9 figure Direct competitive ELISA in bound hapten (**a**) and bound antibody (**b**) formats

the hapten (as an added reagent) for the binding of limited amount of the specific antibody. To bind the hapten to a solid surface such as nitrocellulose or plastic, it can again first be linked to a protein that binds to these hydrophobic surfaces. However, the protein used for binding the hapten to the surface is different than the carrier protein binding the hapten used for immunization of the animal, since the animal also has developed antibodies against the carrier protein used for injection, and only the hapten-specific antibodies are desired for the competitive immunoassay. Since all types of competitive immunoassays involve a reduction in absorbance with respect to a control (containing no small molecule or analyte), data often are presented as a ratio of sample absorbance to the absorbance of the control. The concentration of the inhibitor (target antigen) required to reduce the assay absorbance by 50 % (defined as $\mathbf{IC_{50}}$) is a useful value to be determined for a competitive immunoassay because this is the region of greatest change in response compared to concentration changes and therefore the lowest coefficient of variation.

To increase the sensitivity of a competitive immunoassay, the amount of limiting antibody should be reduced. Note that this is the reverse of what one would do to increase the sensitivity of a noncompetitive immunoassay such as a sandwich immunoassay. Theoretically the most sensitive competitive immunoassay would be between one antibody binding site and one hapten, with either of the two labeled with an enzyme. It is for this reason that the ability to detect the presence of the enzyme is so important for a competitive immunoassay. The more sensitive the system is to detect the enzyme, the more sensitive the competitive immunoassay. Two competitive ELISA procedures are described here:

1. **Bound Hapten Format**. In the bound hapten competitive immunoassay format (Fig. 27.9a), the protein-bound hapten is first immobilized to a solid surface by hydrophobic interactions. Excess material is washed away. Next a competition is created between the protein-bound hapten and the free small antigen molecule in a food extract, both competing for binding to the limited binding sites on the antibody labeled with an enzyme. It is important to realize that the free small molecule in the food extract is not completely identical to the immobilized hapten since the latter is covalently linked to a protein. However, if properly designed, the free molecule in the food extract is so chemically similar to the bound hapten that the competition for the limited number of antibody binding sites is nearly equal. The primary antibody bound to immobilized hapten remains after a subsequent washing step. The more small molecules in the food extract, the more antibody is bound to these free small molecules, and this unbound antibody (and its attached enzyme) will be washed away in the subsequent washing procedure. Finally, the amount of bound antibody is identified by adding the enzyme substrate and observing the amount of color developed. Therefore, there is an inverse relationship between the amount of small molecules or analyte in the food and the amount of color developed in the final step.
2. **Bound Antibody Format**. The other variation for a competitive immunoassay is to bind a limited amount of antibody to the solid phase and create a competition between enzyme-labeled conjugate antigen and free small antigen molecules in the food extract (Fig. 27.9b). It is generally believed that this second format is somewhat valthough it can require the use of more antibody reagent. Again after a washing step, the final procedure is a color development to determine the amount of antibody-bound antigen-enzyme. This competitive format also results in an inverse relationship between the amount of color and free small molecules in the food extract.

27.3.3 Immunoblots

27.3.3.1 *Western Blot*

As one of the immunoassays that uses the specificity of the antigen-antibody interaction to indicate the presence of particular proteins in a sample, **Western blot** is a laboratory-based method that combines two techniques: **polyacrylamide gel electrophoresis** (PAGE) and **immunoassay**. In the first part of a Western blot, proteins in a complex mixture are separated by PAGE according to their molecular mass. In the second part, the separated proteins are subjected to an immunoassay to detect the presence of antigenic proteins. Using this combination of techniques makes it possible to identify target proteins and confirm their identity by molecular mass. The detection reagent in a typical Western blot is an enzyme-labeled antibody conjugate directly or indirectly labeled to the detection antibody. If the original protein mixture was labeled with a radioactive material, then autoradiography is used to visualize the radioactive signal. The primary antibody used largely determines the specificity and sensitivity of the method. Specific proteins in picogram quantities can be detected in a highly sensitive Western blot.

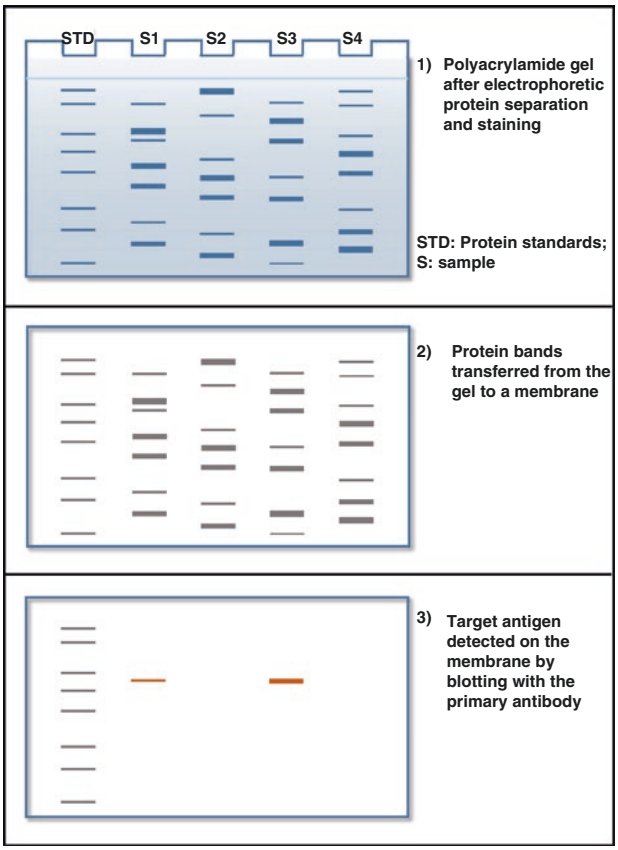
To prepare protein samples for the initial separation by PAGE, they are typically boiled in a buffer solution containing a reducing agent (usually mercaptoethanol) and detergent (e.g., sodium dodecyl sulfate), to unfold the protein peptide chains. The treated sample is applied to a polyacrylamide gel and

separated by electrophoresis based on **molecular mass**. To prepare for the immunoassay portion of the method, the separated protein bands are transferred from the polyacrylamide gel to a **nitrocellulose** or **PVDF membrane**. After blocking the nonspecific binding site, for a direct detection format, this membrane is then incubated with a solution of primary antibody-enzyme conjugate. After washing away the excess conjugate from the membrane, the enzyme substrate is added. A colored band forms at the site on the membrane where the protein that reacted with the antibody was immobilized (Fig. 27.10). The enzyme substrate used in Western blot is different than the ones used in ELISA because the intent is to form an insoluble colored product that stays on the membrane. The color intensity and width of the protein band together indicate the concentration of the target protein in the sample extract. The molecular mass of the protein bound by the antibody can be estimated by its position relative to standard proteins of known molecular weight.

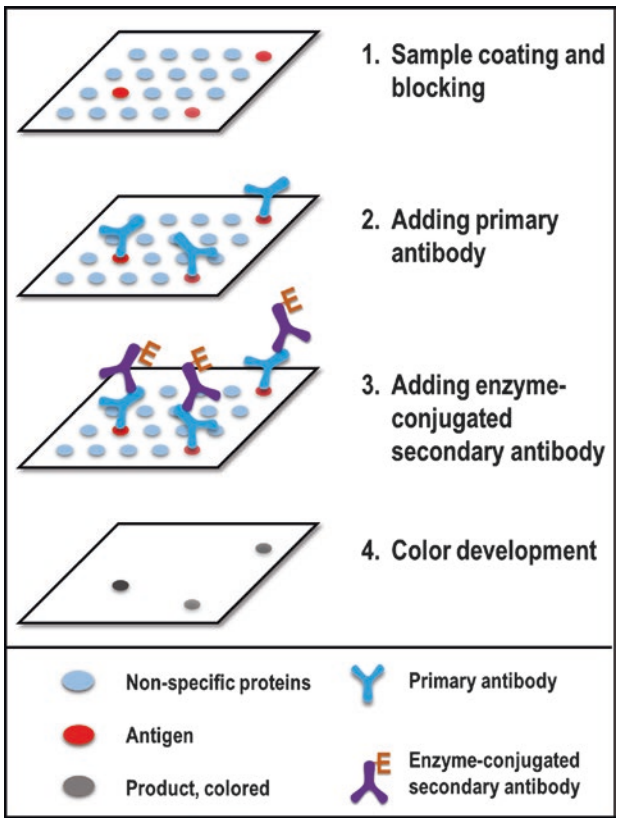
The Western blot procedure is technically complex, so it requires highly trained personnel working in a laboratory setting. However, the Western blot method is especially well suited to analysis of food samples that have been subjected to processing conditions. The Western blot method requires that the antibody used binds to a linear epitope on the protein, or is reactive to **denatured protein** (which would occur under the denaturing conditions in PAGE sample preparation). Because the Western blot method is highly sensitive and specific, and the antibodies used recognize denatured proteins, it is uniquely well suited to detect the presence of target food proteins at low concentrations in processed food.

27.3.3.2 *Dot Blot*

Dot blot represents a simplified version of Western blot. It is usually performed by depositing a drop of sample extract containing the molecule to be detected directly onto a piece of nitrocellulose or PVDF membrane as a dot. Unlike Western blot, protein samples in a dot blot assay do not need to be separated electrophoretically. The target protein molecule is directly detected by specific antibodies. The following general guideline describes a commonly used antigen bound dot blot assay for detecting a particular protein in food samples (Fig. 27.11):



27.10 figure Western blot technique. Protein profile before (*1*) and after (*2*) transferring and immunodetection of the antigen on the membrane (*3*)



27.11 figure Dot blot technique

1. A drop of the food sample extract is applied to the membrane.
2. The remaining binding sites on the membrane are "blocked" using a protein unrelated to the target antigen to minimize the nonspecific binding.
3. Following a rinsing step, the bound molecules in the sample spot are incubated with the primary antibody (for indirect detection) or with the primary antibody-enzyme conjugate (for direct detection). An additional step using an antispecies antibody-enzyme conjugate is required for the indirect assay.
4. After washing and adding the substrate solution, the reaction signal can be revealed either visually by color or measured by chemiluminescence imaging apparatus depending on the enzyme label and the substrates used. The spot signal (i.e., color) intensity positively related to the amount of target protein present in the sample.

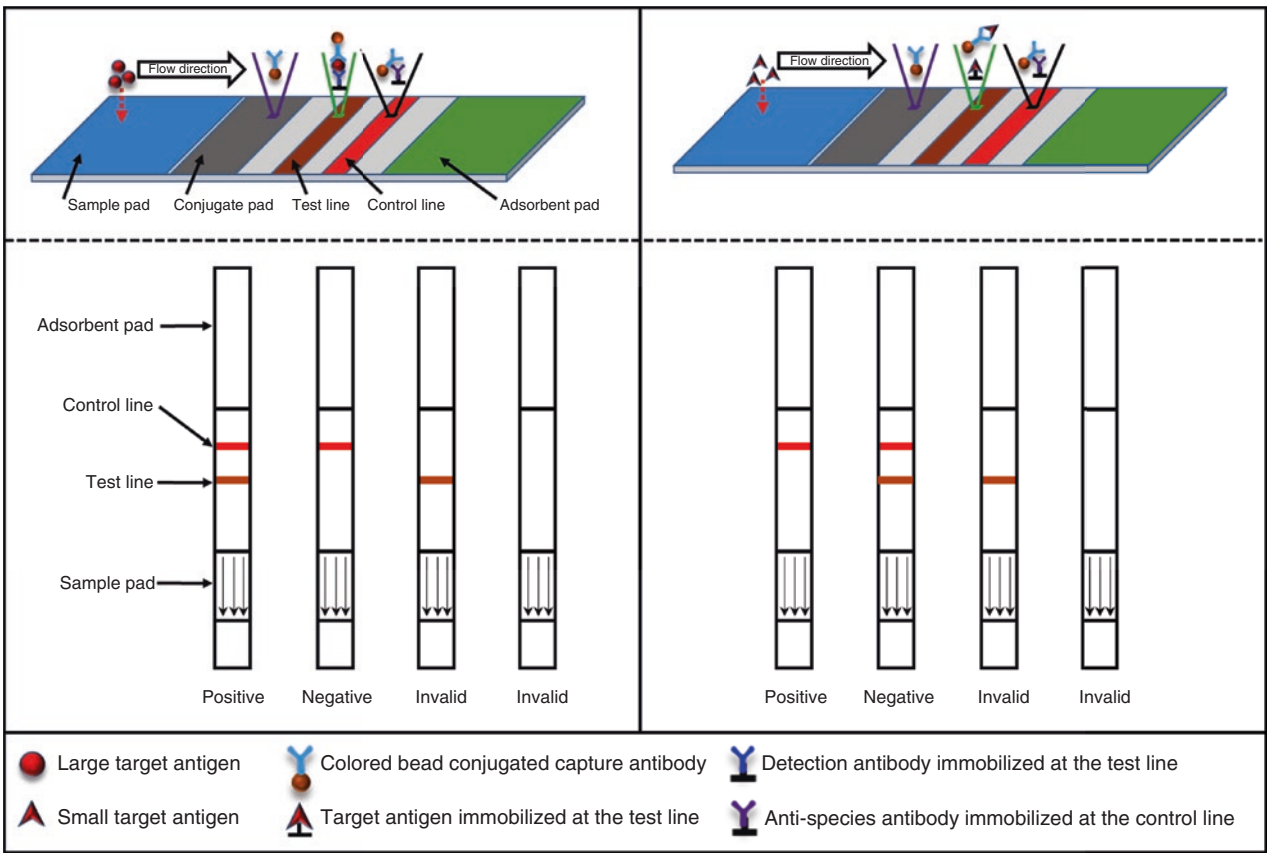
This blot dot technique does not offer molecular weight information of the antigen but is useful as a qualitative method for rapid screening of a large number of samples to probe the presence or absence of a protein target in food samples. This technique is also commonly used to evaluate the quality of the antibodies and testing the suitability of experimental design parameters.

27.3.4 Lateral Flow Strip Test

27.3.4.1 *Overview*

The **lateral flow strip** (LFS) test is a simple immunoassay format, commonly used for proteins to determine if their concentration is above or below a specified threshold (cutoff) (Fig. 27.12). The home pregnancy test is the best known LFS method. Results with LFS usually can be visualized in 10–20 min, and no washing steps are required to separate bound and unbound molecules. The characteristics of LFS methods – simplicity, low cost, ease of use, and reliability – make them ideal for use outside a laboratory setting, i.e., **field testing**, where supplies and equipment are limited.

Just as with other immunoassays, LFS methods are configured in a **competitive assay format** for detection of small molecules such as toxins or chemical residues or in a noncompetitive **sandwich immunoassay format**



27.12 figure Schematic view of a sandwich-type (noncompetitive) lateral flow test strip (**a**) and a competitive-type (**b**) lateral flow test strip

for detection of large molecules. While the color in an enzyme immunoassay comes from enzyme action on its substrate to create a colored reaction product, the LFS methods instead use very small, spherical, colored particles (colloidal gold or colored latex) attached to antibodies to generate a positive colored signal. The capture antibody is immobilized in a zone on a porous membrane (usually nitrocellulose). By capillary action of the membrane, the test sample travels past the zone of immobilized antibody. The target protein in the test sample binds to the capture antibody. This type of sample movement and separation explains why LFS is called an **immunochromatographic assay**.

27.3.4.2 *Procedure*

Figure 27.12a depicts a typical LFS sandwich immunoassay and shows the various regions of the test strip. A primary antibody capable of binding the target protein is coated onto the surface of very small, colored particles (usually 20–40 nm diameter, colloidal gold or colored latex). These antibody-coated colored particles are dried in a porous pad. When these particles come in contact with liquid samples, they get reconstituted and are able to flow with the sample, moving across the **sample pad** of the strip by capillary action. Any large particulates in the sample liquid can be filtered out by a fiber filter placed at the front of the strip in the sample pad area. A second primary antibody, also capable of binding to the target protein, is immobilized at the **test line** (zone) on the surface of the fibers of the porous nitrocellulose membrane. A **control line** above the test line, containing an antispecies antibody capable of binding antibody-colored particle conjugates, serves to indicate that the test ran appropriately. Sample gets drawn through the test strip by an **absorbent pad** placed at the back of the strip.

Both liquid and solid samples can be analyzed by the LFS assay. A solid sample must be dissolved or dispersed in a liquid solution to extract the target protein (antigen) from the sample. To do the assay, the strip is put in contact with the sample solution at the end of the sample pad. The sample solution is drawn into the test strip by capillary action. The sample first passes through the filter, and the target protein is bound by the colored particle-antibody conjugate. Then the antigen-antibody-colored particle conjugate complex gets drawn into the membrane and is captured by the immobilized antibody in the test zone. A colored line becomes evident as more colored complexes are captured. The color intensity generally correlates with the amount of antigen present in the sample. A dedicated device can be used to measure the color intensity of the test line if a quantitative result is required. Samples containing no target protein show no color at the test line. The control zone binds the excess antibody-colored particle conjugates that pass through the test zone, and then forms a colored line for any complete test. If no colored line is formed at the control zone, the assay is invalid and needs to be repeated on a new strip.

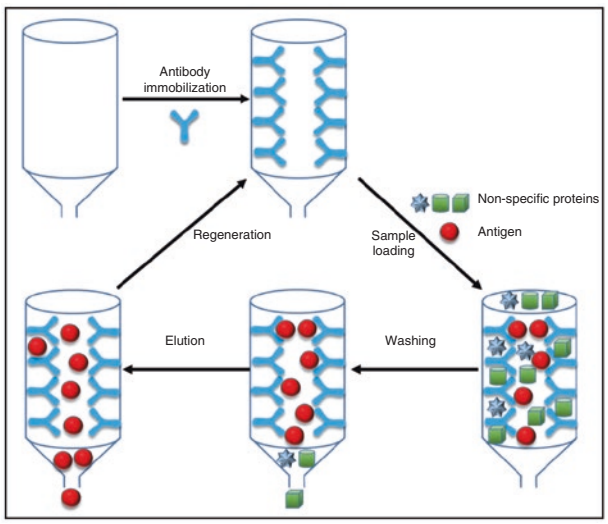
In the case of competitive LFS immunoassays, the target antigen that is immobilized on the test line and the free antigen from the sample solution will compete for the antibody-colored particle conjugates. Therefore, no colored line is formed for samples containing an antigen concentration above the cutoff value of the LFS test (Fig. 27.11b).

27.3.4.3 *Applications*

Current generation of LFS methods are made into versatile formats and are useful in applications for which their primary attributes (i.e., speed, simplicity, low cost, etc.) are important aspects of the testing (e.g., field testing). LFS methods have been developed for qualitative and to some extent quantitative monitoring of food allergens, foodborne pathogens, food toxins, hormones, and certain food protein ingredients to ensure food safety and quality. They also have been developed to detect the presence of genetically modified organisms in processed foods for consumers' interest, and to detect prohibited ruminant proteins in ruminant feedstuffs for the surveillance of mad cow disease. These easy-to-use rapid tests allow food processors and regulators to comply with regulations governing the labeling of food and feed products with minimal user training and facilities. Various devices of LFS for food allergen detection have been described in a recent handbook [5], and the strengths, weaknesses, opportunities, and threats of LFS methods have been thoroughly discussed in a review article [6].

27.4 IMMUNOAFFINITY PURIFICATION

Besides the use of antibodies in immunoassays as described above, often antibodies are used in food analyses as complements to other analytical methods. This is due to the remarkable specificity of antibodies and their strong binding to antigen. The most common example of this is **immunoaffinity** purification, which is an antigen capture technique. Basically the antibody is immobilized on some support, most often using a covalent linking method so that there is no concern with "bleeding" of the antibody in later steps (Fig. 27.13). The antibody can be bound to a solid phase such as agarose (e.g., Sepharose®) gel. These antibody-bound solid phases can be used later for purification of antigen via a chromatograaphy method or by the use of these phases on the surface of magnetic beads that are separated using a magnet. A simple purification sequence would involve exposing the antibody-bound solid to a food extract to first bind antigen, then washing the solid phase free of all unbound material, and finally releasing the pure antigen. Even though antibodies have such remarkably strong binding constants,



27.13 figure An example of antibody-bound immunoaffinity chromatography

they can be treated to release antigen by simple procedures such as changes in pH or solvent. Since the antibody is a protein, pH changes or solvent changes result in denaturation that changes the conformation of the binding site, causing the release of the antigen. If these changes are carefully selected, denaturation can be reversed by reestablishing moderate conditions so that the valuable antibody-bound solid phase can be reused repeatedly. For sensitive antigens, like enzymes, these elution conditions also can be a concern.

These immunoaffinity purification procedures have been used for small molecules like toxins (e.g., aflatoxins) and even materials as large as cells. Different microorganisms contain unique cell surface antigens that can be selectively bound to aid in purification and differentiation.

27.5 APPLICATIONS

Immunoassays are a well-developed area in food analysis and there is a plethora of literature available. For all sorts of laboratory techniques, Harlow and Lane [7] wrote one of the best books on the use of antibodies. The theory and practice of immunoassays is well handled in several books [8–10]. There are even entire journals, such as *Food and Agricultural Immunology*, devoted to describing methods for preparing food immunoassays.

Because of the speed, simplicity, sensitivity, and selectivity of immunoassays, they are used widely as screening tests for pesticide [11, 12] and drug residues [13, 14] in food (see Chap. 30). Besides chemical analysis, immunoassay techniques are used in microbiology to rapidly detect foodborne pathogens [15, 16] and bacterial and fungal toxins [17, 18]. Immunoassays also are commonly used for meat and fish species identification [19]. Since immunoassays can easily be developed to detect trace amounts of specific proteins, they are among a number of methods used to detect hidden food allergens [20–22] and genetically modified organisms in foods [23]. In fact, immunoassay can be developed to detect almost any organic substances in a food system. The flexibility is limited only by the availability and quality of the specific antibody used. Immunoassays are being automated for higher analytical throughput and for improved data quality [24]. Food immunoassays can be prepared using very simple and rapid formats, making them ideal for kits used in the field. While every effort is made to control the specificity of these rapid tests, they can suffer from false positives and false negatives. For this reason, immunoassay kits are used most often as rapid screening tests, while food samples that test positive for the target analyte by immunoassay are often confirmed using another, more sophisticated analytical method. Immunoassays are also applicable in situations for which analysis by conventional methods is either not possible or is prohibitively expensive.

The research in the immunoassay area continues to develop the following: (1) specific antibodies, especially monoclonal antibodies for providing the ultimate specificity for the antibody-antigen interaction, (2) different solid-phase configurations to provide simpler means to carry out the assay, (3) new detection systems to further improve the assay sensitivity, and (4) alternative methodologies, such as immunosensors and immunoarray chips for multiple analyte detection and pattern recognition [16, 25].

27.6 SUMMARY

Almost any organic molecule in food can be determined using immunoassays as long as the specific antibodies are available. Both polyclonal antibodies and monoclonal antibodies or a combination of them can be used in an immunoassay. The remarkable selectivity and sensitivity of these assays are the result of the strong binding affinity between antibodies and their antigens. While the precise protocols of immunoassays can vary a great deal, all immunoassays use either a noncompetitive or a competitive format. The competitive format is the only one that can be used for quantification of small (about 1000 Da or less) molecules. ELISA has become the most popular immunoassay that uses an enzyme as the label to reveal the primary antibody-antigen binding through a color reaction catalyzed by the enzyme. In a noncompetitive ELISA with enzyme-derived color development, the more antibody-bound molecules (analyte) in the food sample, the more color develops, while in a competitive ELISA,

the reverse is true. The most common labels used for food immunoassays are enzymes, and the most two common enzymes used are alkaline phosphatase and horseradish peroxidase. The reaction signals of both competitive and noncompetitive immunoassays can be detected using direct or indirect labeling methods, although indirect methods are used in most detection applications due to their various advantages over direct methods.

Two other commonly used immunoassays are immunoblot and LFS test. Two types of immunoblots, Western blot and dot blot, are commonly used in food analysis. Western blot is a laboratory-based immunoassay that combines PAGE and immunoassay to reveal the presence and the molecular mass of the antigenic protein on a membrane. Western blot is the most commonly used method for identifying and characterizing an antigen. A positive result also indicates the antigen conferring a linear epitope with the antibody. Dot blot is a simplified version of Western blot without the protein separation procedure, hence does not offer the molecular weight information of the antigen. The LFS, on the other hand, is by far the simplest form of immunoassay. The assay is easy to use and is ideal for outside-the-laboratory applications for which access to equipment and supplies is limited. While the general immunoassay procedure of the immunoblot is similar to ELISA, the LFS assay is a one-step assay based on one-direction movement of the antigen in a sample solution toward immobilized capturing antibodies at different zones. The separation of bound and unbound molecules by washing steps is not required. Because ELISA involves a signal amplification activity of the enzyme, it is generally regarded as inherently more sensitive than LFS methods. However, protein concentrations of less than a part per billion can be detected with a highly sensitive LFS.

Besides being the required constituent in immunoassays, antibodies also can be used to purify specific compounds in food for other analysis methods. These immunoaffinity purification methods allow for rapid purification of analytes from complex food matrices.

27.7 STUDY QUESTIONS

1. What is the relationship between an antigen and an antibody?
2. What is an epitope? What are the two types of epitopes?
3. What is the difference between monoclonal and polyclonal antibodies?
4. All immunoassays have two conditions that they must satisfy; what are they?
5. What is a hapten and what is a conjugate antigen?
6. What are five general steps for an ELISA procedure?
7. What is the rationale for the blocking step in an immunoassay protocols?
8. What is the difference between direct and indirect immunoassays? What are the advantages and disadvantages associated with each type of assay format?
9. Two common immunoassays are the sandwich assay and the competitive assay. Which molecules are best detected by each? Why?
10. What are two types of competitive ELISA procedures? Describe the main differences between these two.
11. Give four common applications of immunoassays in food analysis.
12. What is a Western blot? What is the major difference in reaction signals between Western blot and ELISA?
13. Compare and contrast ELISA and LFS, by identifying the similarities and differences in their characteristics, principles, and applications.
14. Describe, in general terms, how you would use immunoaffinity purification to isolate a protein for which you have developed antibodies.
15. All commercial potatoes contain the toxic glycoalkaloids α-solanine and α-chaconine. Both of these glycoalkaloids have the same large alkaloid portion, known as solanidine. Therefore polyclonal antibodies can be developed in rabbits against solanidine by chemically linking it to a foreign protein (foreign to the rabbit) and injecting the protein-conjugated hapten (solanidine linked using a succinic acid derivative) into rabbits. The antibodies that develop in the rabbit against the hapten bind to the alkaloid portion of both toxic glycoalkaloids. The rabbit antiserum containing primary polyclonal antibodies can be made highly specific to solanidine without cross-reactivity with other similar molecules by removing all cross-reactive antibody components using an immunoaffinity column procedure.

    To develop an appropriate competitive ELISA, solanidine is again linked to a protein, but this time a different protein, and this conjugate is used to coat plastic microtiter plates. After excess conjugate is washed away, the plates are ready for the competitive ELISA procedure.

    The glycoalkaloids in potatoes are extracted with methanol, and this extract is further diluted with water for use in the ELISA procedure. A standard curve is prepared by diluting standard solutions of α-chaconine at low, medium, and high concentrations with

similar aqueous methanol solutions. In addition, a negative control is prepared using methanol and water at similar concentrations to the diluted potato extracts and standards, but without any glycoalkaloid present. Now the various extracts, standards, and negative controls are placed in individual wells with equivalent amounts of diluted rabbit serum containing the specific polyclonal antibodies. After incubation for 30 min at room temperature, all of the wells on the plate are again washed. Next a solution of commercially available goat anti-rabbit antibody conjugated to peroxidase is added to each well. After another 30-min incubation, the wells are again thoroughly washed.

Finally, phenylenediamine substrate solution is added to each well along with peroxide and again the plate is incubated for 30 min. After 30 min, the plate is rapidly read (in under 1 min) using an ELISA plate reader. The wells all contain differing amounts of yellow color:

(a) Tomatidine is a glycoalkaloid found in tomatoes and contains the alkaloid portion tomatine. Would the polyclonal antibodies detect tomatidine?
(b) Why is the protein that the hapten is attached to different for the ELISA procedure than for the injection?
(c) Is the ELISA protocol direct or indirect?
(d) Which wells would you expect to contain the most color, standards, potato extracts, or negative controls?
(e) Would you be concerned if a potato extract gave almost no color at the end of the ELISA procedure?

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