Chapter 12

Immunological Methods

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12.1 INTRODUCTION

There are multiple infectious agents including viruses, bacteria, fungi and parasites that can infect a host and cause disease. However, in many cases, infections within healthy individuals are transient in nature and do not cause permanent damage. The reason for this is due to an individual's immune system which fights off and overcomes the infectious agents. Immunology is the study of the immune systems of higher organisms in relation to disease. In this chapter, we will describe the structure and function of the immune system, and how immunological methods can be used in environmental microbiology via antibody-based laboratory techniques or immunoassays.

The immune system is divided into two functional divisions: the innate and the adaptive immune systems. The innate immunity system acts as a first line of defense against infectious agents or pathogens by recognizing whether or not an entity is "native" to or "foreign" to the body before an infection is established. If determined to be foreign, the adaptive immune system can be triggered into action resulting in the evaluation of the foreign agent. Interestingly, once the body has been exposed to a specific foreign agent, frequently the immune system remembers the agent providing life-long immunity against subsequent infection. The innate and adaptive immune systems involve multiple molecules and cells distributed throughout the body. The important adaptive immune responses are categorized as cell mediated or antibody mediated. The cell-mediated response is produced when a subset of sensitized white blood cells or lymphocytes directly attacks material such as a bacterial cell or virus, which has been determined to be foreign to the body. The antibody-mediated response involves the transformation of a subset of lymphocytes into cells that produces and secretes specific antibodies that target the foreign material, which is termed the antigen. Overall, the two immune responses are triggered when foreign material is introduced into the host as depicted in Figure 12.1.

Here we do not deal with all aspects of immunology or the immune responses *per se*, but instead adapt immunology-based research technologies or immunoassays for the study of microorganisms and chemical contaminants found in the environment. The primary immunological tool used in environmental microbiology is the antibody. In this chapter, an introduction to

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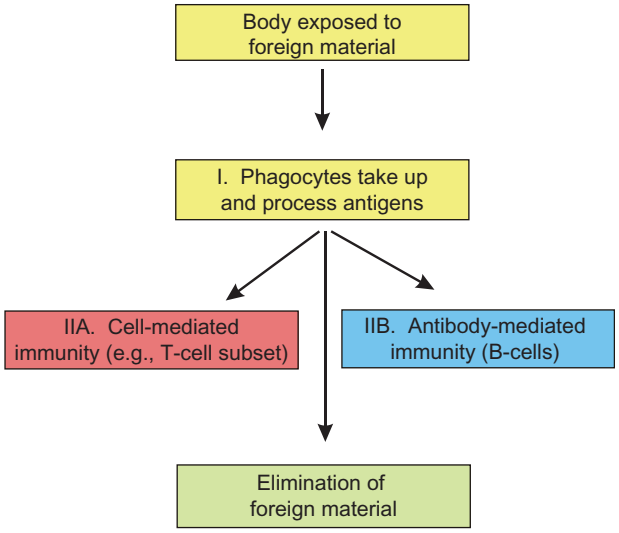


FIGURE 12.1 Flow chart showing the two major immune response systems that comprise the host response to foreign materials. Together these two branches of the immune system work together to create immunity. The cell-mediated response is designed to directly attack and destroy cells determined by the body to be nonself. The antibody-mediated response is the branch of the immune system involved in the formation of antibodies.

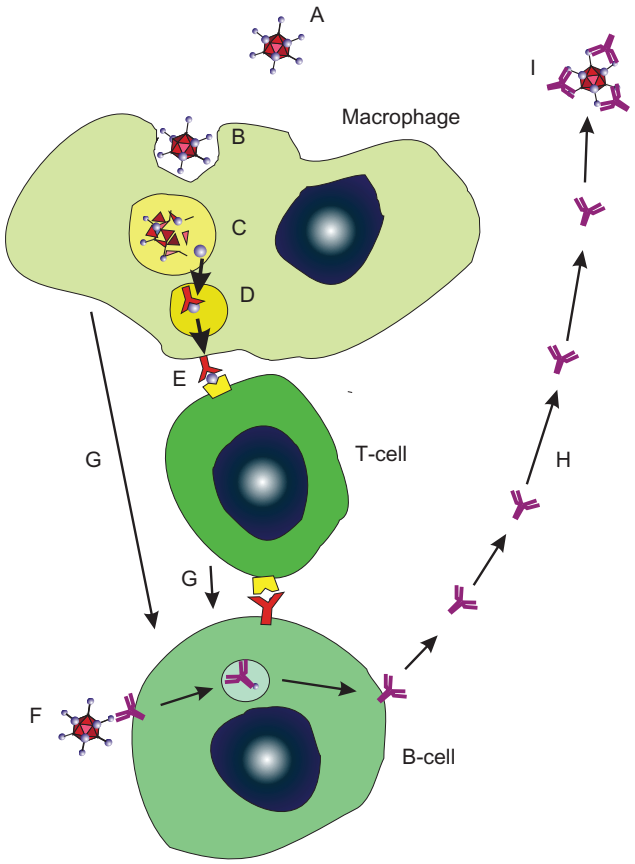


FIGURE 12.2 Schematic representation showing the processes that lead to the formation of antibodies. (A) Foreign material represented here by a virus that has gained access to the body; (B) the virus is phagocytosed by a macrophage; (C) the virus is broken down into subunits by enzymes contained in the phagocytic vacuole; (D) an antigen-presenting molecule escorts antigenic subunits from the virus to the macrophage surface; (E) the antigenic molecule derived from the virus is presented to a T cell; (F) the B cell encounters the virus and expands its antibody production specific to the viral epitope; (G) the macrophage and the T cell release chemicals that stimulate B cells; (H) antibodies are released into circulation; (I) antibodies neutralize the virus in circulation by binding to the viral epitopes inhibiting viral attachment to target cells.

antibodies is given, with respect to the structure of antibodies, the various classes of antibodies and the interaction of antibodies with antigens. Following this introduction, we discuss several of the basic immunological methodologies or immunoassays that are widely used in environmental microbiology. These immunoassays include fluorescent immunolabeling, enzyme-linked immunosorbent assay (ELISA), magnetic bead antigen capture, Western immunoblotting, immunoaffinity chromatography, immunoprecipitation and lateral flow immunoassay. Finally, in order to provide perspective and illustrate how these immunoassays and immunosensors can be used in the field of environmental microbiology, an example of each immunoassay and immunosensor is provided in relation to current research topics such as bioremediation and pathogen detection.

12.2 WHAT IS AN ANTIBODY?

Antibodies, also known as immunoglobulins, are produced by the immune system of higher life forms that help defend the host against foreign invasion. When a host is challenged by an antigen such as bacteria or viruses, the first response of certain host immune cells called macrophages is to engulf these invaders and process them biochemically. This biochemical processing essentially creates a blueprint that is used for the development of an immune response that results in the production of antibodies (Figure 12.2). The unique feature of antibodies produced in response to an antigen is that they are synthesized in such a way that they are highly specific for that antigen. Thus, they can chemically interact and bind only with that particular antigen, neutralize it, and/or aid in its destruction and removal from the body.

There are five different classes of immunoglobulins (Igs): IgA, IgD, IgE, IgG and IgM. These immunoglobulins differ in many ways including their overall structures (Figure 12.3). The most common type of antibody used for immunoassays is the IgG class of immunoglobulins (Figure 12.4). IgG antibodies are Y-shaped proteins composed of four peptide chains that are joined together by disulfide linkages. There are two major structural fragments or regions of IgGs called the Fc and Fab regions. The antigen-binding fragments (Fabs) of the IgG immunoglobulins are the two identical regions at the top of the molecule, which, as indicated,

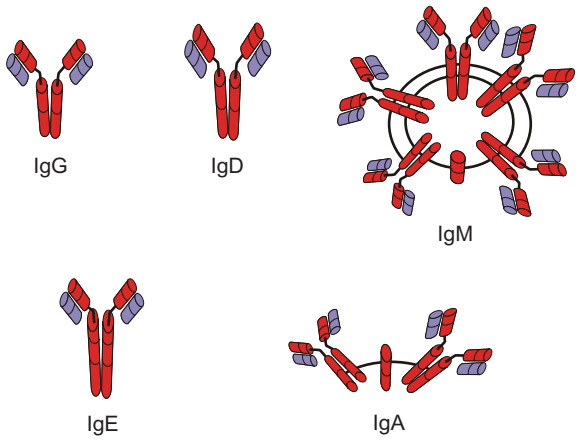


FIGURE 12.3 Schematic representation of the five classes of immunoglobulins. IgG (molecular weight 146,000), IgD (184,000) and IgE (188,000)) are all comprised of one basic subunit. IgG is the major immunoglobulin involved in the humeral response. IgM, which consists of five subunits (970,000), is the second major antibody involved in the humeral response. IgA (385,000) is commonly found in body secretions such as saliva, milk and intestinal fluid. IgD is found in low concentrations in plasma, and IgE is involved in allergic reactions. IgG and IgM are the antibody classes most commonly utilized in immunoassays.

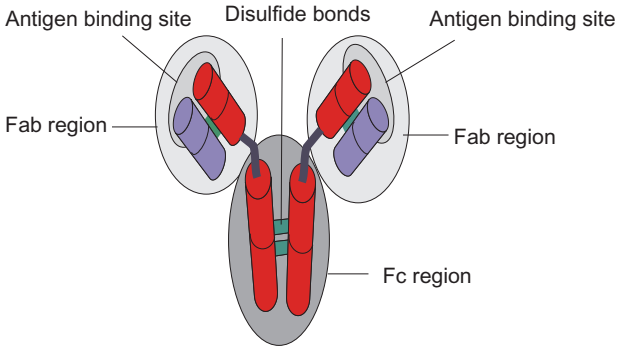


FIGURE 12.4 Schematic representation of an IgG antibody, showing the various regions associated with the antigen–antibody interaction. There are two antigen-binding fragments (Fab), which interact with the antigen. There is also one crystal fragment (Fc), which is the part of the antibody recognized by the host immune system as self. There are two light chains (in purple) joined to two heavy chains (red/orange) by disulfide bonds, and the two heavy chains are in turn joined to each other in a similar fashion.

are the sites of antibody–antigen interaction. The Fc region is the tail of the antibody, and is the fragment that is recognized by the host as "self." Because antibodies are relatively large proteins they can also act as antigens, so the ability to recognize a particular antibody as being "self" prevents the host from responding against its own antibodies. In addition to the major classes of antibodies, minor differences in the protein structure result in subclasses. Variance among different subclasses is less than the variance among different classes. For example, all rabbits and humans have the same classes of antibodies such as IgG or IgM, but do not have the same subclasses such as IgG1 or IgG2, or the same host recognition sites on the immunoglobulins. This species-specific difference makes the immune system of one species recognize another species' antibodies as being foreign. For example, if you immunize a rabbit (purposefully expose it to a foreign antigen) with a human's antibodies, the rabbit will produce different antibodies against them. Antibodies produced in response to another individual's antibodies are termed antiglobulins.

12.2.1 Antibody Diversity

B cells belong to a group of white blood cells or lymphocytes, and are responsible for antibody diversity through the production of a wide variety of antibodies that can interact with a diverse range of antigens. In fact, the vast populations of B cells have been estimated to have the potential to produce up to 1×10^{10} structurally different IgG antibodies, which in theory could recognize 1×10^{10} different antigens. This enormous diversity has been exploited in the field of environmental microbiology. An animal's immune system can adapt and produce an immune response against many different antigens. Therefore, essentially any bacterium, virus, protein or pollutant that can stimulate an immune response can be used as an antigen to produce specific antibodies. These antibodies can then be used to design immunoassays to aid in the study of that particular bacterium, virus or toxic pollutant.

12.2.2 Antibody Specificity

Specificity for a particular antigen is one characteristic that makes immunology-based methodologies such valuable tools. In essence, once they are produced, antibodies are very precise in their recognition of the particular antigen. This discrimination is based on the molecular structure of the antigen-binding sites located on the Fab portion of the antibody, and on the epitopes or chemically reactive sites of the antigen (Figure 12.5). Antigen–antibody binding is the result of specific chemical interactions (i.e., charge–charge, dipole–dipole, hydrogen bonding and van der Waals) that occur between the antigen and amino acid residues of the antibody that are located in the Fab region. This reaction is so specific that even a small change, such as an alteration of one amino acid in the binding site of the antibody, may weaken or nullify the antigen–antibody binding. It should be noted, however, that even with this specificity, there are still instances in which the antibody may react with more than one antigen. This phenomenon in which an antibody reacts with two unrelated epitopes is termed cross-reactivity.

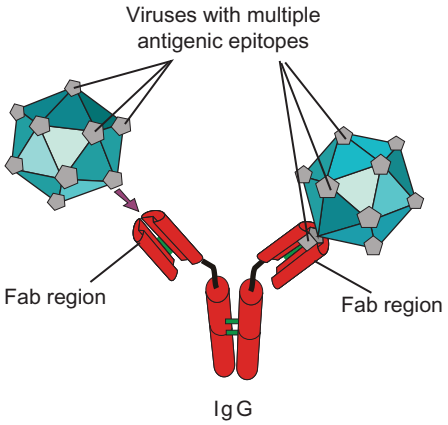


FIGURE 12.5 Schematic representation of the antigen–antibody interaction. This figure shows an IgG antibody binding to an enterovirus. Note that the Fab regions of the antibody are chemically formed to fit perfectly with the antigenic epitopes of the virus.

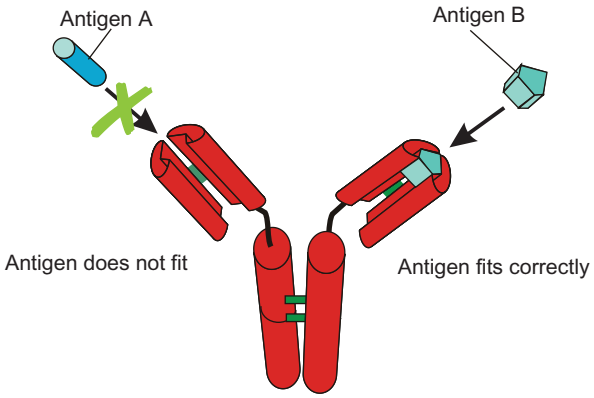


FIGURE 12.6 Schematic representation of an IgG antibody interacting with two different antigens. In the first case, antigen A does not have the correct biochemical conformation so the antibody is unable to interact and bind it. Antigen B has the correct conformation and the antibody is able to interact and bind this antigen tightly.

12.2.3 Antibody Affinity

Affinity is defined as the attraction between an antibody and an antigen. More specifically, affinity is a measure of the strength of this interaction, and is usually expressed as an interaction or association constant. Quantitatively, affinity is the sum of the chemical bonds that form between the antigen and the antibody. These are usually relatively weak interactions such as hydrophobic interactions and hydrogen bonds. Even though individually such chemical bonds are relatively weak, collectively they form very strong and tight interactions. Thus, the strongest binding occurs between epitopes and antibodies only when their shapes are complementary (Figure 12.6). Affinity can be described as the reversible formation of the antigen (Ag)–antibody (Ab) complex by the equation:

$$Ag + Ab \rightleftharpoons AgAb \qquad \text{(Eq. 12.1)}$$

The affinity constant (K) can thus be determined by the mass balance equation, which is expressed in the form:

$$K = [AgAb]/[Ag][Ab] \qquad \text{(Eq. 12.2)}$$

where:

- [AgAb] is the concentration of the antigen–antibody complex at equilibrium
- [Ag] is the concentration of free antigen-binding sites at equilibrium
- [Ab] is the concentration of free antibody binding sites at equilibrium, and
- K is the affinity constant or the measure of the strength of the bond formation between Ag and Ab.

Essentially, the higher the K value, the stronger the affinity of an antibody for an antigen. This is a direct consequence of a stronger molecular interaction. This is important in the development of monoclonal antibodies for immunoassays, especially in the selection of the best hybridoma to use for monoclonal antibody production (see Section 12.2.4). The affinity constant not only gives an indication of which of the antibodies might be best for a certain assay, but it can also indicate the concentration of antibody required for the assay, and the potential for cross-reactivity with other antigens.

12.2.4 Polyclonal and Monoclonal Antibodies

In the past, injecting a laboratory animal with the antigen of interest produced particular antibodies needed for environmental and other research purposes. In response to immunization, the animal produces antibodies that can be collected (in serum) directly from the blood of the animal (Figure 12.7). These blood-derived antibodies are termed polyclonal antibodies because they are not derived from a single B lymphocyte or its progeny, but are instead a product of many different B cells binding in slightly different ways to the same antigen. As a result, this method yields a mixed product that contains a wide variety of antibodies.

To avoid problems associated with use of such antibody mixtures, scientists developed the technology to produce monoclonal antibodies. A monoclonal antibody is an antibody that is the product of a single B cell clone. Production of monoclonal antibodies involves the *in vitro* combination of two types of cells. The first type of cell is a B cell that produces a single, unique antibody. The second type of cell is an immortalized myeloma cell: a cancer cell that is able to thrive and multiply *in vitro*. The specific antibody-producing B cell is fused with the myeloma cell to form a hybrid cell called a hybridoma. This hybridoma

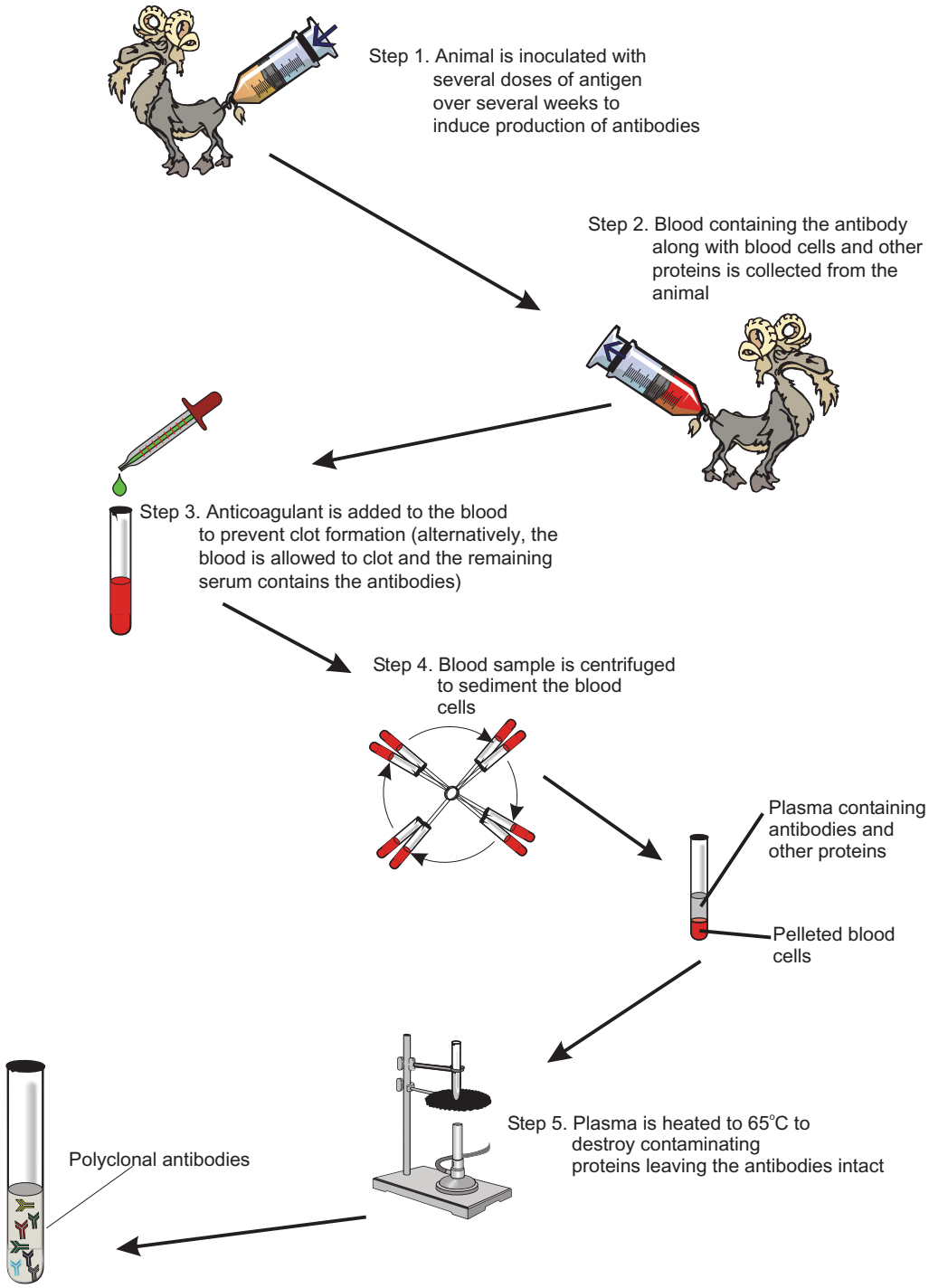


FIGURE 12.7 General outline of steps used to produce polyclonal antibodies. The primary step is the immunization of the animal and the collection of the blood containing the antibodies. Following collection, the blood cells are separated by centrifugation and heating denatures other proteins such as complement. This results in a stable suspension of antibodies that can be used in many types of immunoassays.

combines the characteristic of "immortality" with the ability to produce the desired specific antibody in high concentrations and in pure form. The procedure for the production of monoclonal antibodies is schematically diagrammed in Figure 12.8. As a result of the development of monoclonal antibody technology, scientists are able to produce large amounts of pure and highly specific antibodies.

What are the advantages and disadvantages of using polyclonal or monoclonal antibodies? In general, monoclonal antibodies have higher specificity and lower cross-reactivity

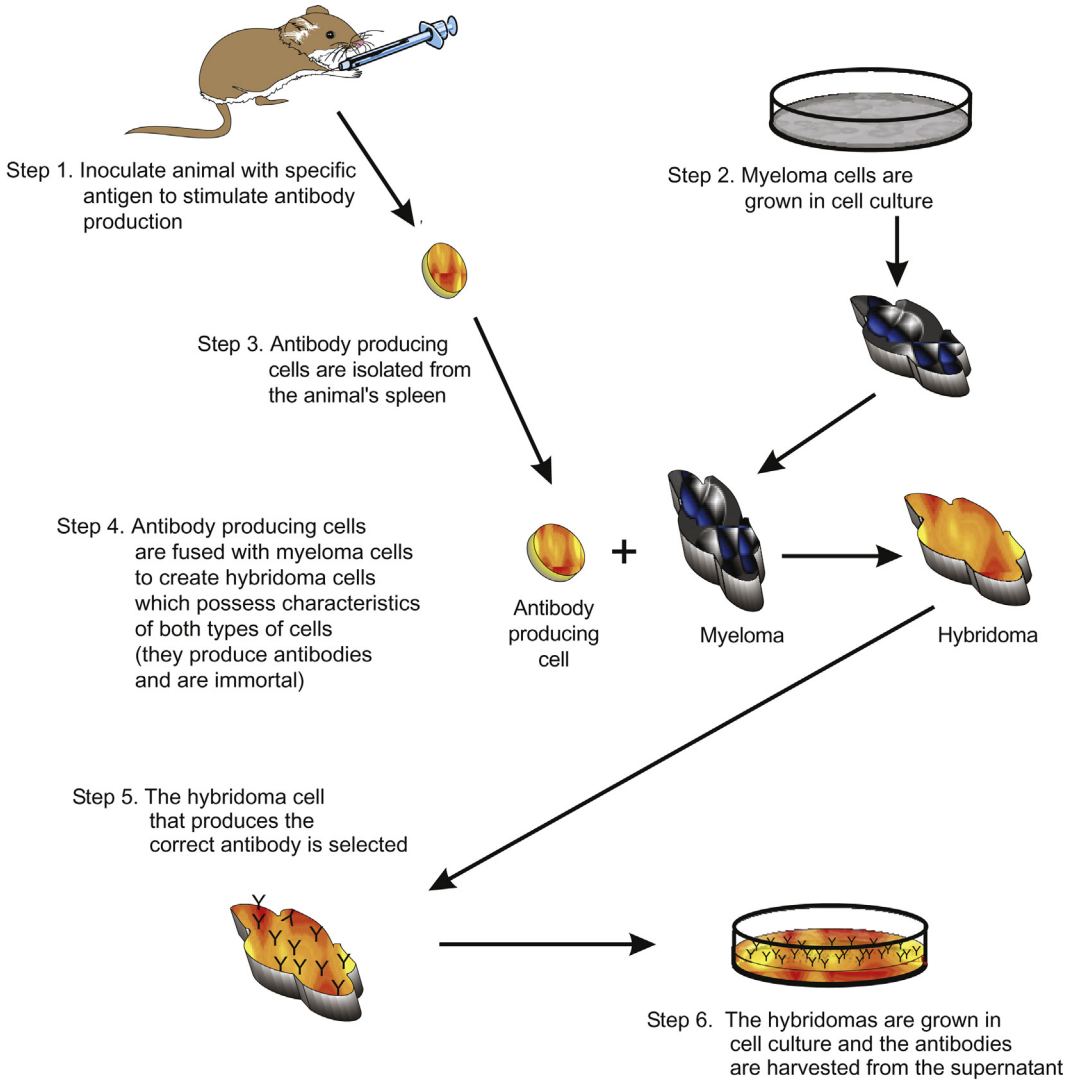


FIGURE 12.8 General outline of the steps used to produce monoclonal antibodies. Essentially, the spleen cells from the immunized mouse are removed and combined with myeloma cells using polyethylene glycol to fuse the two. After this the cells that produce the specific antibody needed are selected and cultured in order to produce large quantities of highly purified monoclonal antibody.

than polyclonal antibodies. They can also be produced indefinitely and in relatively large concentrations (>13 mg/ml). Polyclonal antibodies, on the other hand, can be produced more rapidly and much less expensively because they are prepared directly from the serum of immunized animals. Thus, polyclonal and monoclonal antibodies each have benefits and drawbacks that must be considered during the design of any immunoassay.

12.2.5 Antiglobulins

Antiglobulins, as indicated earlier, are antibodies that are specific (usually targeting the Fc portion) for another foreign antibody. Usually, antiglobulins are developed to recognize a whole antibody class for a specific organism, e.g., a mouse. The antiglobulins can then be attached to a signal molecule, and used as secondary or indirect detection molecules to detect any mouse antibody used in an immunoassay (see Section 12.3). Because antibodies are large proteins with complex structures, they have the potential to be seen as antigens if they do not have the "self" recognition sites common to the host that produced the antibody. For example, an antiglobulin can be raised in a goat by immunizing the goat with mouse IgG antibodies (Figure 12.9). Because the goat does not recognize the mouse antibody as self, it produces its own set of antibodies against the mouse antibodies. Thus, the antibodies produced by the goat are specific for the mouse-derived IgG antibody. Using monoclonal methods for production

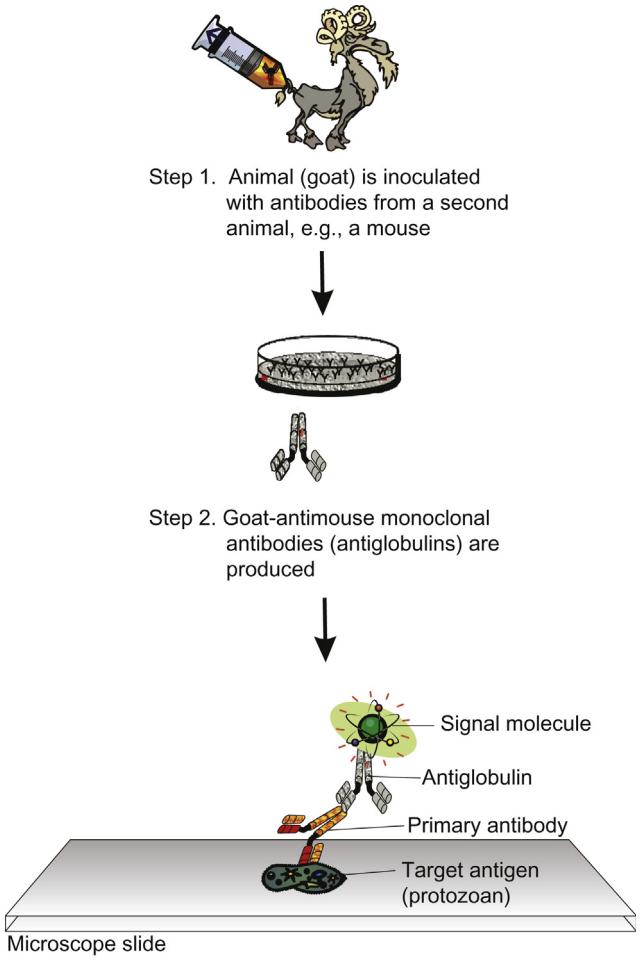


FIGURE 12.9 A general outline of the steps involved in the production of monoclonal antiglobulins. A goat is inoculated with the mouse antibodies. The goat produces antibodies against the mouse antibody. These goat anti-mouse monoclonal antibodies are then cloned and used as secondary labeling antibodies. In this case, a mouse antibody has been made to detect a protozoal target antigen. The labeled goat anti-mouse monoclonal antiglobulin is then used to detect the protozoan–mouse antibody complex.

of specific antibodies, goat antibodies can be derived that are specific against all mouse IgGs, or even against specific IgG subclasses, for example IgG2a.

12.3 IMMUNOASSAYS

Immunoassays are analytical methods used for the detection and/or quantitation of the antigen–antibody interaction. For the most part, the types of immunoassays used in environmental microbiology are based on quantitation or detection of antigens as opposed to the characterization of the antigens. That is, we are usually interested in using immunoassays to determine how much antigen is in an environmental sample, and not in characterizing an antibody–antigen interaction, or the role of the antigen in disease or in the immune response. However, in order to quantitate or detect the antigen there must be a way to visualize the antigen–antibody interaction. This visual signal is produced by the attachment of specific signal molecules to the antibodies or antiglobulins used to detect the antigen within an environmental sample.

For almost all types of immunoassays, attachment of a signal molecule to the antibody and/or antigen is very important. Many types of signal molecules are used in immunoassays, including iodine, enzymes, fluorochromes and radioisotopes. These signal molecules produce a visual signal that allows quantitation of the specific antibody–antigen interaction being investigated. The signal is usually indicated by the production of some type of color change. For example, enzymes such as horseradish peroxidase and alkaline phosphatase act by enzymatically cleaving colorless substrates to produce a colored product. This signal is then detected qualitatively by the naked eye, or quantitatively using an instrument such as a spectrophotometer. For fluorochrome signal molecules, the antigen–antibody interaction can be detected by exciting the fluor with a particular wavelength of light. The fluor will emit energy (light) at a second wavelength, which can then be detected visually or instrumentally. The fluorescent dye most commonly used as an antibody label is fluorescein isothiocyanate (FITC). Other examples of fluorescing chemicals used in this type of assay include R-phycoerythrin, rhodamine and Texas red. Radioisotopes are quantitated by liquid scintillation counting (see Figure 11.11) or by exposing the sample to a photographic emulsion (X-ray film), which produces a qualitative signal on the film that can be visualized as a dark spot.

Attachment of an antibody or antigen to a signal molecule is an almost universal way to allow for visual detection of the antigen via an immunoassay, which can be achieved via direct or indirect labeling (Figure 12.10). For an immunoassay with direct labeling, the primary antibody (antibody specific for the target) has the signal molecule attached to it allowing for one-step detection. Indirect labeling involves two steps. The first is the attachment of a primary antibody to the target, and the second is the attachment of a secondary (antiglobulin) antibody to the primary antibody. In indirect labeling, it is the secondary antibody which has the signal molecule attached. Both methods work well though both have advantages and disadvantages. With direct labeling, the binding and signal are usually more specific because there is a smaller signal-to-background noise ratio. However, the use of indirect labeling allows for one labeled antibody to be used with many different primary antibodies provided they are all of the same type, meaning that each primary antibody does not

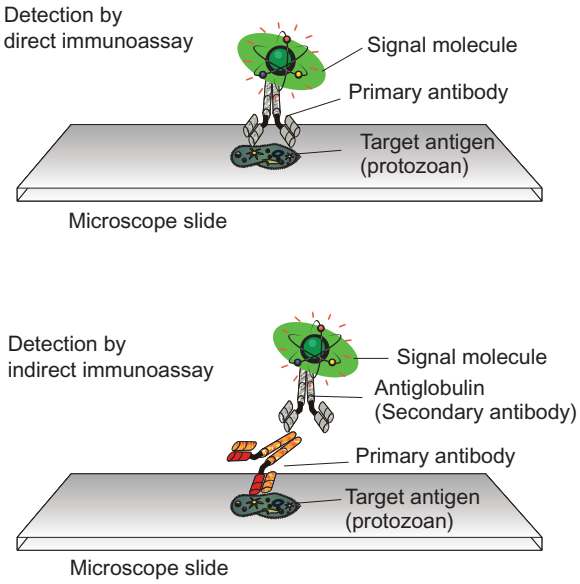


FIGURE 12.10 Direct vs. indirect immunoassay. In the direct assay the primary antibody, also called the detection antibody, is labeled and used to directly detect the antigen. The indirect assay has an extra step. In the first step the primary or detection antibody is bound to the antigen. In the second step a secondary labeled antibody (antiglobulin) is used as the signal molecule. In ELISA the indirect assay is more common because the secondary antibody can be used to detect numerous different primary antibodies and thus the primary antibodies do not have to be individually labeled.

have to be labeled separately. For instance, if you use a mouse to produce monoclonal IgG primary antibodies against four different protozoa (*Microsporidia*, *Giardia*, *Cryptosporidium* and *Entamoeba*), then you can use the same labeled antiglobulin to bind to each of these. Because conjugating signal molecules to antibodies is tedious and often difficult, and because a wide range of antiglobulins conjugated to various signal molecules are available commercially, this is frequently the format of choice. However, if a one-step assay that is slightly more specific is desired, then the use of direct labeling is often preferred.

With this brief introduction to the immunoassays, the following sections provide descriptions of the main types of immunoassays routinely used in environmental microbiology.

12.3.1 Fluorescent Immunolabeling

12.3.1.1 Technique

Fluorescent immunolabeling (immunofluorescence) involves the use of fluorescent signal molecules conjugated to antibodies to interact with and subsequently indicate the presence of a particular antigen by the production of fluorescent light. Detection of the fluorescent signal can be via epifluorescence microscopy as described in Section 9.4.2. Figures 8.10 (protozoa) and 9.13 (rhizobia) provide examples of immunofluorescent microscopy. The basic procedure for immunofluorescence microscopy is to attach the sample antigen to a microscope slide, add a fluorescent chemical/antibody conjugate specific to the antigen and view the sample under a microscope equipped with a fluorescent light source. When viewing fluorescence-labeled samples under the microscope, the labeled antibody-bound antigen appears bright green against a dark background.

12.3.1.2 Application

One of the main current uses for immunofluorescence is to spatially examine the interaction of an antigen of interest with its environment. For example, Rendón *et al.* (2007) used immunofluorescence labeling to study a pilus common to both commensal and pathogenic *E. coli*, and its interaction with human intestinal epithelial cells. Recall that some *E. coli* are commensal or normal inhabitants of the human gut. However, others, such as the enterohemorrhagic *E. coli* strain O157:H7, are highly pathogenic (Section 22.2.2). Thus, it is important to understand how different *E. coli* strains (both commensal and pathogenic) interact with the intestine. One technique that this group used to study this question was immunofluorescence (Figure 12.11). In this study, the authors showed that there is an *E. coli* common pilus (ECP) that aids all strains in colonization of the epithelial cells. It is suggested that ECP production by the pathogenic strain O157:H7 helps it to mimic commensal *E. coli* strains which gives it a competitive advantage and allows it to avoid detection and elimination during colonization of the human host.

12.3.1.3 Advantages and Disadvantages

One of the primary advantages of fluorescent antibody-based techniques over other detection methodologies is the ease of use. Individuals with a minimal amount of training can perform immunofluorescence assays. Another obvious advantage is that results can be obtained usually within a few hours. In many cases, immunofluorescence is also highly specific and sensitive. On the other hand, there are several problems that must be considered when performing immunofluorescence assays. One serious disadvantage is the potential for cross-reaction between the antibody and nontarget antigens. Such cross-reaction can provide false-positive results using immunofluorescence assays. False-negative results are also possible. This could occur if antigenic epitopes on a target cell were damaged or genetically altered, thus preventing antibody recognition, binding and subsequent detection.

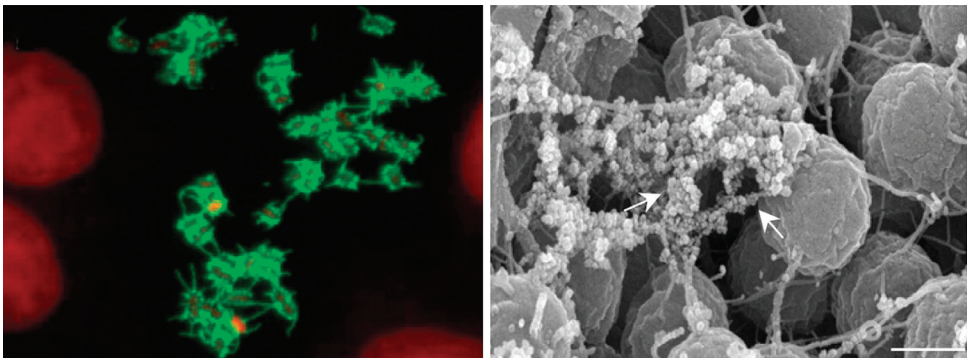


FIGURE 12.11 (Left panel) Immunofluorescent microscopy of the *E. coli* common pili (ECP) which are depicted as green fluorescent structures that mediate the interaction between *E. coli* cells (stained red with propidium iodide). The *E. coli* cells in this sample have adhered to a model epithelial cell. In this case the sample was incubated first with anti-ECP antibody (created in a rabbit) and then with a goat anti-rabbit IgG fluor conjugate. (Right panel) For comparison, we also show an immuno-scanning electron micrograph of the ECP. In this case, the samples were incubated with anti-ECP antibodies followed by incubation with anti-rabbit IgG conjugated to 30 nm gold particles. The white arrows show the antibody−antiglobulin−gold particle complex covering the ECP. (Scale bar: 0.5 μm.) Courtesy Rendón *et al.* (2007).

12.3.2 Enzyme-Linked Immunosorbent Assays

12.3.2.1 Technique

The enzyme-linked immunosorbent assay (ELISA) is a very sensitive method used to detect the presence of antigens or antibodies of interest in a sample. ELISA is typically performed using one of two detection methods: direct or indirect (Figure 12.10), but can also be performed as a competitive assay (see Section 12.3.3). In direct ELISA an enzyme-linked (labeled) antibody is used to directly detect the captured antigen or antibody of interest. In the more common indirect ELISA, a primary antibody is bound to the sample antigen−antibody, and then a secondary labeled antibody (antiglobulin) is used to detect the primary antibody. For any ELISA procedure, the sample antigens−antibodies of interest are concentrated (if necessary) and solubilized in an appropriate buffer. An outline of the steps for an indirect ELISA used to detect an antigen and an antibody is shown in Figure 12.12. For example, for detection of antigen, also called a sandwich ELISA, a capture antibody is attached to a microtiter plate, microcentrifuge tube or other solid support. The antigen is then added and allowed to incubate in order to bind with the antibody (antigen capture). After the antigen is bound, a second detection antibody is added. Usually, the detection antibody recognizes a different antigenic determinant than the capture antibody. Finally, a secondary labeled antibody (antiglobulin) is added which binds to the detection antibody. The secondary antibody is conjugated to a signal molecule, for example, the enzyme alkaline phosphatase. Substrate is then added that causes a color change in response to the presence of the signal molecule. This color change is usually in proportion to the amount of antigen present; thus, the assay becomes quantitative. This makes it possible to quantify the amount of antigen present in a given sample. Once a signal is produced it can be used to visually score the results based on the color change or an automated plate reader can be used. Plate readers provide highly sensitive detection of low-level signals and can determine accurately the strength of a given signal in comparison with a standard curve.

12.3.2.2 Application

Biofilms are specialized environments where microorganisms are firmly attached to surfaces and to one another by exopolymeric substances. Previously, the enumeration of specific organisms within biofilms was done using cultural counts, most probable number (see Chapter 10) or immunofluorescence microscopy. However, the inefficiency of these methods in biofilms due to cells clumping and exopolysaccharide production can lead to significant underestimation of the actual numbers. ELISA has been used as an alternative method to quantifying biomass within biofilms and even protein production in biofilms (Black *et al.*, 2004; Nguyen *et al.*, 2007). In a study by Bouer-Kreisel *et al.* (1996), ELISA was used to quantify populations of *Dehalospirillum multivorans* (*D. multivorans* are bacteria associated with the biodegradation of organic pollutants) in mixed culture biofilms. A standard curve was developed using ELISA and immunofluorescence microscopy to quantify signal from and enumerate serial dilutions of pure cultures of *D. multivorans*. The standard curve related the amount of signal provided by ELISA to the direct counts provided by microscopic enumeration. The two assays were found to be directly proportional. ELISA was then performed on

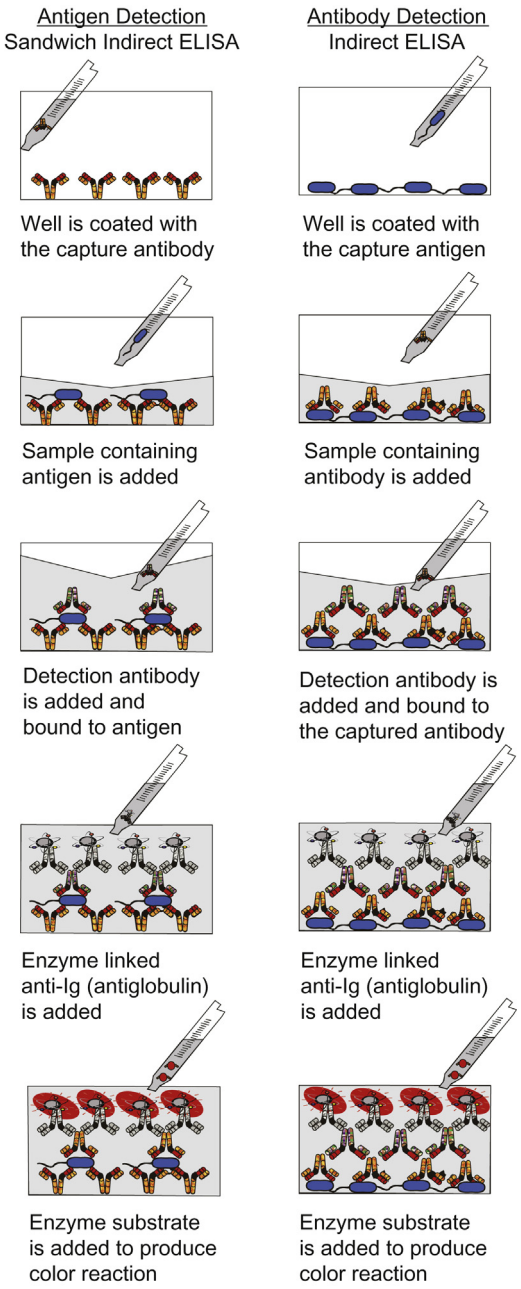


FIGURE 12.12 Schematic representations of direct and indirect ELISA. These reactions are usually carried out in a microtiter plate and the color change shown can be detected and quantitated using a plate reader.

actual biofilms, and the results were used to quantify the total biomass of specific organisms responsible for the degradation of organic pollutants in mixed culture biofilms. Thus, the rates of degradation of these pollutants could be directly correlated with biomass using ELISA methodology.

12.3.2.3 Advantages and Disadvantages

There are many advantages of using ELISA over other detection or quantification methods. ELISA is sensitive and can be quantitative when used in conjunction with standard curves. Disadvantages are similar to all antibody-based methods, and are related to cross-reactivity and nonspecific signal production. ELISAs must also be optimized to provide consistent results especially when using environmental samples. ELISAs, as described above, are also poorly suited for detection of extremely low concentrations of antigens. Other types of ELISAs have been described that are capable of detecting low target concentrations. One of these assays is known as competitive ELISA.

12.3.3 Competitive ELISA

12.3.3.1 Technique

In competitive ELISA, both an enzyme-linked (labeled) control antigen and a sample containing an unknown quantity of unlabeled antigen are added to a sample well coated with antibody (Figure 12.13). In this assay, the sample is added first and the bound antibody captures the unlabeled antigen. The labeled antigen is then added, and a reversible equilibrium is established between the amount of labeled control antigen and sample antigen bound to the antibody depending on their relative concentrations. If the ratio of labeled control antigen to sample antigen is high then the signal will be maximized. If the ratio of labeled control antigen to sample antigen is low, then the signal will be weak. Because the standard curve in a competitive ELISA exhibits the maximum signal at the lowest concentrations of sample antigen, this assay is very sensitive.

12.3.3.2 Application

Various immunoassay test kits based on the competitive ELISA are commercially available for measurement of organic contaminants such as pesticides, petroleum and PCBs in environmental samples (Neilson and Maier, 2001). These kits are approved for use by the U.S. Environmental Protection Agency for detection and monitoring of these contaminants. Several groups have also worked on development of monoclonal antibodies for detection of various metals. Metal detection is problematic due to the small size of the metal atom which does not illicit an immune response and so antibodies are not produced (Neilson and Maier, 2001). This problem has been overcome by using a metal–chelator complex to produce specific monoclonal antibodies (Zhu *et al.*, 2007).

Commercial kits use different approaches to trap the contaminant antigen including antibody-coated tubes, a series of filtering steps or magnetic particles (see Section 12.3.4). Various immunoassay kits have compared favorably in terms of sensitivity with traditional analytical approaches for a variety of organic contaminants including pesticides such as chlorfenapyr (Watanabe *et al.*, 2005) and polychlorinated

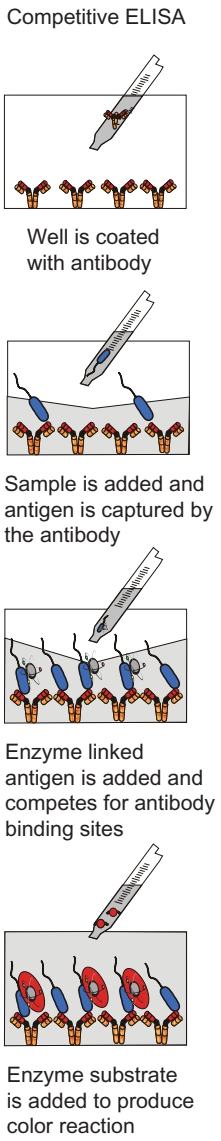


FIGURE 12.13 A schematic representation of the procedure used for competitive ELISA. This variation of a typical ELISA shown in Figure 12.12 can be used to detect very low concentrations of antigen.

biphenyls (PCBs) (Schreiber and Pedersen, 1996) with detection limits ranging from high μg per liter (parts per billion) to low mg per liter (parts per million). For example, Schreiber and Pedersen (1996) compared the use of a commercial immunoassay test kit with traditional gas chromatography-mass spectrometer (GC-MS) analysis for monitoring 161 soil, sediment and waste samples in a PCB-contaminated site. Results showed that in comparison to the GC analysis, the immunoassay had 3.1% false positives and 11.2% false negatives out of 161 samples.

12.3.3.3 Advantages and Disadvantages

The advantage of competitive ELISA lies in its ability to detect extremely low antigen concentrations. This is related to the inverse relationship between target concentration and signal strength described previously. In some cases, such as for highly contaminated samples, these assays are so sensitive that extensive dilution may be required, thereby introducing potential for erroneous analysis. Other advantages include ease and rapidity of analysis, portability, the ability to operate at remote locations and a reduced cost compared to conventional measurement. Perhaps the biggest problem associated with immunoassays is related to their selectivity for a contaminant. This is because the antibody can cross-react with molecules that have a structure similar to the contaminant. Immunoassay of polyaromatic hydrocarbons (PAHs) illustrates this point well. A PAH-specific antibody will react to some extent with many different PAH molecules causing potential overestimation of the amount of contaminant present. This was illustrated by Barcelo *et al.* (1998), who showed that immunoassay results overestimated PAH concentration in river water samples by approximately one order of magnitude in comparison to GC-MS analysis. When the contaminant has a more unique structure (as do many of the pesticides), the problem of overestimation is reduced.

12.3.4 Immunomagnetic Separation Assays

12.3.4.1 Technique

Magnetic immunoseparation is an antigen capture methodology that uses antibodies conjugated to paramagnetic beads to attach to, concentrate and purify antigens. Immunomagnetic separation, especially with the development of immunomagnetic nanoparticles, allows for the specific manipulation of microorganisms, proteins and nucleic acids. In its simplest form, an immunomagnetic separation is accomplished with antibody-coated magnetite beads and a magnet. Essentially, the antibodies coated on the magnetic beads bind with antigens in solution, and are then separated from the solution using a magnet (Figure 12.14). The beads used for magnetic separation are small, ranging from 75 nm to 20 μm, and are typically made of iron oxide (magnetite). Such particles react strongly in a magnetic field but do not retain any magnetism when the magnetic field is removed.

12.3.4.2 Application

Immunomagnetic separation was used to recover thermophilic sulfate-reducing bacteria from oil field waters below oil production platforms in the North Sea (Christensen *et al.*, 1992). These bacteria can proliferate in oil field waters and cause considerable problems for oil companies during oil recovery, so the ability to detect their presence is of great value to the petroleum industry. In this example,

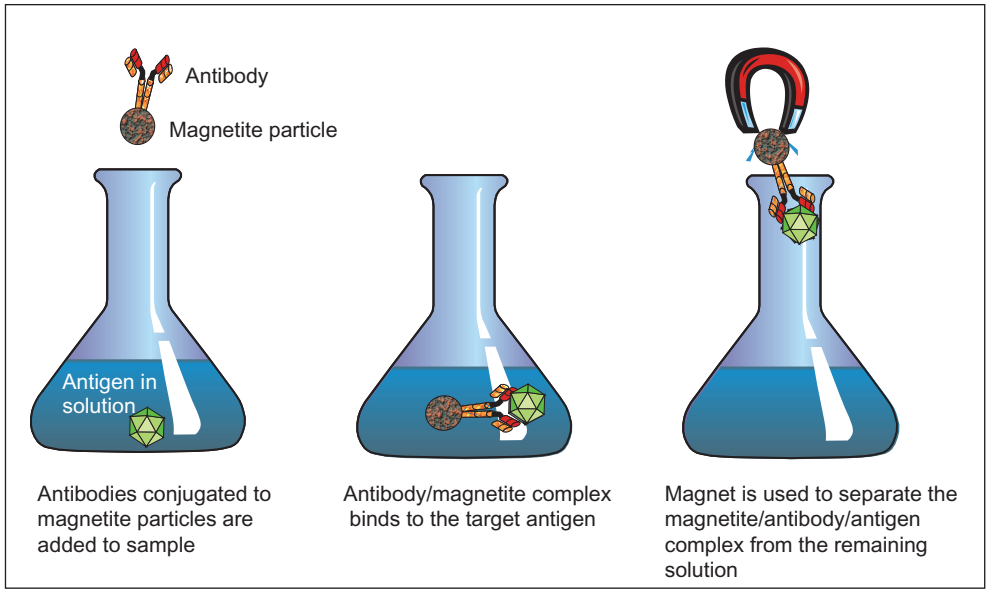


FIGURE 12.14 This is a schematic representation of the principle of immunomagnetic separation. In the first flask is a solution containing the antigen (virus). Antibodies specific to the virus are added to this solution. These antibodies, which have been conjugated (attached chemically) to a paramagnetic particle such as magnetite, then bind the virus. When a magnet is applied to the solution the magnetic particle is attracted to the magnet along with the attached antigen (virus). Immunomagnetic separation is a valuable tool for concentrating and purifying antigens from complex solutions.

immunobeads specific against cell wall antigens of the thermophilic *Thermodesulfobacterium mobile* captured several different isolates from oil-containing strata. Only one of the bacteria isolated by this method was serologically and morphologically identical to the bacterium (*T. mobile*) for which the antibodies were designed. Two other species of bacteria isolated using the immunomagnetic beads were spore forming and similar to *Desulfotomaculum* sp., a sulfate-reducing bacterium that had previously been isolated from oil fields. However, Western blots (see Section 12.3.5) of whole cells showed that the isolates were serologically different from *Desulfotomaculum* sp. This is a good illustration of the fact that an antibody that is designed against one organism can cross-react with other organisms that are serologically different.

Rapid detection of pathogens in food, water and air is one area of current research focus. Yang *et al.* (2007) used immunomagnetic nanoparticles in combination with real-time PCR (Information Box 13.7) to achieve very sensitive detection of the pathogen *Listeria monocytogenes* in milk. With this combined technique, they achieved a sensitivity of 226 CFU per 0.5 ml of milk, which is lower than for most other methods currently available.

12.3.4.3 Advantages and Disadvantages

Advantages of immunomagnetic separation techniques include relatively efficient separation of the target antigen, low cost and potential for automation. In most cases, immunomagnetic separation is also one of the easiest methods available for specific target isolation. Though cross-reactivity issues are a concern, as with any antibody-oriented methodology, in most cases the problems associated with nonspecific signal production can be overcome. As with any microbiology protocol such immunomagnetic separations require optimization and proper choice of format (i.e., microcentrifuge separation or column matrix separation).

12.3.5 Western Immunoblotting Assays

12.3.5.1 Technique

Western immunoblotting is a three step, binding assay used to identify the presence of target antigens in a complex mixture of many other nontarget antigens such as might be found in environmental samples. This assay can be done with simple dot blot hybridization with a labeled antibody, or an electrophoretic separation followed by hybridization with the labeled antibody. In dot blot hybridization, environmental samples are added directly to an immobilizing nitrocellulose membrane, followed by immunolabeling and signal detection. In the second technique, a sample of antigen is added to a gel and separated by size using electrophoresis. After electrophoretic separation, the sample is transferred to an immobilizing

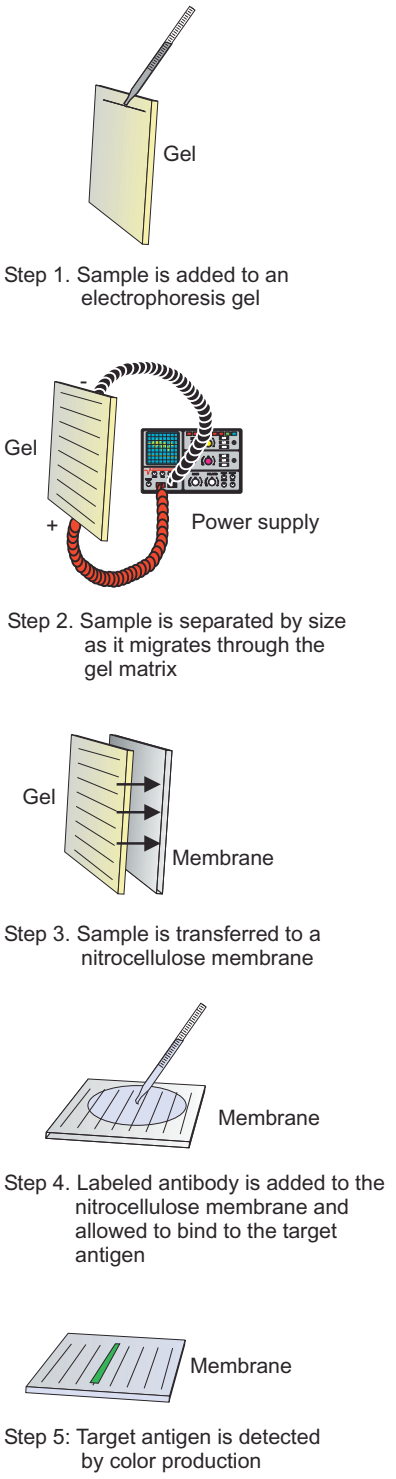


FIGURE 12.15 A schematic representation showing the basic steps involved in Western blot immunoassay.

nitrocellulose membrane. This membrane is then incubated with enzyme-labeled or radiolabeled antibodies that specifically bind to the antigen. After incubation, a substrate for enzyme-labeled or photographic film for radiolabeled substrate is used to detect the presence of the target antigen. Either method (dot blot or electrophoretic separation) indicates the presence and relative quantity of an antigen. If a separation step is used, this also allows molecular size determination of the antigen, which aids in confirming its identity. A schematic diagram of the electrophoretic separation and detection process is shown in Figure 12.15.

12.3.5.2 Application

Methylosinus trichosporium OB3b is a methanotrophic bacterium that has been studied for bioremediation of trichloroethylene (TCE). TCE is a common environmental pollutant (see Section 16.2.3.3). The first step in the degradation pathway of TCE is the enzymatic cleavage of TCE by an enzyme called methane monooxygenase (MMO). Because little was known about the environmental factors that influence the rates of TCE degradation by *M. trichosporium*, studies were undertaken to optimize the cellular expression of the enzyme (MMO). One study used Western blotting to determine the amounts of MMO produced by *M. trichosporium* (Fitch *et al*., 1993, 1996). The amount of signal produced by the Western blot analysis was compared with a standard curve. The maximum amount of signal was then used as an index of optimized MMO expression conditions, which could then be correlated with optimal rates of TCE degradation. A more recent study used Western blotting to help examine the expression of a cadmium binding protein that was engineered into two TCE degraders to help make the degraders tolerate the presence of toxic levels of cadmium (Lee *et al*., 2006b). Results showed that the two engineered strains (*Pseudomonas* and *Rhizobium*) recovered the ability to degrade TCE in the presence of cadmium when they expressed the cadmium-binding protein.

12.3.5.3 Advantages and Disadvantages

The obvious advantage of immunoblotting is its ability to specifically detect a particular antigen (target) within a heterogeneous matrix. Further, immunoblotting can detect extremely low levels of target antigen. However, this procedure is not quantitative, and it is subject to the problems inherent to all antibody-based methods.

12.3.6 Immunoaffinity Chromatography Assays

12.3.6.1 Technique

Affinity chromatography is a very powerful method used in purification and concentration of antigens. In affinity chromatography, the antibody is chemically bound to an inert support matrix (usually a glass, latex or plastic bead)

in a chromatography column. The sample containing the antigen is eluted through the column, and the antigen is selectively retained within the column while the sample passes through. After the sample is run through the column, the purified antigen is eluted, usually by changing the pH of the column, which causes the antigen to detach from the antibody. The antigen can then pass out of the column and be collected in highly purified form (Figure 12.16). This process provides a very efficient means of both concentration and purification. Immunoaffinity chromatography offers several advantages compared with conventional purification techniques. Not only is the process selective and efficient, but it also enables the processing of large-volume samples with relatively few steps.

12.3.6.2 Application

In terms of application, there is currently a virtual explosion in the development of commercial immunochromatographic assays for various pathogens including protozoa (*Giardia*, *Cryptosporidium*), bacteria (*Yersinia pestis*, *Listeria*) and viruses (parvovirus, rotavirus) that allow rapid screening for these organisms (e.g., Magi *et al.*, 2006; Garcia and Garcia, 2006). These assays are done in cartridges or on test strips.

Another example is the use of immunochromatography for recovery of high value enzymes. Pyranose oxidase is an enzyme made by many types of fungi. It catalyzes the oxidation of D-glucose in the presence of oxygen to form 2-dehydro-D-glucose and hydrogen peroxide (H_2O_2). These types of enzymes are important in many industrial processes to aid in catalysis of reactions that are used to synthesize different carbohydrate products. Schafer *et al.* (1996) described the use of immunoaffinity chromatography for the highly efficient purification of a pyranose oxidase produced by *Phlebiopsus gigantea*. They used antibodies specific for other pyranose oxidases to construct an immunoaffinity column to purify this enzyme from mycelial extracts. The researchers were able to get yields of 71% of highly purified enzyme. Thus, immunoaffinity chromatography is one of the most powerful methodologies for the isolation, purification and concentration of antigens including active enzymes from complex samples.

12.3.6.3 Advantages and Disadvantages

Immunoaffinity chromatography for pathogens is rapid and available at commercial scale. One concern with these assays is that they produce false-positive results. Immunoaffinity chromatography for the purification of high value enzymes is rapid and efficient, but some problems encountered with this technique include a limited degree of sample concentration, and the possibility for column clogging if the sample applied is too turbid.

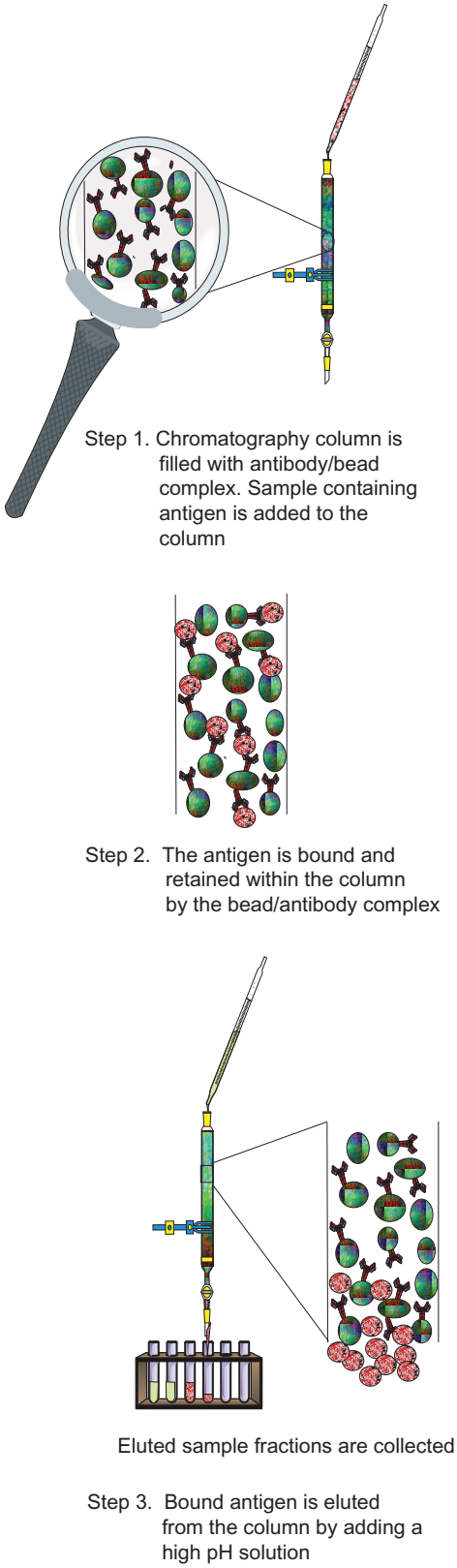


FIGURE 12.16 A schematic representation showing the basic steps involved in antibody-mediated chromatographic separation.

Sample concentration is problematic because usually several column volumes are required for target elution after purification. Further, the column matrix can often retain the target even after elution.

12.3.7 Immunocytochemical Assays

12.3.7.1 Technique

An immunocytochemical assay is used for the detection and determination of the cellular localization of target antigens. The purpose of an immunocytochemical assay is to determine where target antigens are localized within a particular cell. For instance, you can determine whether the target antigen (a particular protein, for example) is localized in the cytoplasm or on the cell surface. In many cases, light microscopes are used to determine the location of antigens within a eukaryotic cell. However, immunocytochemical assays more often involve the use of electron microscopes to increase resolution and magnification of the area being studied. Many types of antibody labels can be used for electron microscopy, including heavy metals such as colloidal gold, enzymes such as horseradish peroxidase and proteins such as ferritin (Figure 12.11, left panel). These labels are visualized by the electron microscope as electron dense regions (Bozzola and Russell, 1992). The process involved in the preparation of a sample for immunocytological analysis is complicated. The sample is fixed with a preservative to maintain the original localization and antigenicity of the target. The sample is then embedded in plastic, sectioned, placed on an electron microscope grid, immunolabeled, poststained and finally viewed under the electron microscope.

12.3.7.2 Application

An example of the use of immunocytochemical techniques in environmental microbiology is in the study of the parasitism of certain amoebae by *Legionella pneumophila*. *L. pneumophila* is an intracellular parasite and is the causative agent of Legionnaires' disease (Section 22.2.7). This bacterium is found in aquatic environments and has been shown to parasitize and multiply within some protozoa (Declerck *et al*., 2009). The sequence of events in the intracellular infection of the amoeba *Hartmannella vermiformis* by *L. pneumophila* was examined by Kwaik (1996) using an immunocytochemical assay. The goal of this study was to compare the intracellular infection of the amoebae with the infection of human alveolar macrophages that occurs during onset of Legionnaires' pneumonia, to aid in understanding the environmental life cycle of this human pathogen. Specifically, these researchers wanted to determine whether the accumulation of ribosomes and rough endoplasmic reticulum (RER) around amoebic phagosomes containing *L. pneumophila* was similar to that observed in human phagosomes. To accomplish this, monolayers of *H. vermiformis* amoeba cells were infected with *L. pneumophila*. The infected cells were harvested, fixed and embedded in plastic. The samples were then thin sectioned, collected on sample grids and incubated with an antibody specific for Bip, a heat shock protein associated with RER. This was followed by secondary incubation with an antiglobulin labeled with colloidal gold particles (indirect immunoassay), and examination by transmission electron microscopy. The results demonstrated that there is considerable similarity in the ultrastructure of phagosomes containing *L. pneumophila* in both amoeba and humans. Using an immunocytochemical assay, it was also found that similar to the human system, RER-specific protein (Bip) was present in the phagosomal membrane of the amoebae. Further, the localization of the RER and ribosomes in the amoebic phagosome was identical to the localization seen in human macrophage phagosomes. This study helped indicate a possible role of the RER in the protection and growth of the *L. pneumophila* within the phagosomes of both hosts.

12.3.7.3 Advantages and Disadvantages

Immunocytochemical assays used in conjunction with electron microscopy provide high resolution of the spatial interactions in the system being studied. This is not a quantitative technique, but rather one that examines qualitative interactions in the sample being studied. Like all the techniques discussed it is subject to the problems inherent to all antibody-based methods.

12.3.8 Immunoprecipitation Assays

12.3.8.1 Technique

Immunoprecipitation is a methodology that uses the antigen–antibody reaction in solution to semi-quantitatively determine the amount of antigen or antibody in a sample by determining the amount of precipitation or clumping of the antigen–antibody complex. Immunoprecipitation can be used to determine the concentration of low levels of antigen, or can be used to quantify or titer antibodies or antigens. Immunoprecipitation can also be used to determine the optimal concentration ratio for precipitation of an antibody and antigen. Most commonly, a series of reaction tubes are set up, each of which contains a constant titer of antibodies. Antigen is then added in increasing concentration to consecutive tubes. In the initial tubes, where the lowest concentration of antigen has been added, there is no obvious precipitation. As the antigen concentration is increased, the

formation of antigen–antibody complexes increases until a visible precipitate is formed. As the antigen concentration is further increased, it will eventually exceed the concentration of antibody present and the amount of precipitate will decrease again (Figure 12.17).

This may seem strange until one entirely understands the nature of antibodies and antigens. IgG antibodies are considered to be bivalent, whereas antigens are most often multivalent; in other words they have multiple antibody reactive sites or epitopes as shown in Figure 12.18A. This means that antibodies are able to bind at least two antigens at once, and antigens can be bound by more than one antibody at a time. When there is excess antibody (Figure 12.18B), multiple antibodies bind to each antigen and no cross-linking beyond this takes place. In this case, there is no visible precipitation and considerable antibody is still found in solution. When antibody and antigen are present in optimal proportions, antigen–antibody cross-linking is much more extensive forming large complexes (Figure 12.18D). This results in precipitation from solution. As the antigen concentration is increased past this optimal antigen–antibody proportion, smaller and smaller complexes form with only one molecule of antigen, no precipitation occurs and excess antigen is found in the supernatant (Figure 12.18C).

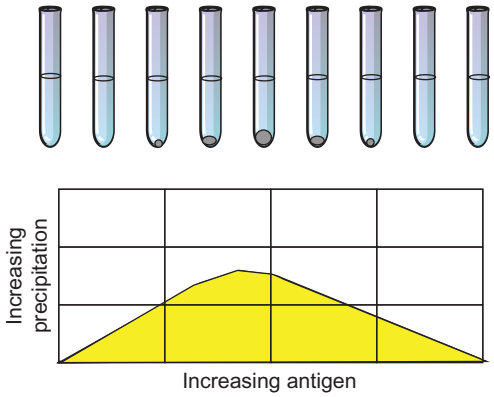


FIGURE 12.17 This is a schematic representation of an immunoprecipitation assay. The test tubes at the top all have constant concentrations of antibody and increasing concentrations of antigen starting from the left. In the middle tubes there is optimum antigen–antibody interaction and precipitation of the antibody and antigen occur. As indicated by the graph on the bottom there is a point where the highest amount of precipitation forms. This type of assay is used to determine optimum antibody to antigen ratios for immunoassays. It is also useful for quantitating either antibody or antigen concentrations in solutions.

12.3.8.2 Application

This technology has been utilized in environmental microbiology to determine the mechanism for the inhibition of certain economically important fungal plant pathogens by another nonpathogenic fungus. It was hypothesized that the fungus *Talaromyces flavus* can control the proliferation of several fungal plant pathogens including *Sclerotinia sclerotionum*, *Rhizoctonia solani* and *Verticillium dahliae* by the production of hydrogen peroxide. *T. flavus* produces hydrogen peroxide as a product of glucose metabolism in the presence of the enzyme glucose oxidase. To determine

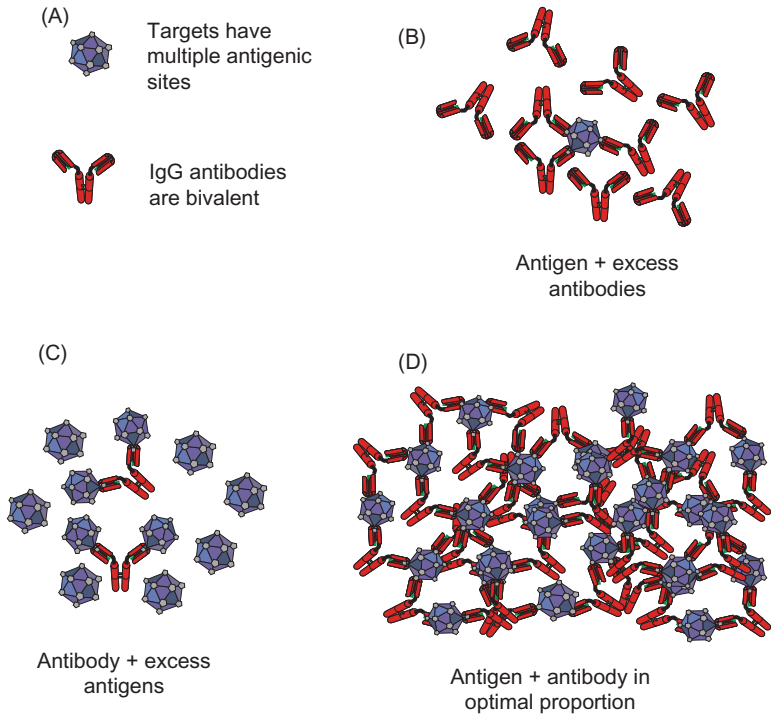


FIGURE 12.18 This is a schematic representation of what occurs in an immunoprecipitation assay. (A) Targets can have multiple antigenic sites. An IgG antibody has two binding sites (bivalent). (B) In this case there is excess antibody so little antibody–antigen binding occurs resulting in no precipitation. (C) In this case there is excess antigen and while antibody–antigen binding occurs, there is little cross-linking and so precipitation does not occur. (D) In this case the amount of antibody and antigen are optimal, there is extensive cross-linking of antibody–antigen complexes and this results in maximum precipitation as shown in Figure 12.17.

whether hydrogen peroxide was responsible for the inhibition, cultures containing the pathogens were incubated with and without glucose oxidase. The cultures without glucose oxidase showed a high percentage of germination, whereas cultures containing glucose oxidase exhibited very low germination (inhibition). To confirm these results, the cultures with glucose oxidase were subjected to immunoprecipitation. Antibody to the glucose oxidase was added to the cultures, effectively removing the glucose oxidase from the culture. After immunoprecipitation of the glucose oxidase, the fungal pathogens recovered and showed high levels of germination. Germination was subsequently halted again when more glucose oxidase was added. This research effectively showed that glucose oxidase is the enzyme produced by *T. flavus* that controls the plant pathogens.

12.3.8.3 Advantages and Disadvantages

In addition to the type of application described, this method is important in the development of immunoassays and the characterization of antigen–antibody interactions. This assay is relatively easy and inexpensive to perform though its use requires careful optimization. Disadvantages vary depending on what type of assay and application is being considered. As with all immunoassays, the possible nonspecific interactions with nontarget antigens are always an issue.

12.3.9 Lateral Flow Immunoassay

12.3.9.1 Technique

Lateral flow immunoassay (LFIA), also known as immunochromatographic assay, is an antibody-based analytical method which uses a prefabricated strip of a carrier membrane containing dry reagents that are activated by applying a fluid sample via capillary action. It can be used as a rapid diagnostic pregnancy test, or to test failure of internal organs (e.g., heart attack or diabetes). Additionally, it can be used to detect contamination of food or water with specific pathogens.

The test strip is composed of a series of capillary zones including a sample pad, conjugate pad, reaction membrane and absorbent pad. These are made of porous polymeric materials, to allow analytes in a sample to travel laterally across the strip and react with reagents on the strip (Figure 12.19A). When a liquid sample is applied onto the sample pad and becomes saturated, the capillary fluid moves to the conjugate pad in which detector regents (target analyte-specific antibody conjugated with label) are stored for immunological recognition of analytes in the sample. Labels are used that generate a visually detectable mark (colored or fluorescent materials), but also allow for an unobstructed flow through the membrane. Labels are generally fluorescent dyes or nanoparticles, colloidal gold particles, magnetic particles, colored latex beads or dyed liposome. Typically, there are at least two lines in the reaction membrane: the test line and control line. The absence or presence of a colored line in the test and control region indicates a negative (absence of analyte in sample), positive (presence of analyte in sample) and test failure (test strip does not work well) (Figure 12.19A). Two predominant approaches to the test are the sandwich and competitive reaction schemes depending on the molecular weight, and the number of antigenic sites for binding target analytes. For analytes with high molecular weight and more than one epitope (antigenic determinant or antigen-binding site), the sandwich format is applicable using two different antibodies to recognize separate epitopes of target analytes, which are utilized for detector reagents and capture reagents (Figure 12.19B). Once detector reagents in the conjugate pad react with target analytes, the mixture migrates to the reaction membrane to react with capture reagents at the test line that involves capturing a target analyte between two layers of antibodies (i.e., detector reagents and capture reagents). This immunological reaction provides a colored line within the test region, which indicates the presence of target analytes in a sample. In addition, detector reagents bind to secondary antibody (anti-immunoglobulin against detector reagents) that are immobilized at the control line, and generate a colored line for test validation. In the sandwich format, the positive response is directly proportional to the concentration of analyte in the sample. When the analyte is of low molecular weight and has only a single antigenic determinant, the competitive format can be utilized by spraying the antigens as capture reagents at the test line to entrap nonreacted detector reagents with target analytes in the sample (Figure 12.19C). Because detector reagents have limited antibody binding sites against target analytes, in occupied sites detector antibodies can bind to capture reagents at the test line and secondary antibodies at the control line. This generates a colored line at the test and control region during sample migration. In the competitive format, the color of the positive mark is inversely correlated to the analyte concentration. After passing these reaction zones, the fluid moves to the absorbent pad, which wicks the liquid to the end of the strip due to capillary force, thus maintaining the flow and acting as a waste container.

12.3.9.2 Application

A lateral flow immunoassay was developed to detect ricin, a cytotoxin found naturally in the seeds of the castor bean *Ricinus communis*, that has been studied for the application of cancer therapy, but has also been considered for potential use as a bioweapon (Shyu *et al.*, 2002). The immunochromatographic assay was based on the sandwich format using two different monoclonal antibodies to recognize the specific binding sites of the

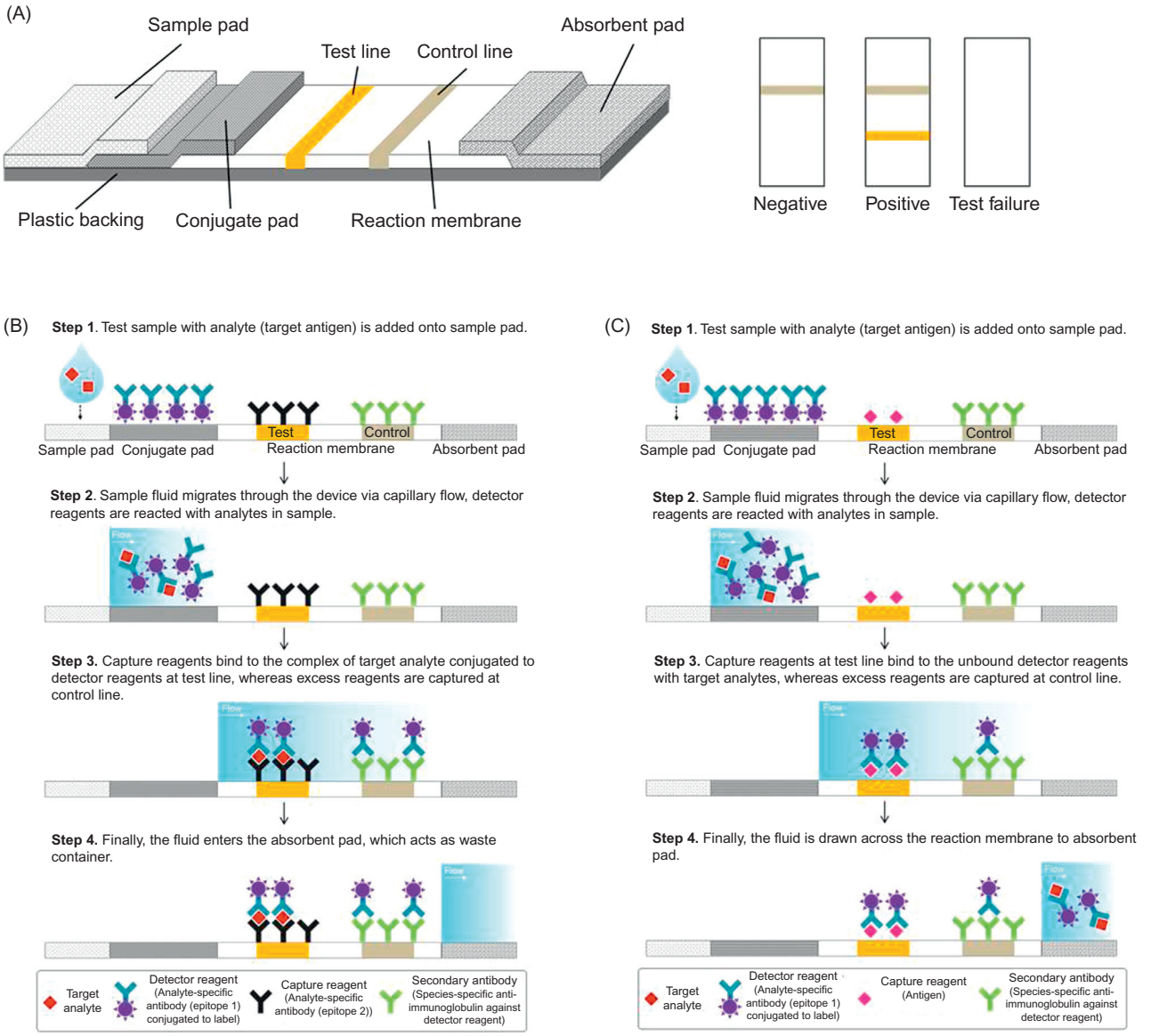


FIGURE 12.19 Typical configuration of the test strip used for lateral flow immunoassay. (A) Sample that is added onto sample pad flows through the strip due to capillary force of fluid, passing each bed and coming into contact with dried reagents to make an immunological reaction sequentially. With color indicators at both the test line and control line, the result can be interpreted as negative (absence of analyte in sample), or positive (presence of analyte in sample). If the test fails, no marks are seen. Depending on immunological properties of analyte, the lateral flow immunoassay can be divided into two types: sandwich format (B); or competitive format (C). Image courtesy H.-W. Yu.

ricin protein. One anti-ricin antibody was immobilized to a defined detection zone on a porous nitrocellulose membrane, while the other anti-ricin antibody was conjugated to colloidal gold particles (25 nm), which served as a detector reagent. Colloidal gold particles at contamination concentrations less than 100 nm develop a red color, such that the test line provides a red color with an intensity proportional to the ricin concentration. With this method, 50 ng/ml of ricin was detected in less than 10 min.

For ensuring the safety of feedstock for animals, a lateral flow dipstick was developed to allow rapid screening of aflatoxin B_1, a secondary metabolite mainly produced by *Aspergillus* spp. This compound, also known as a mycotoxin, is classified as a carcinogenic substance (Delmulle *et al.*, 2005). The test strip was designed using a competitive immunoassay format with aflatoxin B_1–bovine serum albumin conjugate as a capture reagent, and colloidal gold particles (40 nm) coated with anti-aflatoxin B_1 monoclonal antibody as a detector reagent. In the presence of aflatoxin B_1 extracted from the pig feed matrix, the dipstick provides a pink color at the test line, with an inverse intensity relationship against the toxin contents. In this study, the visual detection limit for aflatoxin B_1 was 5 μg/kg within 10 min.

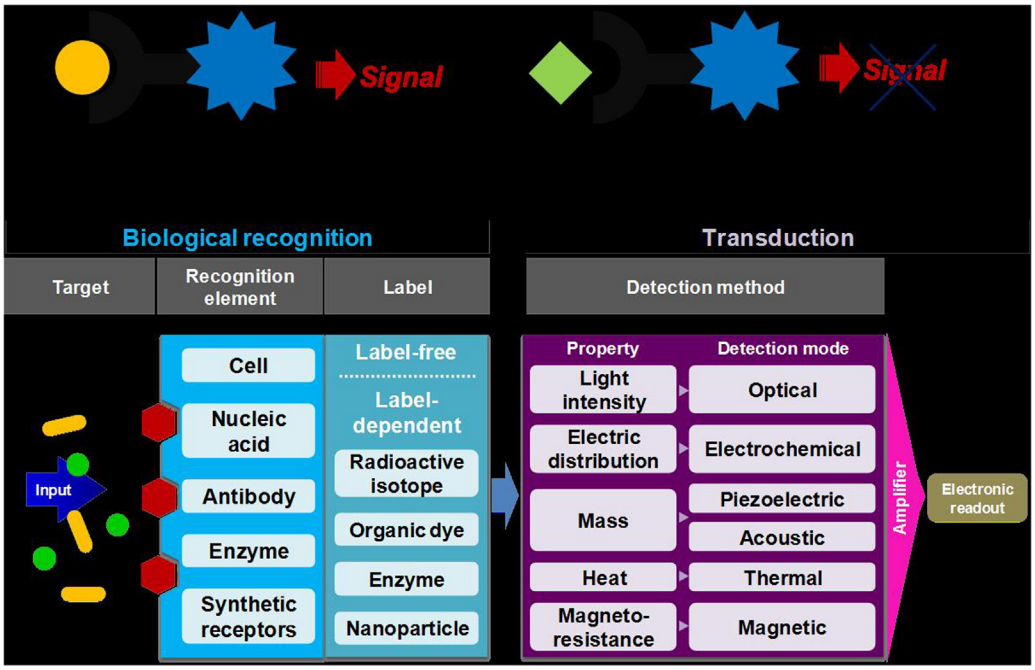


FIGURE 12.20 Conceptual scheme of biosensor. Image courtesy H.-W. Yu.

In addition to toxic compounds, Yan *et al.* (2006) suggested an up-converting phosphor technology (UPT)-based lateral flow immunoassay for quantitative detection of *Yersinia pestis*, which is a pathogenic bacterium that results in plague, a deadly infectious disease. A sandwich immunoassay was employed by using same polyclonal antibodies for detector and capture reagents, in which UPT particles (400 nm) were used as a luminescent label resulting in 10- to 100-fold more sensitivity than assays using conventional reporter systems such as colloidal gold or colored latex beads (Hampl *et al.*, 2001). The signal detection of the strip utilized an infrared laser to excite the UPT particles, then collection of the visible luminescence emitted, and finally its conversion to the voltage as a signal. The detection limit of this assay was 10^4 CFU/ml, taking less than 30 min to perform.

12.3.9.3 Advantages and Disadvantages

Lateral flow immunoassay is a single step assay that requires only the addition of a sample to a prefabricated test strip, with benefits of low cost, commercialization potential, ease of use and rapid detection of various analytes. However, this is not an accurate quantitative technique but rather a presence/absence test for the target analytes in the sample.

12.4 IMMUNOSENSORS

Biosensors are analytical devices that convert a biological response into an electrical signal. They consist of three components including a biological recognition receptor or bioreceptor, which recognizes the target analyte; a transducer, which converts the recognition event into a measurable electrical signal; and some kind of data processing system that allows the data to be displayed in a user-friendly manner. A conceptual depiction of a biosensor is shown in Figure 12.20.

Biosensors can be subdivided into two types based on the kind of biorecognition molecule utilized. Catalytic biosensors employ enzymes and/or microorganisms as the biorecognition molecule to catalyze a reaction with an analyte that results in a product. The other category of biosensors is the affinity biosensors, where the biorecognition molecules can be antibodies, DNA, peptides or lectins. Affinity biosensors are characterized by a binding event between the biorecognition molecule and the analyte (Byrne *et al.*, 2009). A transducer is a device that converts one form of energy to another, in this case the recognition event into a measurable electrical signal (Figure 12.21). The transduction efficiency in turn determines the efficacy of the biosensor including sensitivity, selectivity and signal stability. Generally, the transducer converts the biochemical interactions into measurable electrical signals. Overall, catalytic microbial biosensors are utilized for the assessment of chemical toxicity, whereas affinity biosensors are used for the detection of pathogenic microorganisms and their associated toxins.

Immunosensors are affinity biosensors that utilize antibodies as bioreceptors and rely on the basic Ag−Ab reaction as the recognition event based on the lock and key mechanism of three-dimensional shape fitting and

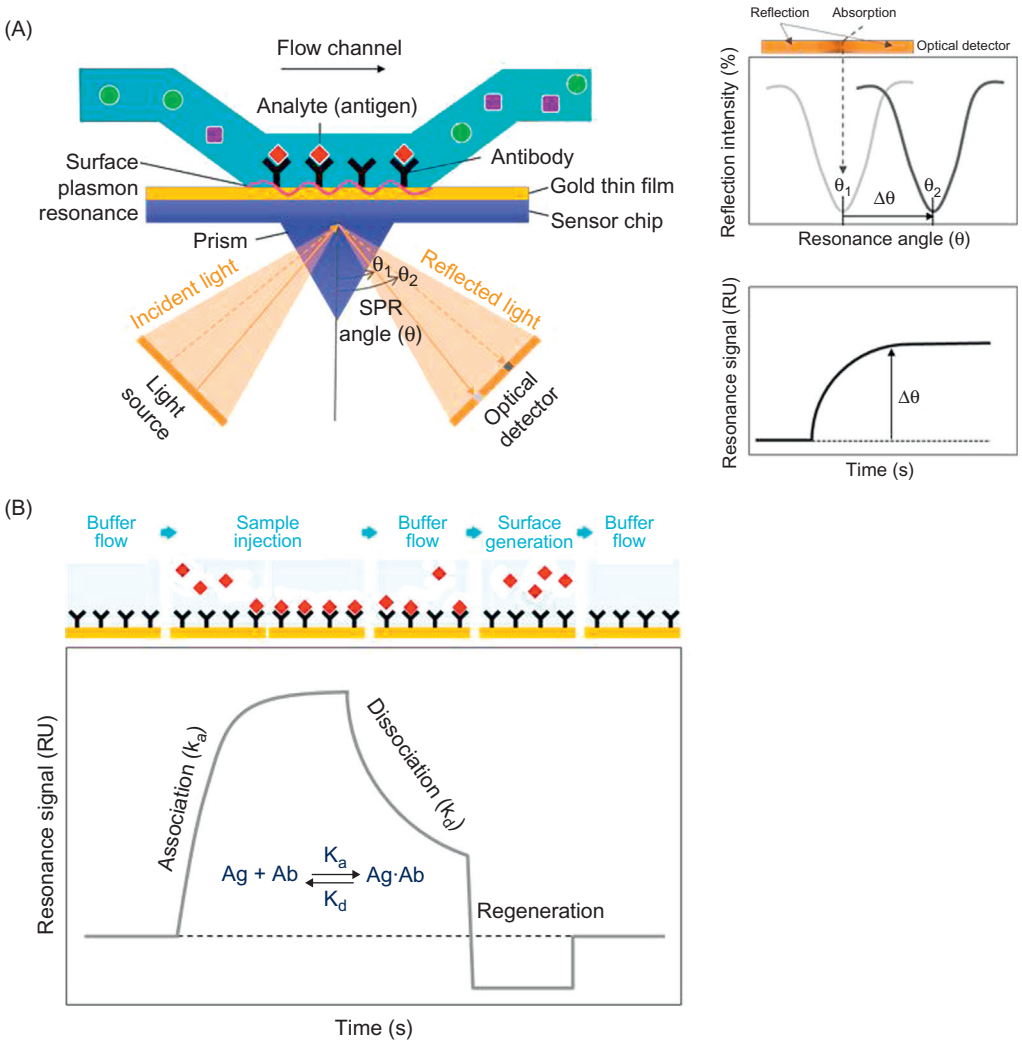


FIGURE 12.21 Typical configuration of surface plasmon resonance (SPR)-based immunosensor and characteristics of a sensorgram. (A) SPR immunosensor can detect the binding of target analyte (antigen) in solution to antibody immobilized on the gold surface of a sensor chip, which alters the refractive index of the medium near the surface. This change can be monitored noninvasively in real time as a plot of resonance signal (proportional to mass change) versus time. (B) Upon injection, binding of the analyte results in an increase in resonance signal (association phase). After equilibrium, the sample is replaced by a running buffer, and the decrease in signal represents the dissociation of analyte from the antigen–antibody complex on the surface. A regeneration solution is used to disrupt binding and regenerate the sensor surface for further measurement. Therefore, the concentration of analyte in the sample and kinetic rate constants (K_a and K_d) can be derived from the sensorgram. Image courtesy H.-W. Yu.

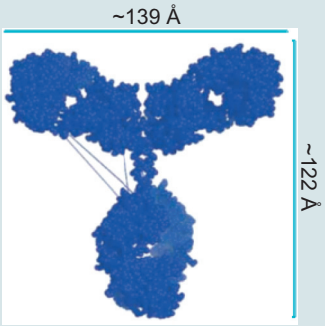
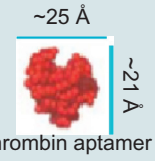
chemical bonds. The transducing agent is the entity that differentiates immunosensors from most standard immunoassays. Immunosensors are becoming increasingly popular for the detection of waterborne pathogens and their associated toxins (Table 12.1) in environmental samples including water.

Aptamers, also known as "artificial antibodies," are single-stranded nucleic acids (RNA or DNA) with defined tertiary structures for selective binding to target molecules (Nutiu and Li, 2003). While aptamers are analogous to antibodies in their range of target recognition and variety of applications (Stoltenburg *et al.*, 2007), they possess several key advantages (Table 12.2) and have been used widely for constructing a new type of affinity biosensor. Mimicking natural evolution, a selection process called systematic evolution of ligands by exponential enrichment (SELEX) has resulted in many aptamers that can bind to a broad range of biological targets with high affinity and with specificity comparable to that of antibodies, such as small organic molecules, proteins, viruses and cells (Li *et al.*, 2012). There have been attempts to identify aptamers that are specific to target analytes including pathogenic bacteria,

TABLE 12.1 Examples of Immunosensors for the Detection of Pathogens and Associated Toxins

Target Analyte	Label	Transducer	Type of Signal	Limit of Detection	Analysis Time	Matrix	Reference
E. coli	Free	Gold screen-printed electrode	Electrochemical (Impedance)	3.3 CFU/ml	60 min	River, tap water	Escamilla-Gómez *et al.*, 2009
Vibrio cholerae	Alkaline phosphatase	Carbon screen-printed electrode	Electrochemical (Amperometry)	8 CFU/ml	55 min	Ground water	Sharma *et al.*, 2006
Cryptosporidium parvum	Alkaline phosphatase and gold nanoparticle	Indium tin oxide electrode	Electrochemical (Voltammetry)	3 oocysts/ml	70 min	Drinking water	Thiruppathiraja *et al.*, 2011
Cholera toxin	Potassium ferrocyanide-encapsulated liposome	Carbon nanotube	Electrochemical (Voltammetry)	10 pg/L	65 min	Tap water	Viswanathan *et al.*, 2006
Microcystin	Quantum dot	Microbead	Flow cytometry (Fluorescence)	0.5 µg/L	30 min	Tap water	Yu *et al.*, 2012

TABLE 12.2 Comparative Properties of Antibody and Aptamer

Characteristic		Antibody	Aptamer
Target molecule		Antigen (protein or peptide)	Amino acid, peptide, nucleic acid, and microbial cell
Affinity		High (K_d: pM – nM)	High (K_d: pM – nM)
Molecular weight		<150 kD	<50 kD
Size[a]			
Chemical stability		Low	High
Reusability		Impossible	Possible
Production	Method	*In vivo* biological process	*In vitro* chemical process
	Development period	<2 months	<6 months
	Cost	High	Low

[a]*Lee* et al. *(2006a).*

viruses and protozoa. Table 12.3 shows examples of a few publications on the utilization of aptamers as a bioreceptor.

Proof-of-concept experiments have been performed under well-controlled laboratory conditions, but relatively little has been done to test these biosensors in actual environmental water matrices. Environmental water samples pose a number of challenges to biosensor development because of the potential for microbial, particulate and

TABLE 12.3 Select Aptasensors Utilized for Detection of Pathogens and Their Toxins

Target	Aptamer			Biosensor Application				
	DNA/RA	Sequence length (nucleotides)	Binding Site	Label	Detection Mode	Limit of Detection	Analysis Time	Reference
Bacillus thuringiensis	DNA	60	*B. thuringiensis* spore	Quantum dot	Fluorescence	10^3 CFU/ml	>20 min	Ikanovic *et al.*, 2007
E. coli O111	DNA	72	Lipopolysaccharide	Alkaline phosphatase	Voltammetry	112 CFU/ml	>3.5 h	Luo *et al.*, 2012
Salmonella typhimurium	DNA	40	*S. typhimurium* outer membrane Proteins	Nanoparticles	Luminescence	5 CFU/ml	>40 min	Duan *et al.*, 2012
Staphylococcus aureus	DNA	88	N.A.	Nanoparticles	Luminescence	8 CFU/ml	>40 min	Duan *et al.*, 2012

other organic and inorganic contaminants (Connelly and Baeumner, 2012). Moreover, although waterborne pathogens and toxins are typically present at low concentrations even in source water, the development of sampling protocols and techniques to concentrate environmental samples is still in its infancy (Vikesland and Wigginton, 2010).

12.4.1 Surface Plasmon Resonance-Based Immunosensor

12.4.1.1 Technique

Surface plasmon resonance (SPR)-based immunosensors provide a nondestructive optical analysis technique, useful for investigating the interaction of thin-layered biomolecules, especially antigen–antibody reactions, on the surface of sensor chip. As shown in Figure 12.21A, when a light beam is focused onto the gold surface of the sensor chip, evanescent wave photons produced by the incident light interact with the surface plasmons, that is, free oscillating electrons propagating along the surface of the gold film. Resonance occurs at a critical angle (SPR angle) of incident light, and light energy is transferred to the electrons in the metal film surface, causing a reduction of the reflected light from the metal film at the interface between two media of different refractive indices. This phenomenon can be utilized by measuring a change in the angle of light reflection, because the reflected beam covers the same angle range of incident light that depends on the refractive index at the gold surface. At resonance, a minimum in reflected light intensity is observed, and a dark line appears in the band of a photodetector, resulting in a sharp drop (also referred to as the SPR "dip") in the plot of reflection intensity of this band versus the SPR angle. The SPR angle shifts when biomolecules bind to the surface and change the mass of the surface layer. This change in resonance angle can be monitored in real time as a plot of resonance signal (proportional to mass change) versus time, and subsequently used to calculate the refractive index and concentration of the adhered target analytes.

A typical sensorgram of an SPR-based immunosensor, a plot of resonance signal (expressed as resonance units (RU) versus time, is shown in Figure 12.21. A specific antibody to an analyte of interest is immobilized on the sensor surface with appropriate coupling chemistry. Before injection of the sample, a running buffer flows through a microfluidic flow cell of the SPR instrument. When a sample containing target analyte (antigen) is injected, binding of the analyte to the surface leads to the increase in refractive index, and consequently the resonance signal (association phase). During this association phase, the association rate constant (K_a in $M^{-1}s^{-1}$) of the antigen–antibody interaction (number of binding events per unit of time), which is a measure of antigen–antibody complex formation, can be determined by interpretation of a sensorgram with different concentrations of injected target antigens. At equilibrium, the signal will reach a plateau when the amount of analyte that is associating and dissociating with the antibody is equal. The response level at equilibrium is related to the concentration of target analyte in the sample. When the analyte solution is replaced with buffer, the antigen–antibody complex is allowed to dissociate as a function of the dissociation rate constant (number of dissociation events per unit of time) (K_d in s^{-1}) that describes the antigen–antibody complex stability. After dissociation, the sensor surface can be regenerated for the next measurement using a regeneration

solution (for example, high salt or low pH) to remove the remaining bound analyte with a denaturing immobilized antibody. Therefore, this immunosensor can determine the analyte concentration in a sample if an appropriate calibration curve is constructed.

12.4.1.2 Application

An SPR immunosensor was developed for the detection of *Salmonella typhimurium* in contaminated water and food (Oh *et al*., 2004). When using the SPR immunosensor, the orientation of antibody immobilized on the sensor surface is crucial to increase the activity of the antibody interacting with the analyte of interest. This improves the sensitivity for the detection of very low concentrations of antigens. Since protein G, an immunoglobulin-binding cell wall protein found in *Streptococci* sp., is capable of interacting with the Fc region of the antibody, the self-assembled protein G layer on the gold surface of the sensor using 11-mercaptoundecanoic acid leads to a well-oriented monolayer of antibody molecules for the target analyte. The shift in SPR angle has a linear relationship with the concentration of *S. typhimurium* over the range of 10^2 to 10^9 CFU/ml. Thus, the limit of detection of this sensor was four orders of magnitude more sensitive than a standard ELISA.

Homola *et al*. (2002) improved the sensitivity of a dual-channel SPR immunosensor based on wavelength modulation. They utilized a sandwich-binding format to detect staphylococcal enterotoxin B (SEB) in milk. SEB is an exotoxin produced by *Staphylococcus aureus*. It is one of the toxins responsible for staphylococcal food poisoning in humans, and has been produced by some countries as a biological weapon. The dual-channel (reference and sample channels) SPR sensor can provide compensation for nonspecific sensor response due to temperature-induced changes in sample refractive index. The sandwich binding of a secondary antibody to an analyte−antibody complex on the gold sensor surface leads to an increase in mass change and refractive index, which can amplify the sensor response by a factor of 10. The SPR immunosensor has been shown to be capable of detecting concentrations of SEB as low as 5 and 0.5 ng/ml, using direct and sandwich modes, respectively.

12.4.1.3 Advantages and Disadvantages

The SPR immunosensor is one of the best-known and commercially available optical label-free biosensors, useful for monitoring the specific binding event of an antigen−antibody continuously in real time with high sensitivity. However, mass transport can affect the kinetic analysis and any artifactual change in refractive index other than from the interaction can also give a false-positive signal.

QUESTIONS AND PROBLEMS

1. Describe an immunological assay for the accurate quantitation of *Rhizobium* around root nodules.
2. Describe an immunological assay for detection of *Giardia* in water samples.
3. Immunomagnetic purification methods are becoming popular in environmental microbiology. Sediment slurries can contain paramagnetic particles, which are copurified along with target antigens, making subsequent assay procedures difficult. Describe the problems that would arise with immunomagnetic analysis in this case, and devise potential methods for solving these issues.
4. How could you determine whether an enzyme is intercellular or extracellular using immunoassay techniques?
5. You have two monoclonal antibodies available from two different commercial companies. Both cost the same and both are specific for the rhizobia you are studying in question 1. After ordering both antibodies, how would you determine which antibody was the best for the assay you designed in question 1? How would you perform these evaluations?
6. Rapid detection of biological warfare agents is an emerging area of research. Design a rapid detection method using an immunoassay that can aid in the detection of *Bacillus anthracis*.
7. What immunoassays described offer quantitative results?
8. What immunoassays described allow for purification of antigens from heterogeneous samples?

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