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Analytical Techniques for Biopharmaceutical Development — ELISA

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INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) is based on the specific reaction between an antibody and an antigen. One of the reagents in the reaction is labeled with an enzyme that generates a colorimetric product that can be measured with a spectrophotometric device. The color intensity correlates with the concentration of specific antibody and the respective antigen. The reaction can be formatted in various ways in a multiwell plate (microtiter plate) with the common formats being the sandwich assay, the competitive assay, and the direct assay. (See Figure 11.1.)

The sandwich assay is the format used most often to quantitate a target antigen or analyte. In the sandwich assay, two antibodies are used that bind to different parts of the antigen. One of the antibodies is bound to, or coated on, the solid surface (mictotiter plate wells), whereas the other has a label attached to it (Figure 11.1a). Alternatively, a secondary conjugated antibody can be used to detect the bound primary antibody (Figure 11.1b). If the antigen is present in the sample solution, it links the two antibodies. Therefore, the label is retained on the plate where it can be detected by use of a colorimetric substrate.

The competitive assay is another format used to quantitate an analyte. An unlabeled analyte competes with a labeled analyte (enzyme-conjugated molecule) for binding to a specific capture antibody (Figure 11.1c).

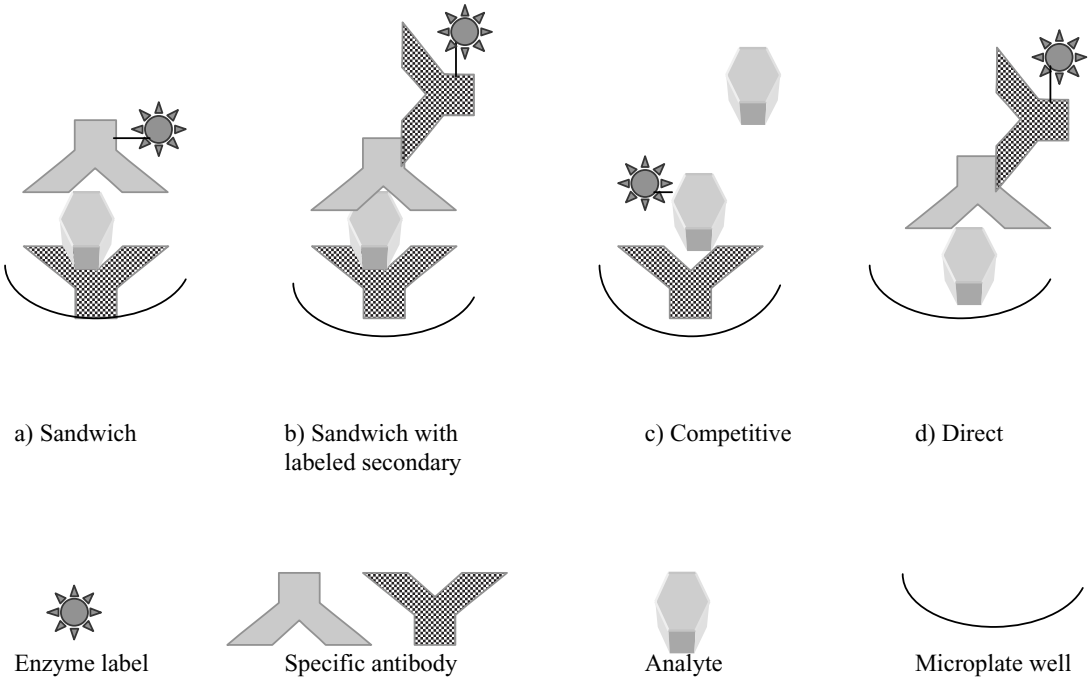

Figure 11.1 (a) Sandwich assay: analyte is captured between an antibody coated on the microplate well and an enzyme-labeled antibody. (b) Sandwich assay: analyte is captured between two specific antibodies and detected with secondary enzyme-labeled antibody. (c) Competitive assay: an enzyme-labeled analyte competes with unlabeled analyte for binding to the antibody. (d) Direct assay: antibodies to the analyte coated directly on the plate are detected with an enzyme-labeled antibody.

The direct assay format is the one used most often to detect antibodies to an antigen, with the antigen coated directly onto the solid phase (Figure 11.1d). This is used mostly for diagnostic purposes. The direct assay can detect whether or not a patient has reacted to an infectious organism by producing an antibody response to an antigenic protein. This format can be applied to biopharmaceutical product development when the drug product itself is an antibody, and the specific antigen is coated onto the plate.

The ELISA can be used as one component of a battery of analyses. Rarely is only one method used in isolation. Other tests include chromatographic methods such as reversed-phase high-performance liquid chromatography (HPLC), size exclusion chromatography, and physical structure analytical methods such as UV spectral analysis, mass spectroscopy, etc.

The ELISA is a versatile method that can be used throughout the biopharmaceutical product development process, from small-scale research to cell-line selection, to monitoring fermentation and downstream processing, and to product release testing. The use of the microtiter plates allows for high sample throughput and various degrees of automation. ELISAs satisfy the biopharmaceutical production requirements for specific, accurate, precise, and reproducible assays.

APPLICABILITY OF THE ELISA METHOD FOR ITS INTENDED USE

Characterization: Characterizing the Protein's Physical–Chemical and Biological Properties

Identification and Quantitation

At an early stage in product development, there must be some material made available for use as ELISA reagents. ELISA requires a "standard" for quantitation and specific antibodies to the drug protein of interest. If these are not already available, either commercially or internally, then time, effort, and expense must be reserved for reagent development as well as assay development activities. The specific antibody is the key reagent in an ELISA. The antibody defines the specificity and sensitivity of the assay. To date there is no successful substitution for the routine production of specific antibodies by immunization of an animal with the antigen target of interest.

The ELISA can be used for identification and quantitation of the protein product (biopharmaceutical) of interest throughout the development, production, and manufacturing process. For example, in the initial development phase, ELISAs can aid in the selection of the best cell line. In the early manufacturing steps, it can be used to identify the appropriate product-containing pools or fractions in process to be subjected to further purification. Because of the selectivity of ELISA, it is a suitable tool to select out the protein of interest from complex protein mixtures, such as cell culture fermentation media or product pools in early steps of protein recovery as well as downstream processing. Even complex mixtures do not require much sample preparation. It is important to determine

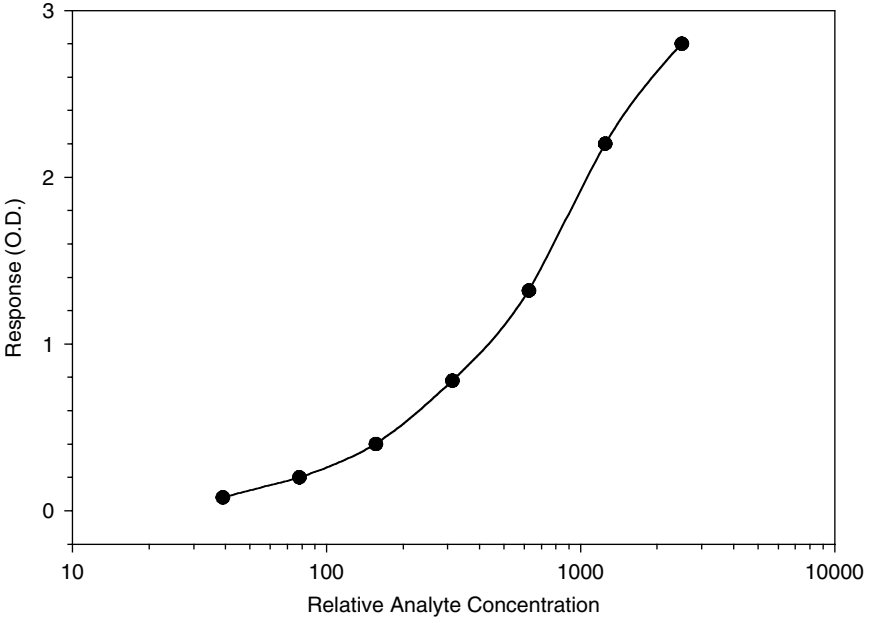

Figure 11.2 An example of a typical ELISA standard curve.

that the sample matrix does not contain any buffer or salt components, surfactants, or protein materials that interfere with the antibody–antigen reactions of ELISA. Often, simple sample dilution is effective in removing or reducing interference. If there is interference, this must be accounted for in the final calculations of sample recovery. The ELISA can still be used, with appropriate compensation.

The most commonly used format for quantitation assays is the sandwich assay format. Typically, a monoclonal antibody (MAb) is used to capture the product. It is then detected by another antibody, usually enzyme-labeled. A reference standard is used from which to compare the response of an unknown test sample. There is a relative increase in measured response (optical density) with increasing analyte concentration. Figure 11.2 is an example of a typical ELISA standard curve.

A competitive assay could also be used for quantitation. In a competitive assay, unlabeled antigen competes for labeled antigen. Examples include ELISAs for vaccine product antigens, such as recombinant proteins from viruses, or nonvaccine antigens such as growth factors or cytokines.

In addition to its use in quantitating the product, ELISA can also be used as a readout tool for cell-based bioassays or other assays that measure a biological activity conferred by the drug product. The end point of the bioassay may be a

particular induced cellular protein (e.g., cytokine) or a reduction of a viral protein that can be quantitated by an ELISA. There are many commercially available reagents as well as complete ELISA kits for various cytokines and cell markers as well as common viral proteins. Sadick et al.[1] developed an assay designated KIRA, for kinase receptor activation. The KIRA assay is capable of quantifying ligand bioactivity by measuring ligand-induced receptor tyrosine kinase activation in terms of receptor phosphorylation. Assays using this method for the ligands IGF-1 and NGF showed excellent correlation with the more classical end point bioassays. Figure 11.3 is a schematic diagram of the IGF-I KIRA.

Product Variants

Product-related substances are molecular variants of the desired product formed during manufacture and storage, which are active and have no deleterious effect on the safety and efficacy of the drug product.[2] Many recombinant protein products are inherently heterogeneous, mixtures of closely related structures or product variants. These variants possess properties comparable to the desired product and are not considered impurities. It is only when they do *not* have properties of the desired product that their presence is problematic.

Product-related impurities are molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture and storage) which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety.[2] Variants may exert different biological effects (potentially uncontrolled or hazardous) and specifically lead to antibody formation in the patient.[3]

Product variants are generated by a number of genetic, chemical, and physical changes:

- Genetic changes can give rise to product variants by mutations in the product-encoding gene itself.
- Chemical changes include posttranslational modifications including glycosylation, phosphorylation, disulfide bond formation and exchange (scrambling), proteolysis or hydrolysis, and deamidation or oxidation of amino acids.[4]
- Physical changes include denaturation, precipitation, adsorption, and aggregation (with like molecules or with excipients).[4]

Product variants can also be the result of action of cellular proteins on the drug molecule, such as proteolytic processing inside and outside cells. Enzymes from the host cell can make modifications of the product protein.

Product variants can also be generated by in-process procedures, such as those used for viral inactivation, for example. These procedures could alter the protein structure, forming new epitopes. These types of changes could potentially be detected by ELISA because of the specificity of the antigen–antibody interaction. In the case of vaccine production, an ELISA could be used to monitor viral inactivation. For this, a panel of MAbs, if available, could be used.

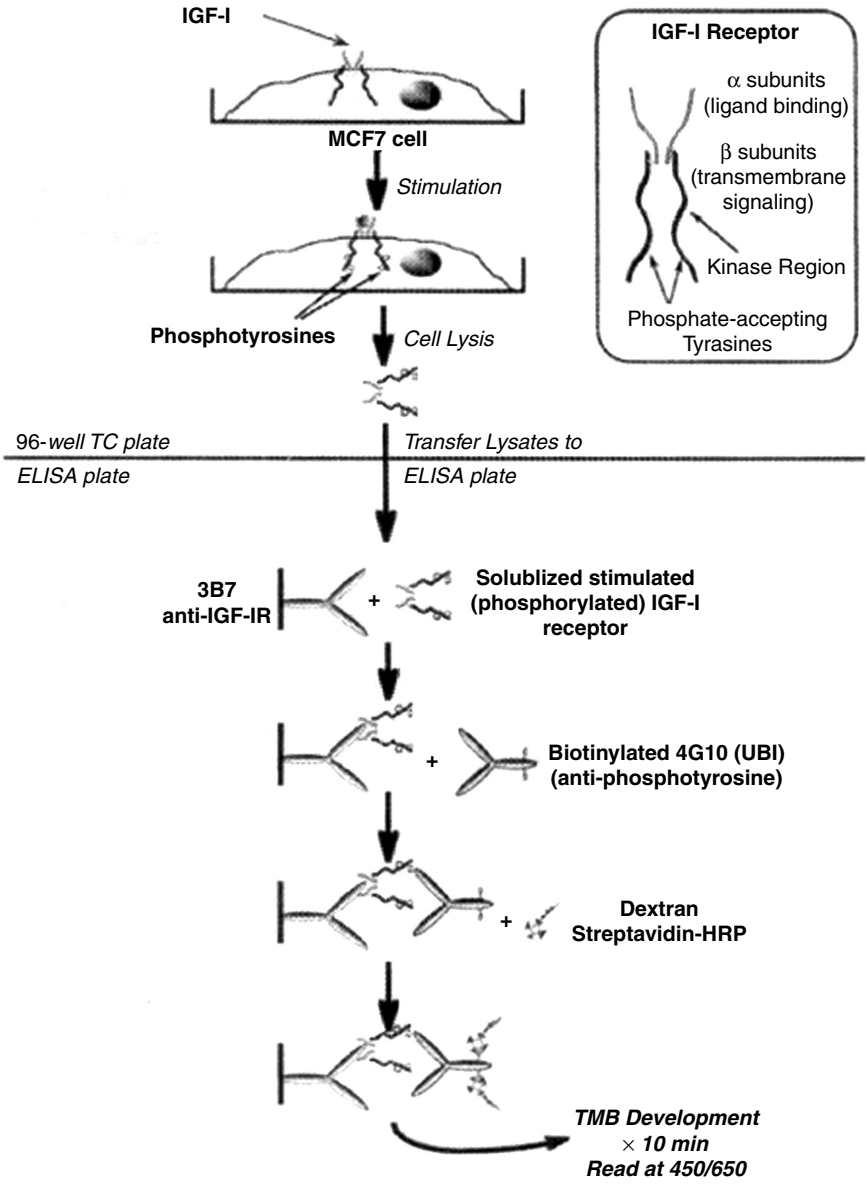

Figure 11.3 Schematic diagram of the IGF-I KIRA.

The most frequently encountered product variants are:[2]

- Truncated forms: hydrolytic enzymes or chemicals may catalyze the cleavage of peptide bonds.
- Other modified forms: deamidated, isomerized, mismatched S-S linked, oxidized, or altered conjugated forms (e.g., glycosylation, phosphorylation).
- Aggregates: include dimers and higher multiples of the desired product.

The ELISA is an appropriate method to detect some, but not all, of these product variants. The reactivity of antibodies is not affected much by glycosylation of the antigen. An ELISA would not be the most appropriate method to analyze product variants due to differences in glycosylation. ELISA would be appropriate for analysis of variants, such as aggregates, that contain a different protein structure that can be specifically recognized by an antibody.

Aggregates: For proper biological activity, proteins must maintain their three-dimensional (native) conformation. Protein aggregation almost invariably leads to severely reduced biological activity.[5] It is desirable to detect protein aggregates because they are likely to be more immunogenic than the monomer. This was shown for aggregated human growth hormone, insulin, and IgG.[6] In general, protein aggregates can be induced by stress conditions, such as exposure to extremes in temperature and pH, introduction of a high air/water or solid/water interface, or addition of certain pharmaceutical additives.[6]

Braun and Alsenz[6] used an ELISA to detect aggregates in interferon-alpha (IFN-α) formulations. They analyzed IFN-α formulations for possible aggregate formation because all marketed interferons are reported to induce antibodies to some extent. Because of its stabilizing effects, human serum albumin (HSA) is used in the formulation of marketed IFN-α at a great excess over IFN-α itself. HSA can also interact with other proteins. Braun and Alsenz developed an ELISA for the detection of both IFN-α–IFN-α and HSA–IFN-α aggregates. A MAb was used for the capture and detection of the IFN-α and a polyclonal for the detection of HSA. The assay is shown schematically in Figure 11.4.

The aggregate-specific ELISAs could be used to monitor the aggregate-inducing processes during IFN-α formulation and storage in an early phase and the development of aggregate-free IFN-α formulations. The ELISAs were highly sensitive, needed low protein concentrations, worked in the presence of excipients, and required no pretreatment.[6]

Proper Protein Refolding: The final protein structure of the recombinant protein is often obtained by the promotion of proper refolding. When the production of recombinant proteins results in inclusion bodies, an ELISA can be used to assure that the final protein product has been properly refolded and to monitor the refolding process. To do this requires antibodies that can only recognize and bind to a particular conformation that is only present upon proper refolding.

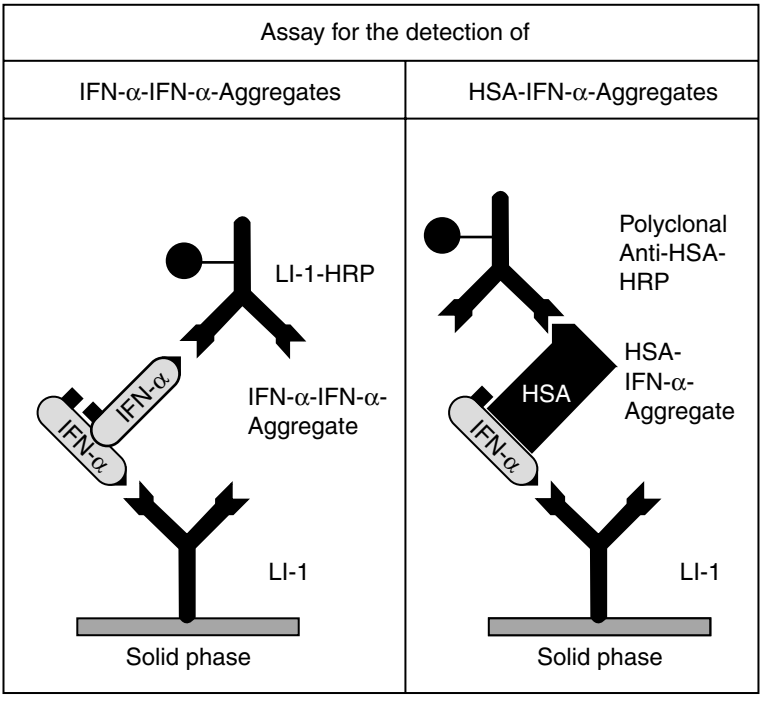

Figure 11.4 Schematic representation of ELISAs for the detection of IFN-α–IFN-α and HSA–IFN-α aggregates. IFN-α–IFN-α aggregates are detected using the same monoclonal anti-IFN-α antibody (LI-1) as capture and detection antibodies. HSA–IFN-α aggregates are captured by the anti-IFN-α antibody LI-1, and IFN-α-bound HSA is identified by a polyclonal and anti-HSA antibody. For simplicity, aggregates are illustrated at a 1:1 molar ratio (HRP = horseradish peroxidase).

Vandenbroeck et al.[7] used an ELISA to determine the recovery of immunoreactive porcine interferon-gamma (IFN-γ) from *E. coli* inclusion bodies. The ELISA used a polyclonal coating antibody with detection by a MAb. The inclusion bodies were solubilized in diluted 6 *M* guanidine/HCl and IFN subsequently refolded by its removal. The antiviral activity of the interferon was measured with a bioassay using the cytopathic effect (CPE) of vesicular stomatitis virus (VSV) on bovine kidney cells. The results of this study showed that the immunoreactivity measured by ELISA matched the biological activity measured by bioassay.

Tsouloufis et al.[8] used an ELISA to assess the refolding of a recombinant subunit of the extracellular domain of the human muscle acetylcholine receptor expressed in *E. coli*. The plates were coated with refolded or unfolded protein and then reacted with conformationally dependent MAbs. The use of specific

MAbs enabled identification of the protein segments required for native conformation.

Merli et al.[9] used an ELISA to optimize refolding of soluble tumor necrosis factor receptor type I (sTNF-RI) in *E. coli.* Native conformation of the molecule is maintained by 12 disulfide bridges. At different time intervals, aliquots of each well in the refolding microplate were diluted and tested by ELISA. The ELISA format used was a double-antibody sandwich assay and was based on the use of MAbs directed, respectively, against a neutralizing and a nonneutralizing epitope on the sTNF-RI molecule. The protein was characterized by biological and functional assays, and a good correlation was observed between all the data (biological assay, ligand-directed ELISA, and double-determinant sandwich ELISA).

Purity: Detecting Impurities and Contaminants

Biopharmaceuticals are subjected to strict regulations to monitor and quantitate impurities to maintain product safety, quality, integrity, and efficacy.[4] The following are definitions that relate to purity:

Impurity: Any component present in the drug substance or drug product that is not the desired product, a product-related substance, or excipient including buffer components. It may be either process- or product-related. According to ICH Guidelines on Impurities in New Drug Substances,[10] impurities at a level greater than 0.1% should be identified. So should degradation products observed in stability studies (for stability identification, the threshold is 1% for a maximum daily dose of < 1 mg to 0.1% for a maximum daily dose of > 2 g[10]). Identification below the 0.1% level is usually not considered necessary.[10]

Process-related impurities: Impurities that are derived from the manufacturing process. They may be derived from cell substrates (e.g., HCPs, host cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing (e.g., processing reagents or column leachables).

Product-related impurities: Molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture or storage) that do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety.

Contaminants:[2] Any adventitiously introduced materials (e.g., chemical, biochemical, or microbial species) not intended to be part of the manufacturing process of the drug substance or drug product.[2]

Degradation products:[2] Molecular variants resulting from changes in the desired product or product-related substance brought about over time or by the action of, e.g., light, temperature, pH, water, or by reaction with an excipient or the immediate container/closure system. Such changes may occur as a result of manufacture or storage (e.g., deamidation, oxidation, aggregation, proteolysis). Degradation products may be either product-related substances or product-related impurities.

Of all the possible contaminants and impurities of a biopharmaceutical product, organisms (bacteria, virus, mycoplasma) and their products (DNA, endotoxin, host protein), media components, and raw materials, it is most appropriate to use an ELISA for the HCP impurities and some of the process residuals (media components and raw materials). Impurities from media components are known or expected unlike those from the host cell.

Host Cell Impurities: Various organisms have been used to produce recombinant proteins: yeast, bacteria (e.g., *E. coli*), insect cells, and mammalian cells such as Chinese hamster ovary (CHO) cells. During the purification process, some HCPs can copurify with the protein product. Because of the specificity of the antigen–antibody interaction, an ELISA can be used to detect and quantitate the contaminating HCPs. Detecting host impurities is important for quality process control as well as for product safety issues. The intent is to avoid "unsafe" levels of residual HCPs which might lead to adverse reactions.[11]

The final purity required depends on final use of the product: e.g., vaccine with one immunization vs. hormone with chronic use. A detection range of 1 to 100 ppm of residual HCPs has been quoted as a regulatory (and analytical) benchmark for therapeutic proteins.[11] Many biotech companies have limited the range to 1 to 10 ppm. The sensitivity and specificity of any unique HCP assay that is used to support such a target should be demonstrated accordingly.[11]

Because ELISA is based on the specificity of antibody recognition of the antigen, the assay is only as good as the antibodies used. Proprietary reagents and assays must be developed and validated for the quantitation of host cell-specific proteins. Prolonged time frames must be anticipated when planning for the manufacture and characterization of complex HCP immunogens, the elicitation, purification, and qualification of anti-HCP immunoreagents, and the development and validation of multianalyte HCP immunoassays.[11] A commitment of time and expense must be made as it may take 3 to 6 months to elicit, purify, and characterize an acceptable polyclonal anti-HCP immunoreagent.[11] There are some commercially available antibodies to some of the most commonly used host cell expression systems. The commercial antibodies should be extensively evaluated before use and would be acceptable if they detected most of the specified host cell impurities. The evaluation should be done by another immunological-based assay, such as Western blot analysis.

There are two approaches to developing a host cell impurity assay, each with advantages and disadvantages. Both approaches require the production of polyclonal antibodies for use in an ELISA, but the immunogen used to elicit those antibodies is what differentiates the two approaches. The first approach is to use the host cell homogenate preparation as the immunogen. The host cell homogenate is produced from null host cells or the same host cell and vector systems as the production cell line, only lacking the cDNA of the product. All other characteristics (i.e., fermentation conditions) should be identical to the production process.[12] This is referred to as a "generic" assay and the goal is to

measure all the HCP impurities that could be present.[13] The second approach is to use a "purified" host cell preparation or only those HCPs that typically copurify with the product as the immunogen. This is referred to as a "process-specific" assay.[13] It also uses host cell homogenate from null host cells, but the homogenate is then subjected to the same final purification process or to some of the purification steps as the recombinant DNA product.

The primary disadvantage of the generic assay is that the antibody may not detect all HCPs because some proteins are not sufficiently immunogenic or present in high enough concentrations to be detected by antibodies. The detection of HCPs should be differentiated from the detection of process impurities such as media components. Some proteins are at a low level, and there could be an immunodominant protein (such as albumin, a common media component). This can be avoided by preparing the host cell homogenate in serum-free media. To increase the response to proteins that are at a low concentration, or to weakly immunogenic proteins, there are methods available, such as chemical modification, passive immunization, and cascade immunization.[11,13] Passive immunization[11] involves immunization of antigen combined with purified IgG from previous postimmunization bleeds. Cascade immunization uses immune IgG to adsorb major antigens from the immunogen preparation. This facilitates the antibody response to weaker or minor antigens.

The host antigen preparation is used in several capacities of assay development as well as routine analysis:

- As an immunogen to generate specific antibodies
- For immunoaffinity matrix, to affinity-purify the antibodies generated
- As an assay standard

The antigen preparation used for immunogen and the antibody reagent generated should be evaluated using a combination of other techniques such as acrylamide gel and Western blot. The Western blot can identify those proteins seen on an acrylamide gel that are reactive with the antisera. This method can be used to evaluate the bleeds obtained over time during the immunization process. The analysis of the antibodies can also yield the specific identity of HCP impurities, especially particularly troublesome or recurring impurities, and differentiate among other process impurities such as media components.

The primary disadvantage of the process-specific assay is that it is specific for a particular purification process, and if any changes are made to this process, the antibodies originally developed are obsolete. Many factors can alter the final population of HCP, expressed, including a change in fermentation that affects host cell viability or a failure in one of the purification steps during a production run. If any unanticipated changes occur and change the host cell impurity load, this could result in a contaminated final product in which the contaminants cannot be detected by the process-specific assay. The impurity could be from a different population of HCPs than the population from a process that did not "fail".[13]

Hoffman advises, "Relying solely on a process-specific assay is ill advised and can result in failure to detect atypical process contaminants. In cases with a defined, persistent, and problematic host cell protein impurity, a down-stream process-specific assay may be justified. It is critical that the immunoassay be capable of detecting every possible host cell protein contaminant."[13]

In the development of an ELISA for host cell impurities, you also have to consider copurification of a HCP that is homologous to the product; the host species version of the recombinant protein, e.g., urokinase, is known to be present in many continuous cell lines.[3] Due to the similarity in structure, it is possible that an endogenous homologous protein molecule could copurify with the desired product.[3]

Because the quality of the impurity assay depends on the quality of the antibodies, there are several important points to consider regarding the immunization. The animals may have to undergo substantial boosting, especially for a "generic" assay. This is to elicit antibodies to proteins of lower concentration or to those that may be less immunogenic. The animal species chosen for antisera production is not critical. Rabbits and goats are often used as the species of choice. The antisera generated from the host immunogen should be pooled from several animals and subjected to immunoaffinity (ideally to the same antigen preparation used as the immunogen) or purified before assay development. The resultant antibody can be used as a capture antibody to coat the plate, and as a detecting antibody by conjugating it with avidin or biotin.[12]

There are many examples of ELISAs used for detecting host cell impurities in the literature. Pauly et al.[12] developed an ELISA to detect impurities in erythropoietin that had a detection limit of around 0.05 ng/ml. SDS polyacrylamide gel and Western blot analysis were used to confirm the spectrum of proteins detected and to demonstrate the specificity of the antibody preparation. Anicetti et al.[14] describe an assay for the detection of *E. coli* proteins in recombinant DNA-derived human growth hormone. Whitmire and Eaton[15] report on an immunoligand assay for quantitation of process-specific *E. coli* host cell contaminant proteins in a recombinant bovine somatotropin.

The host cell impurity assay can be used as a tool for detecting impurities in the various in-process purification steps as well as the final product. When using the assay for the in-process steps, it is important to test the samples in the appropriate buffers or matrix. Any sample recovery problems from buffers need to be determined and accounted for in the analysis.

Process Residuals: A source of potential impurities can be process residuals as well as host cells. Process residuals include fermentation media components as well as raw materials used in chromatography (immunoglobulin affinity ligands), etc. According to the ICH Guidelines,[2] process-related impurities are impurities that are derived from the manufacturing process. They may be derived from cell substrates, cell culture, or downstream processing.

Cell Culture-Derived: Media-Derived Protein Impurities. Immunoassays can detect low impurity levels (<1 ppm).[4] The ELISA is probably one of the most sensitive analytical methods. If bovine serum is used as a media component, then testing should include ELISAs for bovine serum albumin (BSA), bovine transferrin, bovine fetuin, and bovine IgG. Often hormones and growth factors, such as insulin or insulinlike growth factor, are used as media components. ELISAs should be used to detect and quantitate these residuals in the various production steps as well as in the final product. There are commercially available antibodies to most commonly used media components. If proprietary media components are used, then the same investment in time and effort is required for the production of specific antibodies, as described above for host cell impurities.

If the product is an antibody, then it is essential to distinguish the immunoglobulin product, e.g., mouse IgG, from any media immunoglobulin components, e.g., bovine IgG. Lucas et al.[16] developed an immunoassay to measure nanogram quantities of bovine IgG in the presence of a large excess of a structurally homologous protein, mouse MAb. The bovine IgG was a contaminant that copurified with the product from a protein A column. For the bovine IgG assay, whole IgG and protein A-purified IgG reacted differently in the assay. It is important to evaluate these types of assays for cross-reactivity. For other media components, such as chemicals or antibiotics, ELISA is probably not the most appropriate method due to the low immunogenicity of chemicals. Techniques such as HPLC would be better to detect these chemical components.

Downstream-Derived: Column Leachates. If a chromatography medium used in purification contains lectins, an ELISA can be used to detect contaminating lectin. For example, for a downstream column using a lectin such as the *Galanthus nivalis* (snowdrop) lectin, the concentration of unbound *G. nivalis* lectin in bulk solutions can be determined with an ELISA. Reagents for lectins are commercially available. A quantitative sandwich ELISA could be used to quantitate the lectin: a capture antibody (goat anti-*Galanthus nivalis* lectin, Vector Labs, Burlingame, CA) is adsorbed to the surface of a microtiter plate. Biotinylated goat anti-*Galanthus nivalis* lectin (Vector Labs) is used to detect bound samples and standards, followed by a streptavidin-HRP conjugate for detection.

Other common impurities, such as immunoglobulins and protein A, result from the immunoaffinity purification of recombinant proteins or MAbs.[16] If affinity chromatography is used to purify an antigen, then an ELISA can be used to detect contaminating levels of MAbs leached from the column. An assay for the antibody needs to detect the antibody in the presence and absence of its specific antigen.

Lucas developed an assay for protein A contamination.[16] Protein A from *S. aureus* is commonly used for purifying immunoglobulin because of its specificity for binding immunoglobulins of several species. For an assay to accurately quantitate amounts of contaminating protein A, it must be able to measure it in the presence of a large excess of another protein with which it interacts (because

bound antibody might reduce the exposed regions of protein A available for recognition in the immunoassay). For a protein A assay, it was necessary to use F(ab)2 fragments for the capture and detection of antibodies because of the known reactivity of protein A with the Fc portion of antibodies.[16] MAb was added in the diluent for the standard curve of protein A. A standard ELISA can quantitate 50 pg/ml protein A in samples and is excellent for monitoring protein A impurities in process intermediates and final product.[4]

Stability

A variety of analytical methods, such as ELISA and HPLC, can be used to evaluate the effect of excipients or lyophilization on the stability of the biopharmaceutical product. Some parameters the analytical methods should evaluate are degradation, chemical and physical changes, aggregation, adsorption, and loss of biological activity.

Identifying Stable Formulations: Detecting the Effect of Excipients and Lyophilization

Proteins must be stable during processing, storage, and reconstitution (if lyophilized). Although liquid formulation is preferred for protein biopharmaceuticals, it may not always be the most stable presentation. The biopharmaceutical protein may need to be lyophilized to maintain stability. Lyophilization involves the removal of water from a frozen substance by sublimation and water vaporization under vacuum.[17] But in some cases, this process may itself cause protein instability.

Product-specific formulations should be developed, taking account of specific degradation pathways to prevent chemical and physical changes, aggregation, adsorption and loss of biological activity, and to provide long-term stability on storage.[18]

Protein instability is caused by both chemical degradation reactions and physical processes, and usually results in loss of potency.[19] High temperatures increase the flexibility and collision frequency of proteins in solution, which can result in aggregation and/or precipitation.[19] The process may adversely affect the tertiary structure of protein, and the protein then may undergo an unfolding process when it loses the surrounding water.[17] Unfolded protein molecules may have a higher tendency to aggregate.[17] The aggregation of the protein often leads to precipitation upon reconstitution.[17] After lyophilization, during storage, the proteins can undergo aggregation.[5] Aggregation can result from covalent and/or noncovalent interactions. Moisture, oxygen, and light all can induce aggregation.[5] Aggregated proteins may exhibit decreased bioactivity, altered half-life, and enhanced immunogenicity.[19]

Inclusion of a cryoprotectant in the formulation can stabilize the protein during the freezing and drying stages of lyophilization.[17] Excipients frequently

used for stabilization include sugars, amino acids, surfactants, fatty acids, proteins such as HSA, and a range of salts and buffers.[18] Solvent additives can affect protein stability by direct interaction with the protein (binding to the protein), by indirect action through effects on the solvent (increasing solvent surface tension or viscosity), or by a combination of both of these mechanisms.[19] Water-soluble synthetic polymers, such as polyethylene glycol (PEG) or poly(vinylpyrrolidone) (PVP),[19] have been used as protein stabilizing agents. The protein–solvent interaction determines the effect of the cosolutes on the stability of proteins, rather than a direct interaction of solutes with proteins.[17]

Vemuri et al.[17] looked at the effects of various cryoprotectants, freezing rates, and buffer systems on the shelf-life of lyophilized recombinant alpha$_1$-antitrypsin (rAAT). Alpha$_1$-antitrypsin (AAT) is labile in solution; therefore, a more stable presentation was required. A competitive ELISA was used to measure total AAT in a sample. The AAT in the sample competed with HRP-labeled AAT for binding to the specific antibody. A stable formulation containing lactose as a cryoprotectant was found that maintained the protein's specific activity.

Recombinant human IL-11 was under evaluation in human clinical trials for use as a thrombopoietin product for chemotherapy support.[18] An ELISA was developed to measure adsorption (residual concentration in various containers over time). The ELISA was formatted with an anti-IL-11 MAb for capture and a biotinylated antibody for detection.[18] A combination of HSA and Tween-20 was required to address both the adsorption of IL-11 to glass and retention of biological activity postlyophilization.

Lyoprotectants are added to the formulation to stabilize the protein both during the freeze-drying process and on storage.[20] Ressing et al. investigated the effect of freeze drying on the stability of a mouse IgG monoclonal (MN12).[20] MN12 is directed against the class 1 outer membrane protein of meningococcal strain H44/76. ELISA was one method used to determine changes in the physicochemical properties of the monoclonal, by measuring antigen-binding capacity.[20] Aggregation of proteins during the freeze-drying process, often leading to insoluble protein, is a common observation.[20] Degradation may occur during storage of the product.[20]

When the product itself is an antibody, ELISA can be used to measure the binding to its specific target antigen. Gombotz et al.[19] studied stabilization of IgM class human MAb used to provide therapeutic protection in animals infected with group B streptococci (GBS).[19] The ELISA was used to study the nature of the interactions between the cryoprotectant (PVP) and the antibody product (4B9). The ELISA for antigen binding measured the ability of the antibody to bind to the GBS group polysaccharide and to quantitate human IgM, which measures the amount of 4B9 antibody bound by an antihuman immunoglobulin. An ELISA was used to determine if the cryoprotectant (PVP) had an effect on the GBS antigen-binding activity of the 4B9 antibody or its ability to be quantitated by binding to an antihuman immunoglobulin.[19]

Evaluating Real-Time and Accelerated Stability

If a MAb was available that could differentiate between the native and denatured forms of a protein product, an ELISA could be used as a stability-indicating assay. The antibody could also be used for purification of the native molecule by removal of the denatured form.[21] A MAb specific for the denatured protein can detect small amounts of the denatured protein in the presence of the native form.[21]

Gu et al.[22] examined the stability of IL-1 in aqueous solution. They used HPLC for separation of degradation products, then analyzed these by ELISA and bioassay. This is a good example of using a variety of methods together to obtain the most information. The stability (of stressed samples) was analyzed at various temperature and pH conditions using HPLC, ELISA, and IEF-PAGE, and bioassay. The results indicated that the degradation mechanism of IL-1β in aqueous solution is primarily aggregation-precipitation at or about 39ºC and possible deamidation at or below 30ºC.

Another example of a MAb drug product is a chimeric mouse–human monoclonal IgG antibody specific for the Lewis-Y antigen found on the surface of tumor cells.[23] The antibody was labeled with ^{131}I to target tumor cells for radioimmunotherapy or cancer therapy, and an ELISA was used to measure binding activity. An antiidiotype-binding ELISA was used to quantitate the binding activity of selected stressed stability samples of chimeric L6.[23] The antiidiotype 1B antibody binds to the murine Fab portion of chimeric L6 and is therefore a potential indicator of chemical and conformational changes in the antigen-binding region of the protein.[23] ELISA can still bind lower-molecular weight fragments or aggregates that may also have binding activity and falsely elevate the remaining concentration using this assay.[23]

Determining Shelf Life

According to current FDA guidelines, an acceptable pharmaceutical product should exhibit less than 10% deterioration after 2 years.[5] Protein biopharmaceuticals usually have to be stored under refrigerated conditions or freeze dried to achieve an acceptable shelf life. ELISAs for aggregates and product variants as discussed previously would be applicable for determining shelf life.

QC and Manufacturing

Product Testing, Identification, Quantitation

ELISAs can be used for identification and quantitation of a biopharmaceutical product or for quantitation of impurities or contaminants as discussed previously. They can be used throughout the manufacturing process as well as in quality control or the product release stage just as they are used in all the other stages of product development. To be used for quality control, GMP practices must be followed. All methods need to be validated so that the assay's performance is documented. ELISAs should have internal quality controls to monitor assay

acceptability and performance over time. The assay must have defined limits, so it will be known if and when an assay is not acceptable.

Monitoring In-Process Steps

ELISAs can be used for identification and quantitation of the product as well as impurities in the various purification steps (as discussed previously). They can be used to document the removal of known impurities and contaminants, and in process validation to demonstrate batch-to-batch consistency of manufacturing.

STRENGTHS AND WEAKNESSES

In general, the strengths of the ELISA are its selectivity and specificity, whereas its weaknesses are related to precision of measurement. Each assay varies — depending on the antibody, enzyme, enzyme conjugate, and measurement, as well as on assay format. Each should be validated so that its unique performance characteristics are known.

Selectivity

The selectivity of ELISA is based on the reaction between antigen and antibody and thus depends on the specificity of the antibody or antibody pairs. It provides the power and uniqueness of this method as an analytical tool. Selectivity can be viewed either as a weakness or a strength depending on the particular application. Degraded forms of products or products with an altered structure may contain immune-specific epitopes that are recognized by the antibody and not differentiated from full-length or unaltered products. In this case, selectivity would be a weakness. This emphasizes the importance of MAbs. The use of MAbs can provide specificity to the epitope of interest. If a MAb recognized an epitope that was only on intact protein and not on degraded protein, selectivity would be viewed as a strength.

Polyclonal antibodies can react with many epitopes, whereas MAbs are restricted to one epitope on proteins that do not have repeating sequences.[24] By definition, polyclonal immunoassays are generally much more sensitive but less specific than monoclonal assays. Bispecific or hybrid antibodies can be used to increase the affinity. Bispecific antibodies are formed by the fusion of two previously established hybridomas to produce antibodies displaying the binding characteristics of both of the antibodies in one molecule.[25]

The binding of the antigen and antibody can be affected by several factors, including the conjugated label chosen for detection and the method used to conjugate the label, as well as the assay format itself. The selectivity of the ELISA can be affected by the assay format. In an ELISA with a two-site sandwich format, independent epitopes are bound by different antibodies.[26] The specificity comes from multiple site recognition. Polyclonal antibodies can react with many epitopes on a complex antigen surface.[24]

Because of antibody-based selectivity, ELISAs are capable of handling samples that are impure or only semipurified. It is possible to perform ELISAs in a variety of matrices. This is in contrast to other methods such as HPLC that require relatively pure material. During the development and validation of the ELISA method, it needs to be demonstrated that the ELISA is not affected by interfering substances that could be in the test sample, such as buffers, salts, contaminating proteins, and excipients. It also needs to be demonstrated that the conjugated antibody does not bind nonspecifically to the coated solid phase.

Sensitivity

Improvements in ELISA techniques over the years have lead to increased sensitivity. Sensitivity is ultimately determined by the affinity (and avidity) of the antibody used, although the label and detection system used contribute to the overall sensitivity as well. The use of some of the recently developed labels and substrates can amplify the signal for increased sensitivity. The level of sensitivity required is dependent on the application. If ELISA is used to quantitate the product or provide identification of the product in final containers, a sensitivity of μgs may be sufficient. However, if ELISA is used to quantitate impurities, a sensitivity of 1 to 2 ng may be required. For impurity ELISAs, it is possible to have good sensitivity when detecting multiple analytes. It is even possible to get in the picomolar range or even lower using techniques such as enzyme amplification, which generates an increased signal. There are a wide variety of labels now available, including those that are fluorescent and chemiluminescent.[26] With ELISA modifications such as immuno-PCR, it is possible to detect as little as 1000 molecules.[25]

Some ways to increase sensitivity include the use of MAbs or the use of a more sensitive substrate for the enzyme. The ELISA's sensitivity is from the inherent magnification of the enzyme–substrate reaction.

Accuracy

Accuracy expresses the closeness of agreement between a measured test result and its theoretical true value.[27] Accuracy is one of the properties that must be evaluated during assay validation. It is affected by the antigen–antibody reaction as well as the error of measurement. The use of plastic for the solid surface can affect the antigen–antibody reaction. There are sometimes steric constraints and the molecule can change when bound to the surface.[24] Native epitopes could be denatured or altered just by binding to the solid phase.[24] There can also be a difference in affinity depending on whether antigen or antibody is bound to the solid phase. All of these parameters can be investigated during assay development.

Accuracy also depends on the error of measurement. During assay development, it should be demonstrated that there are no plate edge effects, that is, the response is the same in every well of the multiwell plate. The adsorbing

capacity of each well in the same plate should not vary; manufacturers today can certify the homogeneity of the wells on the plate. If there are any differences, those usually occur in wells in the outer edges of the plate. If the edge effect cannot be eliminated with further development, these wells must be eliminated from the assay.

One common problem of ELISAs affecting accuracy is the hook effect. This is when the signal does not increase with increasing concentration but actually decreases. Another weakness of ELISA is that, compared to other techniques, it has a limited dynamic range. Extrapolation beyond the limits of the range of the standard curve can lead to inaccuracies.

Precision

Precision is a quantitative measure of the random variation between repeated measurements from multiple sampling of the same homogenous sample under specified conditions.[27] The weakness of the ELISA is its imprecision. The imprecision is related to the nature of the biological reaction — the reaction between antigen and antibody — and its inherent variability. Typically, the precision of an average ELISA is about 20% relative standard deviation, but can be as high as 30% in some circumstances.

Speed — Same-Day Results

Once the reagents are available, the ELISA method is fast and can be modified to accommodate high throughput. But the time to obtain the appropriate reagents must be considered. It may take considerable time to generate the appropriate antibodies by immunization, as discussed previously. Also considerable time must be allowed to develop and optimize the assays.

Over the years, in addition to developments with ELISA reagents such as labels, there have been improvements in automation. This has enabled ELISA to be utilized as a high-throughput tool. Typically, ELISAs can be performed in several hours to days. The most common practice is to precoat the microtiter plate for an overnight incubation period, with the remainder of the steps performed the following day. While ELISAs are fast when compared to other assays such as bioassays, which can take days to weeks, they might be considered slow when compared to methods like HPLC, in which the time from sample injection to chromatogram is a matter of minutes.

Simplicity

The ELISA uses equipment that is commonly accessible in immunochemical laboratories. They are usually formatted using microtiter plates, which have 96 wells. Depending on how many wells are used for standards and controls, many test samples can be run on one plate. It is common practice to run multiple plates

in one assay. This number can be greatly increased if part or parts of the assay are automated, that is, at least with the use of an automatic plate washer or automatic pipetter to dispense solutions or perform serial dilutions. Most of this equipment is relatively inexpensive. Some kind of photometric detector (spectrophotometer) or a plate reader is required. More common than not, the plate reader is controlled by software of various levels of sophistication for data recording and analysis, as well as for reporting.

ELISA is a method that is simple to perform. Ferris and Fischer[28] evaluated the performance of subjects with no formal laboratory training or experience (sixth and seventh graders), and this sample of "analysts" were able to perform the ELISA with success.

COMPARISON TO OTHER METHODS

Other Immunological Methods

Western Blot

The Western blot, also known as immunoblot, is an analytical method in which proteins are separated by polyacrylamide gel electrophoresis, transferred by electroelution to a nitrocellulose membrane, reacted with antibodies, and detected by a labeled detecting antibody that precipitates substrate onto the membrane wherever the specific antigen–antibody reaction occurred. The Western blot is most useful in early biopharmaceutical product development. It is able to associate specific bands on the acrylamide gel with reactivity with a particular antibody. It provides information regarding molecular weight and subunit structure. The ELISA does not. The advantage of the Western blot is that the samples can be complex mixtures; they do not need to be purified. The disadvantage of this method is that it is not strictly quantitative. Also, some antibodies, particularly some MAbs, may not be able to react with a target protein that has been subjected to this method, whereas they may react with a target protein in solution or present as the solid phase in an ELISA. Some proteins are denatured somewhat during the separation and transfer process.

The Western blot method is often used in the analysis of host cell impurities. It can be used to identify a recurring impurity. O'Keefe et al. used a Western blot to identify an *E. coli* protein impurity in the preparation of the recombinant fibroblast growth factor (aFGF).[29] By using specific antisera to the *E. coli* host cell proteins, they were able to isolate the impurity and determine its N-terminus amino acid sequence to confirm its identity. Antibodies could be used to determine the concentration of this impurity in sample preparations.

ELISA with Other Labels

The "E" in the acronym ELISA stands for "enzyme," indicating that an enzyme is linked, or conjugated, to one of the reactants, most commonly to the antibody.

Enzyme labels are currently by far the most common labels in use. Enzymes include HRP, alkaline phosphatase (AP) and, less often, beta-galactosidase (β-gal). The effectiveness of the conjugated antibody depends on the antibody, the label, and the procedure chosen to link the two.[30]

Other immunoassays are based on the same antibody–antigen binding reaction but use a different labeling system for detection. Instead of an enzyme label, there are radioactive isotopes, and fluorescent and luminescent labels. Some important immunoassays are defined below:

RIA: Radioimmuno assay; uses a radioactive label on the antibody or antigen. These were among some of the first types of immunoassays.

FIA: Fluorescence immunoassay; uses a fluorescent tag on the antibody or antigen. Fluorescent labels absorb light of one wavelength and reemit it at another wavelength. The label is excited by UV and emits visible light. Common fluorescent labels are fluorescein, Texas red, and GFP (green fluorescent protein).

CLIA: Chemiluminescent immunoassay; uses variety of light-generating labels. Luminescent labels are widely replacing radioactive ones in some applications because they can provide the same sensitivity without the hazard. Chemiluminescence uses specific chemicals that, when reacted, give out light, or use a blocking group that, when removed, generates light. A phosphate group is a common blocking group used to detect AP. Bioluminescence uses specialized enzyme systems that can also generate light using ATP. The most common is luciferase.

Unlabeled Format: Electrochemical Immunoassays

The immunosensor is a type of immunoassay that does not depend on labels. The antibody or antigen is coupled to a reactive surface, and then responds to the binding of the complementary antigen or antibody. Indirect immunosensors use separate labeled species to facilitate detection after binding. Direct immunosensors are those in which the reaction between an antibody and its corresponding antigen give rise to changes in the reactive surface, which can then be measured. Direct immunosensors can carry out real-time monitoring of the sample.[25]

The BIAcore biosensor measures biospecific interactions in real time.[31] The principle is to immobilize the antigen on a sensorchip surface while the antibody is allowed to continuously flow over the surface or vice versa. The interaction is then detected by surface plasmon resonance (SPR), directly registered and presented as a sensorgram. Association and dissociation rate constants can be calculated from SPR data. The BIAcore has been used in antibody engineering: in screening, selection, characterization, and epitope mapping during the different steps of generating antibodies.

Another antibody-based method is immunoligand assay (ILA) technology. The Threshold system (Molecular Devices, Sunnyvale, CA) shortens assay development and assay turnaround time.[11] It adapts a sensitive detection system originally

designed for quantitation of DNA at picogram levels and commercially available IgG-labeling reagents compatible with the detector technology.[11]

Ghobrial et al.[32] used ILA to measure HCPs impurities. In this method, immune complexes are formed between the HCPs, biotinylated capture antibodies, and fluorescein-labeled polyclonal antibodies. The complexes are then immobilized on a biotinylated nitrocellulose strip in the presence of streptavidin. After washing, an antifluorescein–urease conjugate is added, thereby forming a complex that is directly proportional to the amount of residual HCPs present. The urease hydrolyzes a solution of substrate urea to release ammonia, causing a localized pH change, which is then read by the Threshold electronics.[4] This was used for CHO proteins in recombinant human erythropoietin,[32] *E. coli* HCPs in recombinant human basic fibroblast growth factor, *E. coli* HCPs in recombinant human alpha interferon, and *E. coli* HCPs in recombinant bovine somatotropin.[11]

Protein pharmaceuticals are so complex that multiple methods are required to gain a complete picture of the sample. Methods are often used in combination. For example, a chromatography system may be used to analyze fluorescent reactants from an immunoassay-based method.

Another technology where two methods are combined is tandem liquid chromatography–immunoassay (LC-IA). LC-IA increases the selectivity and the sensitivity of assays by removing interfering species. The LC system provides a wide variety of new, high-sensitivity, high-speed methods for carrying out immunological assays. This is important for monitoring analytes in complex biological matrices.

Other Methods (Not Immunology Based)

At one end of the analytical spectrum is the bioassay, which can demonstrate what biological activity the biopharmaceutical molecule may possess, regardless of molecular structure. At the other end are structural methods that elucidate the molecular structure of the molecule, regardless of biological activity. Somewhere in the middle of the spectrum is the ELISA.

Structural Analytical Methods: HPLC, CE

Methods to measure the structure of biopharmaceuticals include tryptic peptide mapping, HPLC, capillary electrophoresis (CE), mass spectroscopy, and circular dichroism spectra.

Reversed-phase HPLC uses a nonpolar stationary phase and a polar mobile phase. The characteristics are operational simplicity, high efficiency, column stability, and ability to analyze simultaneously a broad spectrum of both closely related and widely different compounds. Separation is based on hydrophobicity.[33] Findlay et al. provide a comparison of chromatography methods with immunoassays (Table 11.1).[27]

Other commonly used techniques include capillary electrophoresis (CE) and mass spectrometry. These techniques are discussed in other chapters in this book.

Table 11.1 Differences between Chromatographic Assays and Immunoassays

	Chromatographic assays	Immunoassays
Basis of measurement	Physicochemical properties of analyte	Antigen–antibody reaction
Analytical reagents	Well-characterized and widely available	Unique and usually not widely available
Analytes	Small molecules	Small molecules and macromolecules
Detection method	Direct	Indirect
Sample pretreatment	Yes	Usually no
Calibration model	Linear	Nonlinear
Assay environment	Contains organic solvents	Aqueous
Time required for development	Weeks	Months (due to time needed for Ab generation)
Intermediate (interassay) imprecision	Low (<10%)	Moderate (<20%)
Source of imprecision	Intraassay	Interassay
Assay working range	Broad	Limited
Cost of equipment	Expensive	Inexpensive
Analysis mode	Series, batch	Batch
Assay throughput	Good	Excellent

Bioassay

Bioassays are used together with other methods to test biopharmaceutical products for potency, identity, purity, and stability. However, only a bioassay can assess the potency or biological effects of a product. Bioassays can be *in vivo* or *in vitro*. *In vivo*, or animal potency assays, measure the drug product's effect on the whole organism. The trend is away from *in vivo* and to *in vitro*, when *in vitro* methods are available. *In vitro* bioassays are typically cell culture-based assays that measure the drug product's effect on the cell. Bioassays monitor biologically active sites, whereas ELISAs monitor immunoreactive sites. These sites may or may not be the same. Bioassays can be used to monitor the desired relevant biological effects of a product and also to detect any undesirable effects of a product for safety concerns. They may be able to distinguish the activity or inactivity of product variants, whereas ELISA may not. One disadvantage of the bioassay is its imprecision. Bioassays may have percent residual standard deviation (%RSD) of 25 to 30%. Another disadvantage is that it often can take days or weeks to complete. They are useful in all phases of product development, from research to release testing for quality control.

Bioassays can be used to assess the effect of molecular heterogeneity due to glycosylation, whereas ELISA cannot. The sensitivity of the bioassay and ELISA are often comparable. Both methods can be used as stability-indicating test methods.

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