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Critical Ligand Binding Reagent Preparation/Selection: When Specificity Depends on Reagents

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Abstract

Throughout the life cycle of biopharmaceutical products, bioanalytical support is provided using ligand binding assays to measure the drug product for pharmacokinetic, pharmacodynamic, and immunogenicity studies. The specificity and selectivity of these ligand binding assays are highly dependent on the ligand binding reagents. Thus the selection, characterization, and management processes for ligand binding reagents are crucial to successful assay development and application. This report describes process considerations for selection and characterization of ligand binding reagents that are integral parts of the different phases of assay development. Changes in expression, purification, modification, and storage of the ligand binding reagents may have a profound effect on the ligand binding assay performance. Thus long-term management of the critical ligand binding assay reagents is addressed including suggested characterization criteria that allow ligand binding reagents to be used in as consistent a manner as possible. Examples of challenges related to the selection, modification, and characterization of ligand binding reagents are included.

Keywords: Ligand binding reagents, reagent characterization, assay specificity

Introduction

Ligand binding assays are the methods of choice to quantify levels of macromolecular analytes in complex biological matrices.[1-7] Macromolecular analytes usually exhibit some degree of heterogeneity and cannot easily be distinguished from other matrix components by standard physical methods such as size, charge, and hydrophobicity. Ligand binding reagents used in an appropriate assay format are designed to uniquely identify the analyte and distinguish it from other matrix components with which the analyte may share some degree of sequence homology or similar biochemical or biophysical characteristics. Availability of assays that incorporate ligand binding reagents with the optimized specificity and selectivity properties will result in a better understanding of pharmacokinetic, pharmacodynamic, immunogenicity, and manufacturing properties of the drug of interest.[7,8]

Common ligand binding reagents are antibodies, receptors, target ligands, synthetic peptides, or oligonucleotide structures known as aptamers. These ligand binding reagents have the potential to recognize specific sequence or structural features that are unique to the analyte, and thus to selectively measure these analytes in the presence of a multitude of other macromolecules. Yet only a minor portion of the macromolecular analyte may actually be recognized by the ligand binding reagent. For example, the antigenic site recognized by an antibody reagent may be only 4 to 8 amino acids in size, encompassing a contiguous linear or conformational binding epitope.[9] Similar structural sites may be present in other matrix components. Thus, specificity and selectivity of a ligand binding assay are highly dependent on the reagents and the assay format in which they are used.[10-13] Consequently, selection of the ligand binding reagents is potentially the most critical step in the assay development process, while long-term management is essential to assure consistent assay performance throughout the life cycle of the assay.

Critical Components of Bioanalytical Assays

In principle, the chromatographic and ligand binding assays used to measure drugs in biological matrices have similar requirements to assure specific and selective measurement of the analyte of interest. However, the methodology used to achieve these requirements is very different. For chromatographic methods, the critical components are extraction, chromatography, and instrumentation technologies that can be used for any analyte. For ligand binding assays, these requirements are largely achieved by the ligand binding reagents that are specific for the particular analyte. Table 1 contrasts the differences in these assay components and the

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Table 1. Critical Components of Chromatographic and Ligand Binding Assays

	Chromatographic Assays	Ligand Binding Assays
Critical Components	Extraction solutions, instruments, columns	Antibody/ligand reagents
Manufacturer	External, multiple sources	Product sponsor
Product-specificity	Not product specific	Unique to each product
Manufacturing process	Well-established, controlled, high throughput	Generate cell lines, purify, modify
Availability	Buy off the shelf	Need long lead time
Reproducibility	Assumed due to vendor quality control	Expect lot-to-lot differences Can dramatically affect method performance

consequences for assay management. The critical chromatographic components are the extraction solutions, instruments, and columns, which are typically produced by well-established manufacturers, can be obtained from multiple vendors, are expected to provide reproducible results when switching to other instruments or columns of the same type, and are used in a similar fashion for multiple small molecule products. The critical ligand binding assay components are the ligand binding reagents, which are usually specific to the particular biopharmaceutical product. Therefore generation of ligand binding assay reagents is the responsibility of the manufacturer/sponsor, often cannot commence until the product lead candidate has been identified, and may take from months to a year or more to generate. Once generated, ligand binding reagent preparations are prone to lot-to-lot variability, requiring frequent re-optimization of assays and necessitating strategies for long-term management of ligand binding reagent supply.

Stages of Ligand Binding Assay and Reagent Development

In this article, phases of assay development are defined to demonstrate that ligand binding assay reagent development and management activities occur throughout the life cycle of a biopharmaceutical product (Figure 1). Ligand binding reagent development activities often begin with formation of a discovery project team responsible for early management of research activities for a novel biopharmaceutical product. Ligand binding reagent development activities are guided by requirements of the project team for ligand binding assays to support preclinical and clinical studies. The Assay Conception phase involves planning the type of research reagents required for these assays, followed by generation, selection, production, purification, modification, and limited characterization. During the Assay Feasibility phase, a variety of individual or mixtures of research reagents and assay formats may be evaluated. Once specific reagents are identified and implemented, assay qualification activities may commence. The selected reagents are reclassified as critical reagents during the Assay Qualification phase. Larger scale production via standardized procedures as well as more extensive characterization may often occur when the assay enters this phase. Since lot-to-lot variability of critical reagents may have profound or unexpected effects on assay performance, it is usually preferred that a large quantity of material be produced during the Assay Qualification phase for continuity of use during the Validation and Maintenance and Transfer phases. Resupply of critical reagents to support long-term maintenance should, to the extent possible, follow the same procedures used for production of previous reagent lots.

Development of Ligand Binding Assay Reagents

Successful ligand binding assay development incorporates generation and selection of assay reagents as an integral part of the process. The most frequently used ligand binding reagents are polyclonal or monoclonal antibodies derived either from immunized animals[14-16] or recombinant antibody libraries.[17-19] Other ligand binding reagents selected from in vitro production methods include aptamers,[20] affibodies,[21] synthetic peptides with cloned and purified functional ligands, and soluble receptors.[22] Since the generation time and characteristics of each type of ligand binding reagents are different, a multi-tiered approach may be pursued, generating different types of reagents either in parallel or in a staggered fashion until the specified characteristics are obtained.

The approach taken for generating the ligand binding reagents may be dictated by such considerations as the presence of immunogenic epitopes, heterogeneity, and the degree of sequence homology with endogenous versions of the analyte (Table 2). Immunogenicity of the analyte may be improved by conjugation to highly immunogenic carrier molecules such as keyhole limpet hemocyanin, ovalbumin or bovine serum albumin, or by immunization of knock-out animals. Attention should also be given to the anticipated assay format, kinetics of ligand binding reagent binding, nature of the assay matrix, and study population, as well as the type and duration of the study being supported that may

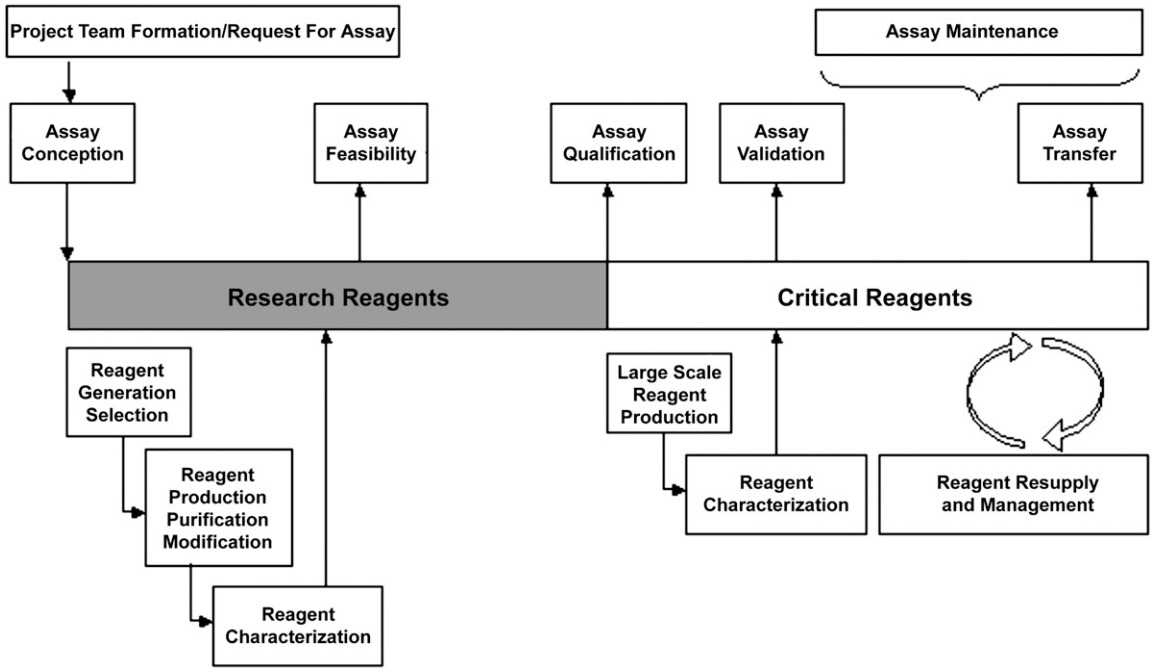

Figure 1. Schematic of management of ligand binding reagents during different phases of assay development.

determine the quantity of ligand binding reagent required. Any potential specific and nonspecific matrix-interfering components and homologous or binding molecules can all be taken into consideration during the selection processes for the ligand binding reagent.

During the Assay Conception phase, the necessary characteristics of the assay reagents (Table 3) should be defined and used to design screening assays to enable selection of such characteristics. Typically the most challenging characteristic to achieve is high binding affinity, which is required to absorb the analyte from a solution phase matrix, and thus evaluation of binding affinity may predominate in the selection process. However, depending on the intended use, other ligand binding reagent characteristics may be equally or more important, such as the ability to detect all or a select few of the various isoforms or to discriminate between free and complexed analyte. If matrix components that interfere with the detection of the analyte are known, these may be included in the ligand binding reagent screening process. Ligand binding reagents can be selected to bind to regions on the analyte that are distinct from the binding site of receptor, carrier, or binding proteins or other potential interacting molecules.

When homologous proteins are known to be present in the assay sample matrix, it is advantageous to have quantities available during the ligand binding reagent selection process to assess potential cross-reactivity and thus select the most appropriate ligand binding reagents. In addition, if sample pretreatment will be required in the assay, the pretreatment conditions may be included in the selection process.

In some cases, ligand binding reagents generated during the Assay Conception phases may be adequate for all later stages of assay development and used throughout the product life cycle. However, as the project progresses, additional information may be learned about the analyte or study population, indicating the need for improved ligand binding reagents. Therefore, for many projects, ligand binding reagent development activities form a continuum throughout the different stages of assay and drug development.

Table 2. Considerations for Determining Ligand Binding Reagent Development Approaches

Parameters For Consideration
Analyte heterogeneity
Presence of immunogenic epitopes
Species sequence homology
Other homologous or binding proteins
Assay format and readout
Assay matrix
Type and duration of study population
Specific and nonspecific interfering matrix components

Management of Critical Ligand Binding Reagents

Ligand binding reagents that support drug development phases become corporate or institutional assets, and thus management of such strategic assets is essential to sustain business

Table 3. Selectable Characteristics of Ligand Binding Reagents

Selectable Characteristics
High affinity capture
Discriminates all analyte isoforms
Discriminates free from complexed analyte
Minimal cross-reactivity with homologous family members
Detects analyte in specific conditions

objectives and timelines. The infrastructure supporting these critical ligand binding reagents is a separate topic and will not be discussed in detail here. As drug pipelines are filled with more biopharmaceuticals, the need to maintain and supply critical reagents in the appropriate quantity and quality increases. The laboratory that develops and supports multiple ligand binding assays may need to develop strategies for supply of these ligand binding reagents over the entire life cycle of the product. The approach of classifying the ligand binding reagents used in Assay Feasibility as research reagents and the reagents used during or after the Assay Qualification phase as critical reagents permits management activities to be prioritized and resourced differently for the 2 reagent classes.

As the research program advances from Assay Feasibility to Assay Qualification, the requirements for characterization and documentation increase. All ligand binding assay reagents are prone to the same issues as macromolecular drug products with regard to heterogeneity in posttranslational modifications such as glycosylation, folding, aggregation, and impurity levels. This heterogeneity increases susceptibility to lot-to-lot variability. For example, changes in a biophysical characteristic of a ligand binding reagent may affect the immunoreactivity but not the biological binding ability and vice versa. The effect of this variability on the ligand binding assay performance may not be known until reagent crossover studies are conducted. Recommended characterization methods for the ligand binding reagents classified in the research reagent and critical reagent categories may include but are not limited to those listed in Table 4, and such characterization information serves to maintain a historical record of lot-to-lot variation.

Batch records documenting the preparation of ligand binding reagents including the source of the raw material, the method of expression, purification, final formulation buffer, and characterization results prove very useful in trending assay performance. Any investigation of assay performance may require evaluation of assay reagents and whether performance trends can be attributed to ligand binding reagent substitutions. The batch record tracks all the information related to the lot of reagent, providing parameters that can be followed and associated with assay performance.

Ligand Binding Reagent Conjugation

In most assay formats, one or more of the critical reagents must be conjugated or modified to facilitate either capture or detecting of the analyte. Commonly used chemicals for creation of ligand binding assay reagent conjugates include biotin and fluorescent tags such as fluorescein isothiocyanate, phycoerythrin, and quantum dots. The most common proteins used in conjugates are enzymes such as horseradish peroxidase or alkaline phosphatase and avidin, the latter of which is used in combination with some other critical reagent that has been biotinylated. In some cases, the critical reagent may be radiolabeled or immobilized on beads or other surfaces. Many of these conjugation reagents are available commercially as individual ligand binding reagents or kits. A variety of functional groups on a protein are available for modification. Table 5 lists some examples. In addition, many useful review manuals on immunoassays serve as good references for multiple strategies and methods that can be used to develop ligand binding assay reagent conjugates.[23,24]

Modification of the protein may result in altered reactivity with the target ligand. Thus it is important to characterize the modified ligand binding reagent, especially with respect to the binding activity. Optimizing the conjugate performance may be accomplished by minor modifications to the procedure including different molar coupling ratios, altered pH, and buffer components, any of which may alter the degree or site of conjugation. Alternatively, different linkers and modification chemistry can be used. In some cases, when the inhibition of binding activity cannot be improved with standard optimization approaches, further measures can be taken to protect the active binding site during the modification process. Active site protection may be accomplished by binding the reagent to the immobilized respective binding ligand, modifying the complex and eluting the ligand binding reagent.

Since heterogeneity is usually already present in the critical reagent prior to modification, it is expected that the modification will add further heterogeneity. Often multiple sites on the ligand binding reagent are susceptible to modification, so that the modified ligand binding reagent may contain a mixture of molecules with varying numbers of modified sites. In addition, some procedures may cross-link the individual components resulting in enzyme-enzyme and antibody-antibody homodimers. Thus lot-to-lot variability is expected for modified ligand binding reagents, and therefore, whenever possible, new and old lots of materials should be characterized and tested together in the assay to ensure consistency of performance.

Surface plasmon resonance (SPR) can be an ideal method to characterize the effects of modification of ligand binding reagents with small chemical groups (eg, biotin or fluorochromes)

Table 4. Recommended Characterization for Research and Critical Reagents*

Characterization	Research Reagent	Critical Reagent
Concentration	Routine	Routine
Purity (SEC or SDS-PAGE)	>80%	>95%
Binding activity assay	Routine	Routine
Aggregate levels	Not routine	Routine
Modification label incorporation level	Not done	Routine
Isoelectric focusing	Not routine	Not routine
Functional bioassay	Not routine	Not routine
Bovine IgG levels	Not done	Not routine
Protein A levels	Not done	Not routine
Host cell protein levels	Not done	Not routine
Endotoxin level	Not done	Not routine
Affinity determination	Not done	Not routine
Formulation buffer assessment	Not done	Not routine
Stability	Not done	Freeze thaws, accelerated stability studies
2-Dimensional SDS-PAGE	Not routine	Not routine
Western blot	Not routine	Not routine
SPR	Not routine	Not routine
Agarose gel electrophoresis	Not routine	Not routine

*SEC indicates size exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IgG, immunoglobulin; and SPR, surface plasmon resonance.

since the signal generated will not be significantly affected by the presence of the modifying group. An SPR analysis was used to assess the biotinylation of a growth factor used in an assay format requiring the intact receptor binding activity. Two distinct sites on the growth factor interact with 2 different types of receptors, designated type I and type II. The receptor binding activity of the growth factor was tested before and after biotinylation using standard N-hydroxysuccinimide conjugation chemistry. The 2 types of receptors were human immunoglobulin (IgG) Fc fusion proteins and were bound to immobilized mouse anti-IgG F on a BIACore chip (Biacore Inc, Piscataway, NJ) followed by addition of either growth factor or biotinylated growth factor. The results showed a complete loss of binding activity for both receptors following the biotinylation procedure, suggesting inactivation of both receptor binding sites by the biotinylation procedure.

A bioassay is also a useful method to characterize effects of modification of reagents because it can determine the effect of large as well as small modifying groups on biological activity. This feature may be especially important when integrity of the sites involved in biological activity is also required for the optimal performance of the ligand binding assay. A dramatic loss of biological activity was observed after biotinylation of a ligand binding assay reagent using multiple ligand:biotin molar coupling ratios ranging from 1:2 to 1:10. Even when modification levels were as low as 1:2, no improvement in retention of biological activity was observed, suggesting that the highly reactive residues on the ligand are at, or close to, the receptors' active binding sites.

Table 5. Recommended Sites for Conjugation to Ligand Binding Reagents

Sites For Conjugation
Amino groups of N-terminal and amino groups of lysine
Carboxyl groups of aspartic, glutamic acid, C-terminal amino acids
Sulfhydryl of cysteine
Phenolic group of tyrosine
Indolyl group of tryptophan
Carbohydrates (oxidized)

Critical Ligand Binding Reagent Stability

For assays used over an extended duration, large lots of ligand binding assay reagents are often created to avoid potential problems of lot-to-lot variability and to minimize resources required for resupply of reagents. Therefore, strategies to ensure long-term reagent storage stability should be considered. In general, immunoglobulins and most other ligand binding assay reagents tend to be stable when stored frozen for long durations (ie, at −20°C or lower). Formal stability studies using standard biochemical

and biophysical characterization methodology are not typically conducted but may be necessary for some ligand binding reagents. Default expiration dates should be set for most ligand binding reagents. Trending of assay performance parameters with ligand binding reagent substitutions or extended durations of usage often provides sufficient information to determine whether modification of the stability period or additional stability investigations are warranted. Investigations may result in modifications to formulation or storage conditions or implementation of standard stability studies for a particular ligand binding reagent.

Ligand Binding Assay Reagent Implementation and Optimized Use in Assays

Optimizing Ligand Binding Reagents for Specificity and Selectivity

Even though ligand binding reagents may appear to be specific during the initial selection, the assay format in which they are implemented may further affect specificity and selectivity. The development of an immunoassay for detection of recombinant human bone morphogenetic protein-2 (rhBMP-2) provides an example of how selection of ligand binding reagents and assay format may be used together to address analyte heterogeneity and selectivity issues. rhBMP-2 is a member of the transforming growth factor (TGF)-β family of proteins and shares a high degree of homology with 1 or more other family members.[25,26] The molecule is expressed in a mammalian cell as a 120 kd dimeric precursor protein that is subsequently cleaved intracellularly to an ~30 kd dimeric mature protein product. As shown in Figure 2, the cleavage may take place at 2 distinct locations resulting in heterogeneity at the N-terminus. Further N-terminal heterogeneity is contributed by an N-terminal glutamine that may cyclize to form pyroglutamic acid. The mature rhBMP-2 is a highly basic protein containing a heparin binding domain and tends to nonspecifically interact with matrix components under conditions of a normal ligand binding assay. TGF-β family members may also interact with a major serum component, α-2-macroglobulin.[27]

Monoclonal antibodies were initially generated to *Escherichia coli*–expressed monomeric rhBMP-2. Two antibodies (MAbBMP2-3 and MAbBMP2-9) deemed suitable for use in a ligand binding assay for the mammalian cell-derived mature dimeric rhBMP-2 bound primarily to 2 distinct epitopes. The epitope recognized by MAbBMP2-3 was internal and common to both rhBMP-2 and rhBMP-4 but was not detected on other BMP family molecules. The epitope recognized by MAbBMP2-9 was located at the N-terminus and only reacted with rhBMP-2. A sandwich immunoassay was developed using MAbBMP2-3 as the capture ligand binding reagent and MAbBMP2-9 as the detector. To maintain the solubility and reduce nonspecific matrix effects, the assay was designed to capture rhBMP-2 in a buffer containing high salt concentration (1.5 M NaCl). Ideally, the antibody with the highest level of specificity (MAbBMP2-9) would be selected as the capture reagent. However MAbBMP2-9 was not a suitable capture reagent because of its low affinity and high dissociation rate, especially in the presence of high NaCl concentration. The cross-reactivity of MAbBMP2-3 with BMP4 was not an obstacle because BMP4 was not expected to be present in serum, and therefore this antibody was selected as the capture reagent. However, further characterization of ligand binding reagent specificity using Western blot and peptide mapping indicated that MAbBMP2-9 bound to the mature BMP-2 containing N-terminal glutamine but not to BMP-2 containing N-terminal pyroglutamic acid. Thus, the assay did not recognize all isoforms present in the analyte and additional ligand binding reagent development efforts were required. The original *E coli*–derived immunogen had not induced antibodies with specificity for the pyroglutamic acid-containing form of rhBMP-2 because of the presence of formyl methionine on the N-terminus, which inhibits glutamine cyclization. A synthetic peptide containing the N-terminal sequence with a portion of the N-termini containing pyroglutamic acid was used to generate a polyclonal antibody with a high degree of specificity for the pyroglutamic acid isoforms. The final assay format used a detector ligand binding reagent containing a mixture of monoclonal antibody MAbBMP2-9 and polyclonal antibody. Table 6 summarizes the characteristics of the 3 ligand binding reagents that were required to ensure that all isoforms of the rhBMP-2 product could be measured.

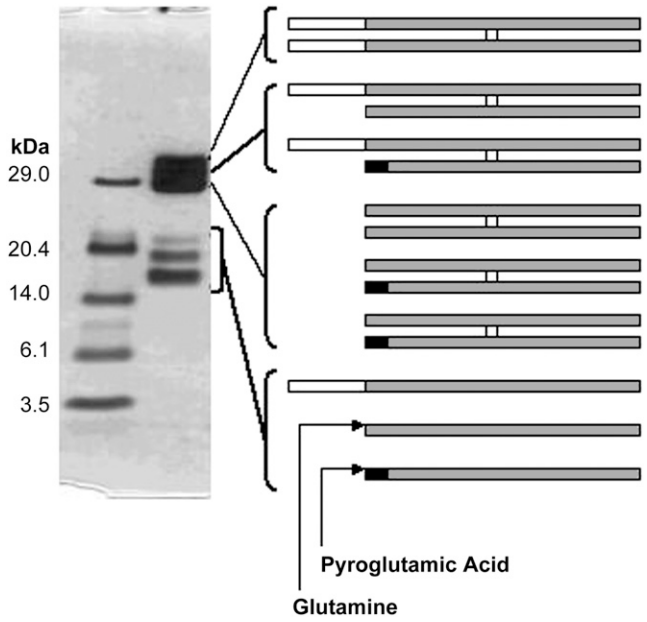

Figure 2. Western blot of rhBMP2 using MAbBMP2-3.

Table 6. Characteristics of Reagents for Optimal Detection of Isoforms*

	MAbBMP2-3	MAbBMP2-9	Pab (antipeptide)
Immunogen	*Escherichia coli*–derived f-met N-term	*E coli*–derived f-met N-term	N-terminal (<GLN-containing) peptide
Binding to isoforms	All	Some (not <GLU)	Some (<GLN)
Epitope	Internal	Mature N-terminus	Mature N-terminus
Specificity	BMP-2 BMP-4	BMP-2	BMP-2
Affinity	Moderate-High	Low-Moderate (fast off)	Moderate
NaCl tolerance	1.5 M	0.5 M	Not tested

* GLU indicates glutamic acid; GLN, glutamine.

Optimizing Ligand Binding Reagents to Measure Analyte Modifications

Macromolecule and small molecule pharmaceuticals can undergo changes in vivo that affect interactions with ligand binding assay reagents. For example, protein analytes may complex with other molecules or may undergo limited or complete proteolysis. The development of an immunoassay for detection of a growth factor binding protein that forms complexes and undergoes proteolysis in vivo provides an example of how ligand binding reagents can be selected to measure analyte after in vivo modification. At the start of the Assay Feasibility phase, it was known that the binding protein circulates either uncomplexed or as a complex with 2 other proteins. Therefore, a sandwich enzyme-linked immunosorbent assay (ELISA) using 2 monoclonal antibodies was developed and shown to detect the binding protein in either complexed or uncomplexed forms. Subsequently it was shown that the binding protein undergoes proteolysis.[28] The degree of proteolysis may vary under different physiological conditions. For example, levels of proteolytic fragments are increased in pregnancy because of increased levels of proteolytic enzyme activity. This finding correlates with the observation that the binding protein was unstable when spiked into serum or plasma from different stages of pregnancy. The monoclonal antibody sandwich ELISA was not suitable for measurement of the proteolyzed binding protein. Therefore, polyclonal antibodies to the binding protein antibodies were substituted in the assay and shown to measure all species of the binding protein.[29]

Identification of metabolic pathways and development of assays that distinguish between metabolite(s) and the small molecule parent drug are standard elements of most small molecule pharmaceutical development programs. In some cases, liquid chromatography-mass spectrometry (LC-MS) may not have the sensitivity afforded by ligand binding assays and thus reagents and immunoassays are assembled in preference to the traditional LCMS approach. The development of a sensitive immunoassay for detection of a small molecule parent drug in the presence of its metabolites provides an example of how selection of ligand binding reagents with minimal cross-reactivity was achieved. At the time of Assay Feasibility, the metabolites had been identified, and therefore the ligand binding reagent selection process incorporated the screening of the metabolite to select reagents with minimal cross-reactivity. This allowed rapid selection from a large panel of hybridomas to identify antibodies with the required specificity. Of 6000 hybridoma-containing wells, 127 had antibodies that reacted with the target small molecule parent drug and 33 recognized binding sites on the small molecule parent drug that were distinct and not present on the metabolite.

Conclusion

The performance of ligand binding assays is highly dependent on the assay reagents. Thus, the reagent selection should be an integral part of assay development. Ideally, reagent screening assays should be designed with the requirements of the ligand binding assay in mind. Once assay ligand binding reagents are selected, managing their long-term usage becomes essential to ensuring consistent assay performance. Different classifications of ligand binding reagents allow varying degrees of characterization, documentation tracking, prioritization, and associated resources during the various phases of assay development. Attention to ligand binding reagent quantity, inventory, and quality throughout the phases of assay development and life cycle of biopharmaceutical drug development ensures optimal performance and application of ligand binding assays.

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References

1. Porstmann T, Kiessig ST. Enzyme immunoassay techniques. *J Immunol Methods*. 1992;150:5-21.

2. Findlay JWA, Smith WC, Lee JW, et al. Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. *J Pharm Biol*. 2000;21:1249-1273.

3. DeSilva B, Smith W, Weiner R, et al. Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm Res*. 2003;20:1885-1900.

4. Mire-Sluis AR, Barrett YC, Devanarayan V, et al. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*. 2004;289:1-16.

5. Geng D, Shankar G, Schantz A, Rajadhyaksha M, Davis H, Wagner C. Validation of immunoassays used to assess immunogenicity to therapeutic monoclonal antibodies. *J Pharm Biol*. 2005;39:364-375.

6. Smolec J, DeSilva B, Smith W, et al. Bioanalytical method validation for macromolecules in support of pharmacokinetic studies. *Pharm Res*. 2005;22:1425-1431.

7. Shankar G, Shores E, Wagner C, Mire-Sluis A. Scientific and regulatory considerations on the immunogenicity of biologics. *Trends Biotechnol*. 2006;24:274-280.

8. Tang L, Persky AM, Hochhaus G, Meibohm B. Pharmacokinetic aspects of biotechnology products. *J Pharma Sci*. 2004;93:2184-2204.

9. Coleman PM. Structure of antibody-antigen complexes: implications of immune recognition. *Adv Immunol*. 1988;43:99-132.

10. Butler JE. *Perspectives, Configurations and Principles: Immunochemistry of Solid-phase Immunoassays.* Boca Raton, FL: CRC Press; 1991:3-26.

11. Tijssen P. *Laboratory Techniques in Biochemistry and Molecular Biology.* San Diego, CA: Elsevier Science Publishers; 1985.

12. Gosling JB, ed. *Immunoassays: A Practical Approach.* Oxford, UK: Oxford University Press; 2000.

13. Burns R. *Immunochemical Protocols.* Totowa, NJ: Humana Press; 2005.

14. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495-497.

15. Howard GC, Bethell DR. *Basic Methods in Antibody Production and Characterization.* Boca Raton, FL: CRC Press; 2001.

16. Subramanian G. *Antibodies. Vol 2. Novel Technologies and Therapeutic Use.* New York, NY: Kluwer Academic/Plenum Publishers; 2004.

17. Bradbury AR, Mark JD. Antibodies from phage antibody libraries. *J Immunol Methods*. 2004;290:29-49.

18. Lipovsek D, Pluckthun AJ. In-vitro protein evolution by ribosome display and mRNA display. *Immunol Meth*. 2004;290:51-67.

19. Konthur Z, Hust M, Dubel S. Perspectives for systematic in vitro antibody generation. *Gene*. 2005;364:19-29.

20. Brody EN, Gold LJ. Aptamers as therapeutic and diagnostic agents. *J Biotechnol*. 2000;74:5-13.

21. Nord K, Gunneriusson E, Ringdahl J, Stahl S, Uhlen M, Nygren PA. Binding proteins selected from combinatorial libraries of an α-helical bacterial receptor domain. *Nat Biotechnol*. 1997;15:772-777.

22. Engelbienne P. *Immune and Receptor Assays in Theory and Practice.* Boca Raton, FL: CRC Press; 2000.

23. Wong SS. *Chemistry of Protein Conjugation and Cross-linking.* Boca Raton, FL: CRC Press; 2000.

24. Niemeyer CM. *Bioconjugation Protocols: Strategies and Methods.* Totowa, NJ: Humana Press; 2004:283.

25. Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev*. 1994;8:133-146.

26. Wozney JM, Rosen V. Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. *Clin Orthop Relat Res*. 1998;346:26-37.

27. Webb DJ, Wen J, Karns LR, Kurilla MG, Gonias SL. Localization of the binding site for transforming growth factor-B in human alpha-2-macroglobulin to a 20-kDa peptide that also contains the bait region. *J Biol Chem*. 1998;273:13339-13346.

28. Blum WF, Ranke MB. Plasma IGFBP-3 levels as clinical indicators. In: Spencer EM, ed. *Modern Concepts of Insulin-Like Growth Factors.* New York, NY: Elsevier Science Publishing Co; 1991:381-393.

29. Harrison D, Celniker A, Reifsnyder D, Sipes D, Schroeder K, Gesundheit N. Measurement of serum insulin-like growth factor binding protein 3 using a two-site ELISA. Abstract 970 presented at: The 74th Endocrine Society Meeting; June 24-27, 1992; San Antonio, TX.