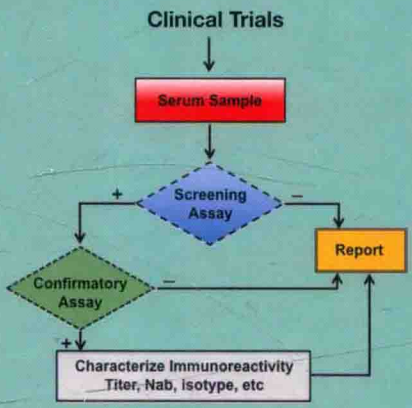
DETECTION AND QUANTIFICATION OF ANTIBODIES TO BIOPHARMACEUTICALS

Practical and Applied Considerations



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DETECTION AND QUANTIFICATION OF ANTIBODIES TO BIOPHARMACEUTICALS

INTRODUCTION: A PERSPECTIVE

MICHAEL G. TOVEY

Recombinant biopharmaceuticals represent an important class of therapeutic agents, as reflected by sales of some $92 billion in 2009 [1]. The safety and efficacy of recombinant biopharmaceuticals can be severely impaired, however, by their immunogenicity. In addition to adversely affecting pharmacokinetics, pharmacodynamics, bioavailability, and efficacy, anti-drug antibodies (ADAs) can also cause immune complex disease, allergic reactions, and in some cases severe autoimmune reactions. It is widely accepted that injection of foreign proteins into humans can elicit an immune reaction leading to the production of antibodies that in some cases may neutralize the activity of the protein. Neutralizing antibodies (NAbs) block the biological activity of a biopharmaceutical either by binding directly to an epitope within or close to the active site of the protein or by binding to an epitope that prevents binding of the drug to a cell surface receptor. It is becoming increasingly apparent, however, that repeated injection of recombinant homologues of authentic human proteins, such as interferon beta (IFN-β) or erythropoietin (EPO), especially when aggregated or partially denatured, can result in a break in immune tolerance to self-antigens, leading to the production of ADAs. This is of particular concern in the treatment of chronic diseases, including certain forms of cancer and autoimmune or inflammatory diseases such as multiple sclerosis or rheumatoid arthritis. ADAs can result in the failure of the patient to respond to therapy and may even prove to be life threatening in the case of NAbs that cross-react with essential nonredundant endogenous proteins such as EPO or thrombopoietin [2, 3]. Drug-induced immunoglobulin IgE antibodies can also cause serious anaphylactic reactions [4]. ADAs can also persist for long periods after cessation of treatment, thereby limiting subsequent treatment with the

same drug [5]. Assessment of immunogenicity is therefore an important component of drug safety evaluation in both preclinical and clinical studies and is a prerequisite for the development of less immunogenic and safer biopharmaceuticals. Immunogenicity is a complex phenomenon influenced by both drug-related factors, including molecular structure, glycosylation, and the presence of degradation products, aggregates, or impurities, and patient-related factors such as genetic makeup, age, gender, disease status, concomitant medication, and route of administration. Currently available techniques do not permit one to predict with a sufficient degree of accuracy, however, whether a product will be immunogenic and in which patients and at what point during treatment an immune response will occur.

The objective of this volume is to provide a single source of information both for those new to the field, seeking a clear understanding of the principal questions involved in the detection and quantification of antibodies to biopharmaceuticals, and for the experienced practitioner, seeking information on a specific topic. Each chapter outlines the principles of the topic covered and, when appropriate, provides sufficient background theory for a clear understanding of the subject together with practical information on how to approach each specific problem. This approach allows the information provided in this volume to be applied to well-established therapeutic proteins or classes of therapeutic proteins as well as to drugs in development or novel classes of molecules.

It is widely accepted by both regulatory agencies and industry alike that a risk-based strategy should be used to assess the potential immunogenicity of a biopharmaceutical. In Chapter 1, Eugen Koren, Erik Foehr, and Charles O'Neill describe a rational basis for the design of appropriate detection strategies and assays for antibodies to biopharmaceuticals. An approach is outlined for assessment of the antibody response to high-risk products such as a recombinant analogue of a nonredundant endogenous protein. Such an approach requires the development of a sensitive neutralization assay, frequent testing, and determination of cross-reactivity of anti-drug antibodies with the endogenous counterpart of the drug. An appropriate approach is also described for assessment of immunogenicity for low-risk products without an endogenous counterpart, where a less rigorous testing procedure may be appropriate. Consideration is also given to the development of risk-based strategies for assessment of the antibody response to multicomponent biopharmaceuticals and to biopharmaceuticals used in replacement therapy for genetic deficiencies.

The following three chapters describe the American, European, and Japanese regulatory perspectives on assessment of the immunogenicity of therapeutic proteins. It emerges from these chapters that, although differences in approach and emphasis certainly exist among the different regulatory authorities, there is nevertheless a large degree of consensus on the type of approach that should be adopted: namely, a risk-based approach that is clini-

cally driven, takes into account pharmacokinetic data, and uses appropriate screening and confirmatory assays for the detection of both binding and neutralizing ADAs

In Chapter 2, the first of the three chapters dealing with regulatory requirements, Susan Kirshner reviews US Food and Drug Administration (FDA) requirements for the assessment of the immunogenicity of a protein therapeutic, including the December 2009 FDA Draft Guidance for Industry [6]. The chapter outlines the current view of the risks of ADAs to safety and efficacy, factors that contribute to the development of ADAs, and strategies for controlling and managing an ADA response from a regulatory perspective.

In Chapter 3, Meenu Wadhwa and Robin Thorpe describe and analyze the implications of the European Medicines Agency (EMEA) Guideline on Immunogenicity Assessment [7], established by the Committee for Medicinal Products for Human Use (CHMP) of the EMEA, that came into effect in April 2008. Topics include factors that may influence the development of an immune response against a therapeutic protein, nonclinical assessment of immunogenicity and its consequences, development of assays for detecting and measuring immune responses in humans, immunogenicity and clinical development, and the establishment of a risk-based management plan. It is emphasized that the guidelines provide a general framework for a systematic and comprehensive evaluation of immunogenicity that can be modified as appropriate, case by case. It is also emphasized that evaluation of immunogenicity is an evolving process that continues for the whole life cycle of the drug and may involve postapproval clinical studies as part of pharmacovigilance surveillance.

In Chapter 4, Takao Hayakawa and Akiko Ishii describe the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) requirements for assessment of the immunogenicity of therapeutic proteins. This includes the PMDA requirements for the evaluation of the immunogenicity of biopharmaceuticals in preclinical and clinical studies and in postmarketing pharmacovigilance monitoring. Although a number of the biopharmaceuticals commercialized in Japan have previously been commercialized in Europe and North America, Takao Hayakawa and Akiko Ishii describe the example of anti-adalimumab antibodies, the incidence of which was substantially greater in the Japanese population than in Westerners, illustrating the fact that the incidence of immunogenicity of a therapeutic protein can vary from one population to another. The chapter also describes the approval process in Japan for recombinant human serum albumin (rHSA) produced in the yeast *Pichia pastoris*. The high dose of the product administered necessitated the reduction of process-derived impurities to as low a level as possible to ensure safety. Furthermore, the presence of anti-yeast IgE in some individuals due to exposure to yeast products in daily life, and hence the risk of severe hypersensitivity reactions in these individuals, led to mandatory testing for the presence of anti-pichia IgE prior to treatment. These examples illustrate how both ethnicity and dietary differences can influence the incidence of immunogenicity of therapeutic

proteins and the importance of taking such factors into consideration when testing biopharmaceuticals in different populations.

Monitoring patients for the presence of ADAs to biopharmaceuticals and correlating immunogenicity with clinical data are key factors in determining the safety of treatment and interpreting clinical data. Whether monitoring for ADAs is carried out in the context of either clinical trials or postmarketing surveillance, numerous samples will be generated that will require the establishment of appropriate screening and confirmatory assays. In Chapter 5, Klaus Bendtzen and Morten Svenson review the importance of using appropriate assays to detect ADAs in order to obtain clinically relevant data that can guide the clinician in the choice of treatment options. This is illustrated by reference to the development of screening assays for the detection of antibodies to the tumor necrosis factor alpha (TNF-α) antagonists currently in clinical use. The advantages of fluid-phase radioimmunoassays (RIAs) or fluid-phase enzyme immunoassays (EIAs) relative to the more common solid-phase enzyme-linked immunosorbent assays (ELISAs) are discussed. Thus, although ELISAs are sensitive and relative simply to use, they are subject to serum matrix effects and interference from the presence of residual drug in a sample. Bridging-ELISAs are less sensitive to the presence of residual drug but are subject to interference by rheumatoid factors or components of complements. Bridging ELISAs also fail to detect monovalent antibodies of the IgG4 subclass. On the other hand, while RIAs are less prone to false positive results due to nonspecific binding, or false negative results due to epitope masking, their application is restricted due to the use of radiolabeled probes.

In Chapter 6, Eric Wakshull and Daniel Coleman review the design of confirmatory assays, for anti-drug antibodies identified in an initial screening assay. The authors emphasize the importance of the use of a tiered strategy in which ADAs identified in a screening assay, designed to detect all ADAs, are further assayed in a confirmatory assay designed to determine their functional significance. Ideally, confirmatory assays should provide information different from that obtained in the initial screening assay. Contrary to conventional wisdom, the authors suggest that using the same assay format as that used in the screening assay, but with samples spiked with excess drug, can provide independent information while minimizing variation due to the use of different assay platforms. An additional advantage of such an approach is that it allows both screening and confirmatory assays to be run on the same plate. Confirmed positive samples can then be further characterized either in a titration assay to determine their potency or in a functional assay to determine their ability to neutralize the activity of the biopharmaceutical.

In Chapter 7, Florian Deisenhammer discusses the difficulty of detecting immunoglobulins and neutralization of biological activity in a single assay. He describes the use of pharmacodynamic parameters, particularly drug-induced biological markers, to quantify *in vivo* or *ex vivo* the activity of biopharmaceuticals such as interferons (IFNs) that are difficult to measure directly due to the very low levels present in the peripheral circulation using current treat-

ment regimens. Thus, IFN-induced gene products such as the myxovirus resistant protein A (MxA) that exhibit favorable pharmacodynamic characteristics are used widely as a biomarker of IFN activity. MxA expression is assessed either by ELISA, using a pair of monoclonal antibodies specific for the MxA protein, or by quantification of MxA mRNA using quantitative reverse transcription polymerase chain reaction (RT-PCR). The activity of IFN biomarkers such as MxA can also be determined directly in peripheral blood mononuclear cells as an indirect indication of the presence of anti-IFN NAbs. Although such an approach has the advantage of simplicity, a loss of IFN bioactivity does not necessarily correlate with the presence of NAbs.

Detection and quantification of NAbs has traditionally relied upon the use of cell-based assays. As essentially any readily measurable activity can be used as the basis for establishment of a cell-based assay for the quantification of NAbs against a particular biopharmaceutical, this has led to a wide diversity of assays. This diversity is illustrated in Chapter 8, where Anthony Meager outlines the principal types of cell-based assays used for the detection and quantification of NAbs to type I IFNs and to TNF-α antagonists. Cell-based assays for IFN-α or IFN-β range from various types of antiviral assays to quantification of the transcriptional activity of IFN-induced genes or detection of IFN-induced proteins, such as MxA, or the use of cell lines stably transfected with various reporter genes. Although IFN assays can be calibrated for quantifying IFN potency by using the appropriate World Health Organization (WHO) International IFN Standard, the polyclonal nature and variable composition of IFN NAbs—in terms of affinity, immunoglobulin class, and isotype composition as well as of epitope specificity, both between individuals and for a particular individual at different times during therapy—precludes the use of antibody standards for potency determinations. The use of cell-based assays to detect antibodies against TNF-α antagonists is confronted with the difficulties associated with the presence of high circulating levels of both free drug and drug–ADA soluble immune complexes in individuals treated with TNF-α antagonists. Provided such difficulties can be overcome, cell-based assays based on induction of apoptosis in TNF-α-sensitive cell lines can be used to quantify NAbs against TNF-α antagonists in an indirect NAb assay based on restoration of TNF-α-induced cytotoxicity. Although reference preparations for NAbs against TNF-α antagonists are not available, TNF-α activity can be calibrated using the WHO International Standard for TNF-α. Ligand binding assays using immobilized cells expressing noncleavable TNF-α may also provide a basis for the detection of NAbs against TNF-α antagonists [8].

In Chapter 9, Francesca Gilli and Antonio Bertolotto describe the use of real-time PCR for the quantification of anti-IFN-β NAbs based on measurement of IFN-β-induced MxA mRNA in interferon-sensitive cells. The advantages of this method relative to the measurement of MxA protein levels or the measurement of interferon antiviral activity using the cytopathic effect (CPE) assay are discussed in terms of savings in assay time, labor,

and materials and quality assurance. The authors also provide an analysis of the relative advantages of the use of SYBER Green to label double-stranded DNA or the use of fluorescent probes to quantify PCR products such as those used in the TaqMan® system. The choice of suitable housekeeping genes or ribosomal RNA for the normalization of results is also discussed, as are different strategies for the quantification of results, such as standard curves or the comparative cycle threshold (Ct) method. The relative advantages of different methodologies, such as manual and semiautomated methods for RNA extraction or direct analysis without RNA purification, or the use of random hexamers or oligo d(T)16 for retrotranscription of RNA, are also outlined.

Detection and quantification of NAbs has traditionally relied upon the use of cell-based assays that are difficult to standardize and subject to interference from the presence of residual drug in a sample. In Chapter 10, Bonnie Wu, George Gunn III, and Gopi Shankar discuss the use of competitive ligand-binding assays for the quantification of biopharmaceuticals, such as certain antagonistic monoclonal antibodies, that exert their action by binding to a soluble ligand, thereby preventing the ligand from interacting with a receptor or other molecule on the surface of target cells. In such instances competitive ligand-binding assays, based on the use of an electrochemiluminescence (ECL) detection system, offer a number of advantages for the quantification of NAb levels, including a wide dynamic range and a relatively high degree of tolerance to the presence of residual drug in samples. The use of an ECL ELISA-based format commercialized by Meso Scale Design is described. The authors also discuss the relative advantages of using a direct format, in which the presence of ADAs in a sample prevents ruthenium-labeled ligand from binding to immobilized drug, or indirect formats based on drug-mediated inhibition of ligand–receptor binding. Examples are also given of the use of cell-based binding assays in which the presence of ADAs in a sample inhibits the binding of labeled drug to the receptor expressed on the surface of immobilized cells. Although such assays are undoubtedly useful, as indicated by their widespread adoption, they do not, however, completely replace the need for cell-based assays. Inhibition of ligand binding does not always equate to the neutralization of biological activity. For example, monoclonal antibodies to the type I IFN receptor have been described that neutralize biological activity without inhibiting binding of IFN to its receptor [9].

Surface plasmon resonance (SPR) can be used to detect ADAs present in a serum sample by measurement of binding to the biopharmaceutical immobilized on a sensor chip. Real-time SPR-based instruments such as the Biacore® biosensor are particularly useful for the detection of low-affinity antibodies produced during the initial stages of an immune response. The immunoglobulin isotype composition of the ADAs' response to a biopharmaceutical can be readily determined using SPR. The Biacore instrument also allows antibodies to be characterized for binding to different domains of the biopharmaceutical or for cross-reactivity with an endogenous protein. In Chapter 11, Steven Swanson and Daniel Mytych describe in detail the use of the Biacore biosen-

sor for characterization of ADAs during the different stages of the development of a biopharmaceutical.

In Chapter 12, Jörgen Dahlström and Lennart Venemalm review current assays for the detection of IgE antibodies to biopharmaceuticals. IgE antibodies specific for epitopes present upon biopharmaceuticals can cause serious adverse reactions, including anaphylaxis, even upon the first treatment exposure. Pre-existing antibodies against similar antigens may cross-react with a biopharmaceutical and thus a medical history of anaphylactic reactions may indicate that a patient is at a higher risk of developing serious allergic reactions against the drug. IgE antibodies are present at low concentration in human serum and sensitive assays are required for the detection IgE ADAs.

In Chapter 13, Daniel Kramer reviews the technical and regulatory considerations involved in the standardization and validation of monoclonal-antibody-based immunoassays such as ELISAs, RIAs, and ECL for the detection of anti-drug antibodies. The author emphasizes the importance of establishing a validated assay that can detect clinically relevant events during the whole course of development of a biopharmaceutical, including postmarketing studies. A strategy and key performance criteria are outlined for the standardization and validation of immunoassays, based upon a tiered testing approach in which samples are first screened for the presence of ADAs using a validated immunoassay; samples that test positive are then further tested in a validated confirmatory assay. Additional analyses, including potency determinations, and/or assays for the presence of neutralizing antibodies, are determined on a case-by-case basis based upon perceived risk to patient safety. Such a strategy ensures an efficient assessment of immunogenicity with optimal safety evaluation, and an efficient use of resources.

In Chapter 14, Deborah Finco-Kent and Amy Grenham review the technical challenges and regulatory considerations involved in the standardization, optimization, and validation of cell-based assays for the detection of neutralizing anti-drug antibodies. The authors outline a tiered testing approach for the assessment of immunogenicity and emphasize the importance of the determination of the neutralizing capacity of ADAs where NAbs potentially pose a risk to patient safety or drug efficacy. The authors point out that it is critical to have a validated assay in place that can detect clinically relevant events that may be related to neutralizing ADAs and outline strategies and key performance parameters for the development, optimization, and validation of cell-based assays to measure NAbs. The authors also discuss the challenges posed by the development of standardized assays for assessment of the immunogenicity of biopharmaceuticals across a product class. Such standardized assays would allow the incidence of NAbs to be compared for different products within a class and allow a better understanding of the correlation between NAb development and clinical effects.

In Chapter 15, Sidney Grossberg, Yoshimi Kawade, and Leslie Grossberg describe a standardized approach to bioassay design for the quantification of neutralizing antibodies to biopharmaceuticals. The authors describe the

theoretical and experimental basis for the use of 10-fold reduction units (TRU) as a measure of NAbs to cytokines such as type I interferons or interleukin-6. This methodology is generally applicable to a wide range of protein effector molecules, and the authors illustrate the use of this methodology applied both to the commonly employed constant antigen method for quantifying NAbs and to the constant antibody method. In the first method a given concentration of an antigen is mixed with serial dilutions of serum to determine the lowest dilution that neutralizes a constant proportion of antigen. In contrast, the constant antibody method is based upon mixing a given dilution of serum with increasing concentrations of antigen. Theoretical neutralization curves based on the established model of the type I interferon antibody reaction are depicted in terms of experimentally observable quantities. As predicted by theoretical studies, the constant antibody method extends the lower limits of antibody detection.

An essential part of assay development is the determination of the assay's performance characteristics, including sensitivity, precision, and dilution linearity. In Chapter 16, Viswanath Devanarayan and Michael Tovey review the establishment of cut points and performance characteristics for anti-drug antibody assays. The authors emphasize the importance of establishing appropriate performance characteristics for assays in order to obtain reliable immunogenicity data. The determination of the different types of assay precision such as sensitivity, low positive control, precision, and in-study acceptance criteria are outlined as well as the establishment of the most appropriate type of cut point for both screening and confirmatory assays.

In Chapter 17, David Lansky and Carrie Wager discuss the importance of assessing dilution linearity for NAb assays in order to establish acceptable limits of bias. The study of dilution linearity allows the effect of sample dilution on the precision and bias of the response measured in the assay to be determined experimentally. Most cell-based assays are characterized by a nonlinear sigmoid dose–response relationship best defined by a four-parameter logistic nonlinear regression model. Although NAb levels are often expressed as a titer, the use of sample-specific ED_{50}s, which provide an estimate of the NAb concentration required to inhibit 50% of the activity of the biopharmaceutical, is preferable since comparisons among them have low variance and they are invariant with respect to slope for curves having similar asymptotes. The authors define five sources of error in the measurement of a NAb assay response (measurement bias, truncation bias, population dilution bias, specimen-specific dilution bias, and other sources of bias) and outline procedures for evaluating the similarity of the dose–response curves for different samples, establishing dilution linearity for NAb assays

In Chapter 18, Arno Kromminga and Michael Tovey discuss the challenges posed by the detection of anti-drug antibodies in the presence of residual drug. This is of particular concern in the case of therapeutic monoclonal antibodies, which are often administered at high doses and are cleared slowly from the peripheral circulation. Accurate quantification of ADA levels is also rendered

difficult by the inability to detect ADA bound to drug in the form of soluble immune complexes. The relative drug tolerance of different types of ADA assays is reviewed, together with procedures for determination of the drug tolerance of a particular assay. Each assay methodology is discussed only briefly with respect to its relative drug tolerance since each assay format is described in detail in the other chapters in this volume.

In Chapter 19, Vera Brinks, Francesca Gilli, Melody Sauerborn, and Huub Schellekens describe the development of assays for the quantification of neutralizing antibodies to human biopharmaceuticals in immune tolerant transgenic mice and the use of these assays to study factors such as changes in drug formulation that can affect the immunogenicity of drugs based on recombinant analogues of human proteins. The authors describe the development of a real-time RT-PCR bioassay, based on the expression of the interferon-induced protein MxA, for the quantification of anti-IFN-β NAbs in mice immune tolerant for human interferon beta. The results of this assay correlate with BAB titers obtained by ELISA.

In Chapter 20, Claudia Berger and Uwe Niesner outline an approach for the detection and quantification of antibodies to mixtures of recombinant enzymes administered as oral replacement therapy in the treatment of pancreatic exocrine insufficiency, a serious and life-threatening condition caused by underlying diseases such as cystic fibrosis and chronic pancreatitis. The authors outline an immunogenicity risk assessment strategy for orally administered microbial digestive enzymes that follows the same overall strategy as that used for parenteral biopharmaceuticals. The potential impact of an adverse immune response is determined for each individual product based on an assessment of a number of product-related and patient-related factors. A preclinical and clinical immunogenicity testing program including assay design and sample timing is then established for each individual product. Although microbial digestive enzymes can for the most part be classified as low-risk products due to their local action in the gastrointestinal tract, trace systemic bioavailability, and low sequence homology to endogenous counterparts, nevertheless some patients may develop an ADA response over time and it is important to have an adequate immunogenicity testing program in place for every enzyme present in a product. Characterization of IgE-mediated responses should also be an integral part of the immunogenicity assessment program for such products.

In Chapter 21, Yao Zhuang and Shalini Gupta describe a novel drug-quantification assay for the detection of anti-drug neutralizing antibodies. The assay is based upon a two-stage approach. The first step involves quantification of the bioactivity of circulating drug present in samples that have tested positive for ADAs in an immunoassay. It is expected that the bioactivity of circulating drug will be reduced in samples containing clinically relevant levels of neutralizing antibodies. In the second step the loss of recovery of the bioactivity of a given quantity of added drug is determined. The drug-quantification assay provides a number of advantages relative to conventional NAb

assays, including enhanced sensitivity, tolerance to the presence of residual drug, and applicability to a large range of biopharmaceuticals.

In Chapter 22, Christophe Lallemand and Michael Tovey describe a novel one-step method for the quantification of anti-drug neutralizing antibodies based on the use of engineered reporter cells. The reporter cells have been co-transfected with the firefly luciferase reporter gene under the control of a drug-responsive promoter together with a second construct consisting of an expression vector for the drug of interest together with a second reporter gene, *Renilla* luciferase, transcribed from a common inducible promoter. Expression of the two reporter genes is determined sequentially in the same sample. Drug concentration is quantified rapidly and with a high degree of precision by quantifying *Renilla* luciferase expression by the measurement of measurement of light emission. Secreted drug will attach to specific cell-surface receptors on the reporter cells, activating the drug-regulated reporter gene. Anti-drug antibody concentration is determined by the constant antibody method from the ratio of light emission from the two reporter genes before and after addition of a serum sample containing ADAs. The advantages of this system relative to conventional assays for the quantification of NAbs against human type I IFNs and the TNF-α antagonists infliximab, adalimumab, and etanercept are discussed. Advantages include the elimination of assay variation due to variations in cell number, the absence of sample or reagent manipulation, and the reduction of serum matrix effects to a minimum.

Assessment of immunogenicity is an important component of the evaluation of drug safety in both preclinical and clinical studies and is a prerequisite for the development of less immunogenic and safer biopharmaceuticals. Assessment of immunogenicity, and in particular detection and quantification of anti-drug antibodies, is evolving rapidly in regard to both technological developments and the expectations of regulatory authorities. This is reflected by the growing number of white papers on the subject and the recent publication of guidance from the US and EU regulatory authorities. Initiatives are also under way for the establishment of international standards for certain human anti-drug antibodies [10]. The objective of the proposed volume is to provide insight into the design, optimization, and qualification of assays, the establishment of sampling strategies, the choice of appropriate assay endpoints, and data analysis for the detection and quantification of antibodies to biopharmaceuticals. It is hoped that the availability of a single volume covering all the principal aspects of the detection and quantification of antibodies, including a review of the expectations of regulatory authorities, will facilitate a more uniform approach to the assessment of immunogenicity of biopharmaceuticals.

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