
Immunogenicity Testing of Therapeutic Protein Products — Developing and Validating Assays for Anti-Drug Antibody Detection

Guidance for Industry

**U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)**

**January 2019
Pharmaceutical Quality/CMC**

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Immunogenicity Testing of Therapeutic Protein Products — Developing and Validating Assays for Anti-Drug Antibody Detection Guidance for Industry¹

This guidance represents the current thinking of the Food and Drug Administration (FDA or Agency) on this topic. It does not establish any rights for any person and is not binding on FDA or the public. You can use an alternative approach if it satisfies the requirements of the applicable statutes and regulations. To discuss an alternative approach, contact the FDA office responsible for this guidance as listed on the title page.

I. INTRODUCTION

This guidance provides recommendations to facilitate industry’s development and validation of assays for assessment of the immunogenicity of therapeutic protein products during clinical trials. Specifically, this document includes guidance regarding the development and validation of screening assays, confirmatory assays, titration assays, and neutralization assays.^{2,3} For the purposes of this guidance, immunogenicity is defined as the propensity of a therapeutic protein product to generate immune responses to itself and to related proteins or to induce immunologically related adverse clinical events. The recommendations for assay development and validation provided in this document apply to assays for the detection of one or more anti-

¹ This guidance has been prepared by the Office of Medical Policy in the Center for Drug Evaluation and Research in cooperation with the Center for Biologics Evaluation and Research at the Food and Drug Administration.

² This document specifically does not discuss the development or validation of anti-drug antibody (ADA) assays for animal studies; however, some concepts discussed are relevant to the design of ADA studies for nonclinical testing. Refer to the International Conference on Harmonisation (ICH) guidance for industry *S6(R1) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals* for more information regarding immunogenicity assessments in animal toxicology studies. Also see the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*, where the topic “Utility of Animal Studies” is covered in detail. We update guidances periodically. For the most recent version of a guidance, check the FDA guidance web page at <https://www.fda.gov/RegulatoryInformation/Guidances/default.htm>.

³ In general, this guidance provides recommendations related to the development of therapeutic protein products intended for submission in a “stand-alone” biologics license application (BLA) under section 351(a) of the Public Health Service (PHS) Act or for submission as proposed biosimilar and interchangeable biological products under section 351(k) of the PHS Act. For additional information on clinical immunogenicity assessment of proposed biosimilar and interchangeable biological products, see the guidances for industry *Scientific Considerations in Demonstrating Biosimilarity to a Reference Product* and *Considerations in Demonstrating Interchangeability with a Reference Product*, respectively.

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drug antibodies (ADAs).⁴ This guidance may also apply to some peptides, oligonucleotides, and combination products on a case-by-case basis.⁵

In general, this document does not discuss the rationale for ADA testing or the subject- and product-specific risk factors that may contribute to immunogenicity.⁶ Also, this guidance, including any discussions of terminology used in this guidance, does not apply to in vitro diagnostic products.⁷

In general, FDA's guidance documents do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

Immune responses to therapeutic protein products have the potential to affect product pharmacokinetics, pharmacodynamics, safety, and efficacy.⁸ The clinical effects of immune responses in subjects are highly variable, ranging from no measurable effect to extremely harmful. Detection and analysis of ADA formation is a helpful tool in understanding potential immune responses. Information on immune responses observed during clinical trials, particularly the incidence of ADA induction or any implications of ADA responses affecting pharmacokinetics, pharmacodynamics, safety, or efficacy, is crucial for any therapeutic protein product development program. Accordingly, such information, if applicable, should be included in the prescribing information as a subsection of the ADVERSE REACTIONS section entitled

⁴ This guidance does not pertain to immunogenicity assays for assessment of immune response to preventative and therapeutic vaccines for infectious disease indications or to cell and gene therapy products.

⁵ General information on combination products is available at <https://www.fda.gov/CombinationProducts/default.htm>.

⁶ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*, where some of these topics are covered in detail.

⁷ Per 21 CFR 809.3(a), "In vitro diagnostic products are those reagents, instruments, and systems intended for use in the diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body. These products are devices as defined in section 201(h) of the Federal Food, Drug, and Cosmetic Act (the act), and may also be biological products subject to section 351 of the Public Health Service Act."

⁸ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*.

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Immunogenicity.⁹ Therefore, the development of valid, sensitive, specific, and selective assays to measure ADA responses is a key aspect of therapeutic protein product development.

III. GENERAL PRINCIPLES

The risk to subjects from mounting an ADA-generating immune response to a therapeutic protein product will vary with the product. FDA recommends adopting a risk-based approach to evaluating and managing immune responses to — or immunologically related adverse clinical events associated with — therapeutic protein products that affect their pharmacokinetics, pharmacodynamics, safety, and efficacy.¹⁰ Immunogenicity tests should be designed to detect ADA that could mediate unwanted biological or physiological consequences such as neutralizing activity or hypersensitivity responses.

A. Assays for ADA Detection

Screening assays, also known as binding antibody assays, are used to detect antibodies that bind to the therapeutic protein product. The specificity of ADA for the therapeutic protein product is usually established by competition with a therapeutic protein in a confirmatory assay. ADAs are characterized further using titration and neutralization assays. Titration assays characterize the magnitude of the ADA response. It is important to characterize this magnitude with titration assays because the impact of ADA on pharmacokinetics, pharmacodynamics, safety, and efficacy may correlate with ADA titer and persistence rather than incidence (Cohen and Rivera 2010). Neutralizing antibodies (NABs) refer to those ADA with the ability to interfere with interactions between the therapeutic protein product and its target. Neutralization assays assess ADA for neutralizing activity. It is important to characterize neutralizing activity of ADA because the impact of ADA on pharmacokinetics, pharmacodynamics, safety, and efficacy may correlate with NAB activity rather than ADA incidence (Calabresi et al. 2007; Goodin et al. 2007; Cohen and Rivera 2010; Wang et al. 2016; Wu et al. 2016). Similarly, in some cases it may be useful to establish NAB titers in addition to NAB qualitative results (for example, positive or negative), depending on immunogenicity risk assessment. Additional characterization assays, including isotyping, epitope mapping, and assessing cross-reactivity (for example, to endogenous counterparts or to other products), may be useful.

The optimal time to design, develop, and validate ADA assays during therapeutic protein product development depends on the risk assessment of the product (Mire-Sluis et al. 2004; Gupta et al. 2007; Shankar et al. 2008; Gupta et al. 2011). The sponsor should provide an immunogenicity risk assessment as well as a rationale for the immunogenicity testing paradigm in the original investigational new drug application (IND). FDA encourages sponsors to test samples during

⁹ Among other requirements, prescription drug labels must include information about the drug's adverse reactions (21 CFR 201.57(c)(7) and 21 CFR 201.57(a)(11)). Adverse reaction is defined in 21 CFR 201.57(c)(7) as "an undesirable effect, reasonably associated with use of a drug, that may occur as part of the pharmacological action of the drug or may be unpredictable in its occurrence."

¹⁰ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*.

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phase 1 and phase 2 studies using suitable screening, confirmatory, and in some instances neutralization assays. Samples derived from pivotal clinical studies should be tested with fully validated assays.¹¹ When immunogenicity poses a high clinical risk and real-time data concerning subject responses are needed (for example, when there is an endogenous counterpart with non-redundant function), FDA may request that assays suitable for their intended purpose be developed before initiating clinical studies and that testing be performed in real time. In such instances, timing and reporting of ADA assessment should be discussed with the Agency. In other situations, the sponsor may store subject samples so they can be tested when suitable assays are available. At the time of license application, the sponsor should provide data supporting full validation of the assays (see section VIII). Recommendations regarding the timing of ADA sample collection are provided in section VII.A.¹²

B. Limitations in Comparing ADA Incidence Across Products

Results from assays for detection of ADA facilitate understanding of the immunogenicity, pharmacokinetics, pharmacodynamics, safety, and efficacy of therapeutic protein products. However, detection of ADA is dependent on key operating parameters of the assays; for example, sensitivity, specificity.¹³ Although information on ADA incidence is typically included in the prescribing information under an *Immunogenicity* subsection of the ADVERSE REACTIONS section, FDA cautions that comparison of ADA incidence across products, even for products that share sequence or structural homology, can be misleading because detection of ADA formation is highly dependent on the sensitivity, specificity, and drug tolerance level of the assay. Additionally, the observed incidence of ADA is influenced by multiple factors including method, sample handling, timing of sample collection, concomitant medications, and disease condition. Therefore, comparing immunogenicity rates across therapeutic protein products with structural homology for the same indication is unsound, even though fully validated assays are employed. When a direct comparison of immunogenicity across different therapeutic protein products that have homology — or across similar therapeutic proteins from different sources — is needed, the comparison data should be obtained by conducting a head-to-head clinical study from which samples obtained are tested using an assay demonstrated to have equivalent sensitivity and specificity for antibodies against both therapeutic protein products.

The recommendations on assay development and validation provided in this guidance are based on common issues encountered by the Agency upon review of immunogenicity submissions. Sponsors should contact FDA for any product-specific advice, particularly for high-risk products; for example, products with endogenous counterparts that have non-redundant

¹¹ Pivotal clinical studies may be used to evaluate and establish the efficacy of the product.

¹² See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*, where immunogenicity risk assessment and mitigation considerations are covered in detail. Guidance on appropriate assay development and validation for immunogenicity testing is also available in the ICH guidances for industry *Q2A Text on Validation of Analytical Procedures* and *Q2B Validation of Analytical Procedures: Methodology*.

¹³ See the United States Pharmacopeia (USP) General Chapters 1106 *Immunogenicity Assays — Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* and 1106.1 *Immunogenicity Assays — Design and Validation of Assays to Detect Anti-Drug Neutralizing Antibody* for a broader discussion of various assay types.

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function.¹⁴ Assay designs for isotyping, epitope mapping, and cross-reactivity with endogenous counterparts should be discussed with FDA. Other publications may also be consulted for additional insight (Mire-Sluis et al. 2004; Gupta et al. 2007; Shankar et al. 2008; Gupta et al. 2011).¹⁵ In general, FDA recommends that sponsors develop assays that are optimized for sensitivity, specificity, selectivity, drug tolerance, precision, reproducibility, and robustness (see sections IV.C through H).

IV. ASSAY DESIGN ELEMENTS

This section applies to all types of assays for detection of ADA, unless specified otherwise. The bioanalytical scientist should evaluate the applicability of these factors and others based on emerging science. FDA's thinking on this matter may change as the science evolves.

A. Testing Strategy

1. Multi-Tiered Testing Approach

FDA recommends a multi-tiered ADA testing approach (see Appendix). In this paradigm, a sensitive screening assay is initially used to assess clinical samples. To gain a more accurate understanding of the natural history of the ADA response, the screening assay should be sensitive and designed to detect low levels of low- and high-affinity ADA; for example, by minimizing wash steps. However, in most cases it is not necessary to empirically determine the affinity of antibodies that are detected by the initial screening assay. Samples testing positive in the screening assay are then subjected to a confirmatory assay to demonstrate that ADAs are specific for the therapeutic protein product. For example, a competition assay could confirm that an antibody is specifically binding to the therapeutic protein product and that the positive finding in the screening assay is not a result of non-specific interactions of the test serum or detection reagent with other materials in the assay milieu such as plastic or other proteins.

Samples identified as positive in the confirmatory assay should be further characterized in other assays, such as titration and neutralization assays. In some cases, assays to detect cross-reactivity to other proteins, such as the corresponding endogenous protein, may be needed. For example, assessment of cross-reactivity may be needed when the therapeutic protein product belongs to a family of proteins with high homology and it is important to know whether other family members are affected by ADA. Further, in some cases tests to assess the isotype of the antibodies or their epitope specificity may also be recommended once samples containing antibodies are confirmed as positive. Epitope specificity determination of the ADA response is not frequently performed, although it is common to perform a more general assessment of domain specificity for multi-domain products such as pegylated proteins, antibody-drug conjugates, and bispecific antibodies (see section IV.A.3).

¹⁴ Ibid.

¹⁵ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*.

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2. Immunoglobulin Isotypes or Subtypes

The initial screening assay should be able to detect all relevant immunoglobulin (Ig) isotypes. For non-mucosal routes of administration and in the absence of a risk of anaphylaxis, the relevant ADA isotypes are IgM and IgG. For mucosal routes of administration, IgA isotype ADAs are also relevant.¹⁶ Although FDA expects that all relevant isotypes be detected in screening assays, it is not necessary that the screening assay establishes which isotypes are being detected. For example, the bridging assay format can theoretically detect antibodies of most isotypes but does not provide information on which isotypes are being detected.¹⁷

In some circumstances the sponsor should develop assays that discriminate between antibody isotypes. For example, for therapeutic protein products where there is a high risk for anaphylaxis or where anaphylaxis has been observed, results from antigen-specific IgE assays may be informative.

Assessment of ADA subtype may be informative in some situations. For example, the generation of IgG4 antibodies has been associated with immune responses generated under conditions of chronic antigen exposure, such as factor VIII treatment, and in erythropoietin-treated subjects with pure red cell aplasia (Matsumoto et al. 2001; Aalberse and Schuurman 2002). Consequently, depending on the clinical concern, assessing for specific isotypes or subtypes may be needed.

3. Domain Specificity

Some proteins possess multiple domains that function in different ways to mediate clinical efficacy. An immune response to one domain may inhibit a specific function while leaving others intact. FDA recommends that sponsors direct initial screening and confirmatory tests against the whole therapeutic protein product. For multi-domain therapeutic protein products, the sponsor may need to investigate whether the ADA binds to specific clinically relevant domains in the protein. For example, to adequately understand the risk of ADA to subjects for therapeutic protein products with modifications such as pegylation, sponsors should develop assays to determine the specificity of ADA for the protein component as well as the modification to the therapeutic protein product (Gorovits et al. 2014).

The domain specificity is generally assessed in ADA samples confirmed positive using the whole molecule. Examination of immune responses to therapeutic protein products with multiple functional domains such as bispecific antibodies may require development of multiple assays to measure immune responses to different domains of the molecules (see section IV.L.4).

¹⁶ Mucosal routes of administration include oral, respiratory, vaginal, ocular, and rectal, where the drug is delivered across a mucosal barrier.

¹⁷ Bridging assays may not be adequately robust for detecting IgG4 antibodies, which may underestimate the levels of antibodies.

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B. Assay Cut-Point

The cut-point of the assay is the level of response of the assay that defines the sample response as positive or negative. Information specific to establishing the cut-point for the respective assay types is provided in sections V and VI. Establishing the appropriate cut-point is critical to minimizing the risk of false-negative results.

The cut-point of the assay can be influenced by a myriad of interfering product or matrix components.¹⁸ These components should be considered early on in assay development when defining the cut-point and are discussed in detail in section IV.K. Because samples from different target populations and disease states may have components that can cause the background signal from the assay to vary, different cut-points may be needed for discrete populations.

Where feasible, the cut-point should be statistically determined using samples from treatment-naïve subjects.¹⁹ By performing replicate assay runs with these samples, the variability of the assay can be estimated. The statistical approach employed to determine the cut-point may entail various processes, such as removing statistical outliers from analyses, and using an approach to account for pre-existing antibodies. During assay development, a small number of samples may be used to estimate the cut-point.

The sponsor should consider the impact of statistically determined outlier values and true-positive samples when establishing the cut-point. The sponsor should provide justification for the removal of any data points, along with the respective method used to determine their status as outliers. Sponsors should consult with FDA if there is a concern regarding the exclusion of outliers.

Apparent positive values and samples may derive from the presence of pre-existing antibodies or other serum factors in subject samples (Ross et al. 1990; Turano et al. 1992; Coutinho et al. 1995; Caruso and Turano 1997; van der Meide and Schellekens 1997; Boes 2000). Although pre-existing antibodies to a variety of endogenous proteins are present in healthy individuals, these can be much higher in some disease states. The sponsor should identify those samples with pre-existing antibodies (for example, through competition with drug) and remove them from the cut-point analysis. If subjects in the study have pre-existing antibodies, it may be necessary to assign positive responses using a cut-point based on the difference between individual subject results before and after exposure to identify subjects in whom ADA increases following treatment, also known as treatment-boostered ADA. A common approach to evaluating treatment-boostered ADA responses is to assess changes in antibody titers. If it is not possible to use the methods described earlier in section IV.B for establishing the cut-point, sponsors should consult with the Agency to explore alternative methods.

¹⁸ The term *matrix* when used in this guidance may include serum, plasma, saliva, etc.

¹⁹ Treatment-naïve subjects could be healthy individuals or a patient population not exposed to a therapeutic protein product, depending on the stage of assay development or validation and the availability of samples. Sponsors should provide justification for the appropriateness of the samples used.

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C. Sensitivity

1. Assay Sensitivity

Assay sensitivity is the lowest concentration at which the antibody preparation consistently produces either a positive result or a readout equal to the cut-point determined for that assay. The assays should have sufficient sensitivity to enable detection of ADA before they reach levels that can be associated with altered pharmacokinetic (PK), pharmacodynamic (PD), safety, or efficacy profiles. Assay sensitivity is assessed using positive control antibody preparations that may not represent the ADA response in a specific subject. For example, positive controls are frequently developed under conditions that enrich for high affinity antibodies. Such high affinity positive controls may overestimate the sensitivity of the assay. Because of this, the assay sensitivity determination contributes to the overall understanding of how the assay performs rather than setting an absolute mass of ADA that will be detected in any given subject. Because the measurement of assay sensitivity can be affected by onboard drug, it is also important to determine assay sensitivity in the presence of the expected concentration of onboard drug (see section IV.C.2).²⁰ FDA recommends that screening and confirmatory IgG and IgM ADA assays achieve a sensitivity of at least 100 nanograms per milliliter (ng/mL) although a limit of sensitivity greater than 100 ng/mL may be acceptable depending on risk and prior knowledge. Traditionally, FDA has recommended sensitivity of at least 250 to 500 ng/mL. However, recent data suggest that concentrations as low as 100 ng/mL may be associated with clinical events (Plotkin 2010; Zhou et al. 2013). It is understood that neutralization assays may not achieve that level of sensitivity. Assays developed to assess IgE ADA should have sensitivity in the high picograms per milliliter (pg/mL) to low ng/mL range.

The sensitivity should be expressed as mass of antibody detectable/mL of undiluted matrix; for example, plasma, sera, saliva. Assay sensitivity should not be reported as titer. Assay sensitivity should be reported after factoring in the minimal required dilution (MRD). For example, an assay with 50 ng/mL sensitivity and an MRD of 20 would be reported as 1000 ng/mL. Testing of assay sensitivity should be performed with the relevant dilution of the same biological matrix as will be used to test the clinical samples. For example, assay sensitivity should be determined using the same anticoagulant as the diluent used with clinical samples.

During development, sensitivity may be assessed by testing serial dilutions of a positive control antibody of known concentration, using individual or pooled matrix from treatment-naïve subjects. The dilution series should be no greater than two- or threefold, and a minimum of five dilutions should be tested. The sensitivity can be calculated by interpolating the linear portion of the dilution curve to the assay cut-point.

A purified preparation of antibodies specific to the therapeutic protein product should be used as the positive control to determine the sensitivity of the assay so that assay sensitivity can be reported in mass units/mL of matrix. Positive control antibodies used to assess sensitivity can take the form of polyclonal preparations affinity purified against the therapeutic protein product or monoclonal antibodies (mAb).

²⁰ See the USP General Chapters 1106 and 1106.1 for a discussion on *Relative Sensitivity*.

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During routine performance of the assay, a low positive system suitability control should be used to ensure that the sensitivity of the assay is acceptable across assay runs. Additionally, the low positive control should be consistently demonstrated as positive in both screening and confirmatory tiers (see section IV.J.1). Both positive and negative controls are discussed in detail in sections IV.J.1 and IV.J.2.

2. Drug Tolerance, Sensitivity, and Assay Suitability

The therapeutic protein product or its endogenous counterpart present in the serum may interfere with the sensitivity of the assay. The assessment of assay sensitivity in the presence of the expected levels of interfering therapeutic protein product, also known as the assay's drug tolerance, is critical to understanding the sensitivity and suitability of the method for detecting ADA in dosed subjects.²¹ FDA recommends that sponsors examine assay drug tolerance early in assay development. The sponsor may examine drug tolerance by deliberately adding different known amounts of positive control antibody into ADA-negative control samples in the absence or presence of different quantities of the therapeutic protein product to determine whether the therapeutic protein product interferes with ADA detection. Results obtained in the absence and presence of different quantities of the therapeutic protein product under consideration should be compared. Drug tolerance may be improved using approaches such as acid dissociation that disrupt circulating ADA-drug complexes. The selectivity of the assay, the nature of the target, and the type of positive control should be taken into consideration when developing the assay because these factors impact the assessment of drug tolerance. For example, acid dissociation may not be appropriate when antibodies are acid labile or the drug target is soluble. Interference from the therapeutic protein product can be minimized by collecting subject samples at trough drug levels. See section VII.A for recommendations regarding the timing of ADA sample collection.

D. Specificity

Specificity refers to the ability of a method to exclusively detect the target analyte, in this case the ADA.²² Lack of assay specificity can lead to false-positive results, which could obscure relationships between ADA generating immune response, pharmacokinetics, pharmacodynamics, and clinical safety and efficacy measures. Demonstrating the specificity of antibody responses to mAb, Fc-fusion proteins, and Ig-fusion proteins poses challenges because of the high concentration of Ig in human serum. The assay should specifically detect anti-mAb antibodies but not the mAb product itself, soluble drug target, non-specific endogenous antibodies, or antibody reagents used in the assay. Similarly, for subject populations with a high incidence of rheumatoid factor (RF), the sponsor should demonstrate that RF does not interfere with the detection method or that the assay can differentiate between RF and specific antibodies. RF is discussed in detail in section IV.L.2. In cases where ADA demonstrates cross-reactivity with host cell proteins and other product-related impurities, the specificity of these reactions may need further evaluation.

²¹ See the USP General Chapters 1106 and 1106.1.

²² Ibid.

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A straightforward approach to addressing specificity is to demonstrate that binding can be blocked by soluble or unlabeled purified therapeutic protein product. One approach is to incubate positive and negative control antibody samples with the purified therapeutic protein product or its components under consideration. Inhibition of signal in the presence of the relevant therapeutic protein product or its components indicates that the response is specific. Establishing the specificity of multimeric antibodies such as IgM by competitive inhibition may be difficult, so establishing assay capability for these circumstances requires careful development or additional approaches. For ADA to mAb products, inclusion of another mAb with the same Fc but different variable region can be informative. If the assay is specific and selective for ADA to the therapeutic protein product being studied, generally the addition of that therapeutic protein product or its components in solution will reduce the assay signal. Conversely, addition of the therapeutic protein product or its components should have little effect on antibodies of other specificities.

E. Selectivity

The selectivity of an ADA assay is its ability to identify ADAs specific to the therapeutic protein product in the presence of other components in the sample. Assay results may be affected by interference from the matrix or onboard therapeutic protein product. It is important to note that most assay matrices contain significant amounts of proteins of various sizes and charges. Failure to establish selectivity can contribute to non-specific signal, thereby obscuring positive results.

1. Matrix Interference

An important consideration is how the sample matrix (for example, plasma, serum, saliva) can affect assay performance. Some degree of signal suppression is expected when comparing assay performance in diluent versus matrix. Endogenous and exogenous components in a matrix may influence assay results, and it is usually necessary to dilute subject samples for testing to minimize such effects. The sponsor should define the matrix and dilution factor that will be used for preparation of subject samples before performing validation studies assessing potential interference of this matrix on assay results (see section IV.E.2 on MRD).

Various substances in the matrix, such as free hemoglobin (hemolysis), lipids (lipemia), bilirubin (icterus), and presence of concomitant medications, can interfere with assay results. For example, the anticoagulants used during sample collection may have different effects on the assay, potentially affecting the assay sensitivity. The sponsor may examine matrix interference by spiking different known amounts of positive control antibodies in the presence or absence of matrix. Comparing the recovery of ADA in buffer alone with that in the matrix can provide input on the degree of interference from matrix components. Furthermore, such analysis may guide decisions on the MRD recommended for sample testing. This information may be useful to understanding assay sensitivity.

Buffer components that are chemically related to the therapeutic protein product may also cause interference in the assay. For example, polysorbate is chemically similar to polyethylene glycol

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(PEG) and therefore may interfere in the detection of anti-PEG antibodies. The chemical composition of the buffer should be carefully considered during assay development.

2. *Minimal Required Dilution*

Matrix components can contribute to non-specific signal, thereby obscuring positive results. Therefore, there is frequently a need to dilute subject samples to maintain a reasonable ability to detect ADA. Multiple definitions of MRD have been proposed, including the sample dilution that yields the highest signal-to-noise ratio; the sample dilution that results in a signal closest to assay diluent; and the sample dilution that results in the highest signal to variability ratio (Mire-Sluis et al. 2004).²³ Sponsors may use any of these definitions, but for the purposes of calculating assay sensitivity and titer, the MRD should take into consideration the final dilution of the sample in the assay, which typically ranges from 1:5 to 1:100 (that is, 1/5 to 1/100).

FDA recommends that sponsors determine the MRD from a panel of appropriate number of samples from treatment-naïve subjects. Determination of MRD usually involves serially diluting treatment-naïve ADA-negative samples, as well as testing known amounts of purified antibody at high, medium, and low concentrations in serially diluted matrix in comparison to the same amount of positive control antibody in diluent. This ensures a reasonable signal-to-noise ratio throughout the range of the assay. The MRD should be calculated using an appropriate number of individual serum samples. The appropriate number of samples will depend on various factors, including the variability of the individual samples; however, at least 10 samples are frequently recommended (Mire-Sluis et al. 2004).²⁴

Although the MRD ultimately selected by the sponsor will depend on the assay design and subject population, FDA recommends that MRD not exceed 1:100. Higher MRD may result in false-negative responses. However, in some instances higher MRD may be required, and the overall effect of such MRD on assay sensitivity and immunogenicity risk assessment should be considered.

F. Precision

Precision is a measure of the variability in a series of measurements for the same material run in a method. Results should be reproducible within and between assay runs to assure adequate precision.²⁵ Demonstrating assay precision is critical to the assessment of ADA because assay variability is the basis for determining the cut-points and ensuring that low positive samples are detected as positive. To provide reliable estimates, the sponsor should evaluate both intra-assay (repeatability) and inter-assay (intermediate precision) variability of assay responses. In cases

²³ Ibid.

²⁴ Ibid.

²⁵ For more information on precision, see the guidance for industry *Bioanalytical Method Validation*. Also see the USP General Chapters 1106 and 1106.1.

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where a floating cut-point is needed, inter-assay precision may be calculated using normalized values.

G. Reproducibility

Reproducibility is an important consideration if an assay will be run by two or more independent laboratories during a study, and a sponsor should establish the comparability of the data produced by each laboratory.²⁶ Comparable assay performance, including sensitivity, drug tolerance, and precision, should be established between laboratories.

H. Robustness and Sample Stability

Assay robustness is an indication of the assay's reliability during normal usage²⁷ and is assessed by the capacity of the assay to remain unaffected by small but deliberate variations in method and instrument performance that would be expected under relevant, real-life circumstances in routine laboratory practice. For example, changes in temperature, incubation times, or buffer characteristics such as pH and salt concentration can all impact assay results. The complexity of bioassays makes them particularly susceptible to variations in assay conditions, and it is essential to evaluate and optimize parameters such as cell passage number, incubation times, and culture media components. The sponsor should examine robustness during the development phase, and if small changes in specific steps in the assay affect results, precautions should be taken to control that step. Some aspects of robustness may be included in the assay validation exercise (see section VI.A). Because it is generally not feasible to establish the stability of subject samples, FDA recommends storing subject samples in a manner that preserves antibody reactivity at the time of testing. FDA recommends that sponsors minimize freeze-thaw cycles by appropriately aliquoting subjects' samples because freezing and thawing such samples may also affect assay results. However, studies evaluating short-term stability, including, as relevant, freeze-thaw cycle and refrigerator- and room-temperature stability of positive control antibodies, may be useful.

I. Selection of Format

Different assay formats and instrumentation are available that can be used for detection of ADA. These include, but are not limited to, direct binding assays, bridging assays, and soluble-phase binding assays; for example, radioimmunoprecipitation assay. Each assay format has advantages and disadvantages, including throughput, sensitivity, selectivity, dynamic range, ability to detect various Ig isotypes, ability to detect rapidly dissociating antibodies, and availability of reagents. Bridging assay formats may be subject to false-negative results when the antigen (for example, PEG) has repetitive motifs. One of the major differences between these assay formats is the number and vigor of washes, which can influence assay sensitivity. Epitope exposure is also

²⁶ For more information on reproducibility, see the guidance for industry *Bioanalytical Method Validation*. Also, see the USP General Chapters 1106 and 1106.1; the USP General Chapter 1225 *Validation of Compendial Procedures*; and the ICH guidance for industry *Q2B Validation of Analytical Procedures: Methodology*.

²⁷ For more information on robustness, see the ICH guidance for industry *Q2B Validation of Analytical Procedures: Methodology*. Also see the USP General Chapters 1106 and 1106.1.

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important to consider because binding to plastic or coupling to other agents (for example, fluorochrome, enzyme, or biotin reporters) can result in conformational changes of the antigen that can obscure, expose, modify, or destroy relevant antibody binding sites on the therapeutic protein product in question.

J. Selection of Reagents

Many components of the assays for ADA detection may be standard or obtained from commercial sources; for example, microtiter plates. Other components, however, including positive control antibodies, negative controls, and system suitability controls, may need to be generated specifically for the assay. Qualification and stability of critical reagents is important for ensuring consistent assay performance.

1. Development of Positive Control Antibodies

Sponsors may use the same or different positive control antibodies to develop, validate, and monitor system suitability during routine assessment of assay performance. For system suitability controls, a positive control antibody, either mono- or polyclonal, used at concentrations adjusted to ensure assay sensitivity and detect hook effects, should be included.²⁸

Different approaches may be used to generate a positive control. Most frequently, positive control antibodies are generated by immunizing animals in the absence or presence of adjuvants. FDA recommends that positive control antibodies generated by immunizing animals be affinity purified using the therapeutic protein product. This approach enriches the polyclonal antibody preparation for ADA, which enables a better interpretation of sensitivity assessment results. The selection of animal species when generating positive control antibodies should be carefully considered. For example, if an anti-human Ig reagent will be used as a secondary reagent to detect antibodies in subjects, the positive control antibodies and quality control (QC) samples ideally should be detectable by that same reagent. When the positive control antibody is not detectable by that same reagent (for example, if the positive control is generated in a rabbit and a different secondary reagent is needed to detect the positive control antibody), a positive control antibody for the secondary reagent used to detect human antibodies in the subject samples also should be included in the assay to ensure that the reagent performs as expected. In some instances, the sponsor may be able to generate a positive control antibody from subjects' samples.²⁹ Such subject-derived positive controls can be very valuable but are generally not available in early trials. Alternatively, individual mAb or panels of mAb may be used as positive control antibodies. For therapeutic mAb, the sponsor should select a positive control antibody that binds to the variable region of the therapeutic mAb. Sponsors should discuss with FDA alternative approaches to assay development and validation in the rare event that a sponsor is not able to generate a positive control antibody.

²⁸ Hook effects are a reduction in signal that may occur because of the presence of a high concentration of a particular analyte or antibody and may cause false-negative results.

²⁹ Proper informed consent from patients is needed and should be planned ahead.

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Once a source of a positive control antibody has been identified, the sponsor should use that source to assess assay performance characteristics such as sensitivity, selectivity, specificity, drug tolerance, and reproducibility. FDA recommends that sponsors generate and reserve positive control antibody for use as a quality control or system suitability control during routine performance of the assay. For assay development and validation, dilutions should generate high, intermediate, and low assay signal values. The intermediate value is useful for assessing precision during assay validation. This is recommended even for development of qualitative assays to understand whether assay performance is acceptable across a broad range of antibody concentrations. Intermediate-value QC samples for detection of ADA are generally not needed for monitoring system suitability during routine assay performance.

2. Development of Negative Controls

FDA recommends that sponsors establish a negative control for validation studies and subject-sample testing. In this regard, a pool of sera from an appropriate number of treatment-naïve subjects can serve as a negative control. Importantly, the value obtained for the negative control should be below but close to the cut-point determined for the assay in the subject population being tested. Negative controls that yield values far below the mean value derived from individual serum samples used to establish the cut-point may not be useful in ensuring proper assay performance.

When possible, negative control samples should be collected from treatment-naïve subjects with the medical condition being studied and should include subjects with similar gender, age, and concomitant medications so that the sample matrix is representative of the study population. Control samples should be collected and handled in the same manner as study samples with respect to, for example, type of anticoagulant used, volume, and sample preparation and storage because these pre-analytical variables can impact the performance of control samples in the assay. It is frequently the case that such control samples are not available for use during development or pre-study validation exercises. In those situations, it is acceptable to use purchased samples or samples from healthy donors, but important parameters of assay performance such as cut-point, sensitivity, and selectivity should be confirmed when samples from treatment-naïve subjects from the appropriate target population become available. If cut-point and selectivity differ when negative controls from different populations are used, re-evaluating other assay parameters (for example, sensitivity) may be needed.

3. Controlling Non-Specific Binding

Every test component, from the plastic of the microtiter plates to the developing agent, can affect assay sensitivity and non-specific binding. One of the most critical elements is the selection of the proper assay buffer and blocking reagents used to prevent non-specific binding. The sponsor should carefully consider the number and timing of wash steps as well as the detergents added to the assay buffer (for example, blocking or wash buffer) to reduce background noise while maintaining sensitivity. A variety of proteins can be used as blocking reagents to provide acceptable signal-to-noise ratio. However, these proteins may not all perform equivalently in specific immunoassays. For example, they may not bind well to all types of solid phases or may show unexpected cross-reactivity with the detecting reagent. Therefore, the sponsor may need to

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test several blocking agents to optimize assay performance. Moreover, including uncoated wells is insufficient to assess non-specific binding. Rather, determining the capacity of ADAs to bind to an unrelated protein of similar size and charge that may be present in the sample may prove to be a better test of binding specificity.

K. Reporting Results for Qualitative and Quasi-Quantitative Assays

Several approaches may be used to report positive antibody responses, and the appropriateness of the approach used should be evaluated on a case-by-case basis. The most common approach is qualitative, with subjects reported as having a positive or negative antibody response.

For subjects who are confirmed to be ADA positive, determining antibody levels can be informative because it allows for stratified assessment of ADAs and their impact on safety and efficacy. Positive antibody levels may be evaluated using a titer. Reporting levels of antibodies in terms of titers is appropriate and generally understood by the medical community. Most frequently titer is determined from the reciprocal of the highest dilution that gives a value at or just above the cut-point of the assay. Alternatively, titer may be determined by extrapolating the dilution to the assay cut-point using the linear portion of the dose response curve. All sample dilutions, such as the MRD and acid dissociations, should be factored into the calculations of titers and provided when reporting titers.

When reporting results for neutralization assays, values may also be reported as amount of mass units of therapeutic protein product neutralized per volume serum with the caveat that these are arbitrary in vitro assay units and cannot be used to estimate in vivo availability of the therapeutic protein product.

Unless the assay method used allows for independent determination of mass per volume of undiluted matrix, antibody levels reported in mass units are generally not acceptable. This is because the mass unit estimations are based on interpolation of data from standard curves generated with a positive control antibody, and parallelism between the positive control and test article cannot be assumed. Furthermore, even if parallelism between the positive control and test article is demonstrated, the absolute mass units cannot accurately be calculated because the samples are likely to contain different populations of antibodies. Thus, FDA does not consider it necessary or desirable for the sponsor to report subject antibody results in terms of mass units unless (1) the results are determined by quantitative means or (2) a universally accepted and accessible source of validated antibody is available as a control and parallelism between the dilution curves of the control antibody and subject samples has been demonstrated.

L. Other Considerations for Assay Development

A myriad of factors can affect the assessment of ADA levels, such as subject-sample variability; therapeutic protein product-dose response of the cells used to generate the standard curve in a cell-based neutralization bioassay; affinity and avidity of the ADA; and concentration of competing product in confirmatory assays. Accounting for such factors is important to understand and analyze assay variability and avoid errors. Common factors that should be considered include the following:

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1. Pre-Existing Antibodies

Pre-existing antibodies may have clinical effects that affect the efficacy of the therapeutic protein product being tested. An alternative to the qualitative screening assay approach may be needed to assess the quantity and quality of ADA when pre-existing antibodies are present. For example, testing samples for an increase in ADA using a semi-quantitative assay such as a titration assay (see sections V.C and VI.D) can provide information on the impact of a therapeutic protein product on product immunogenicity that is not provided by a qualitative assay. When there are pre-existing antibodies and the titer of antibodies increases after exposure to the therapeutic protein product, they can be reported as *treatment-boosted* to differentiate them from *treatment-induced* antibody titers. For example, a boosted ADA response may be defined as a titer that is two dilution steps greater than the pre-treatment titer, when twofold dilutions are used to determine the titer.

2. Rheumatoid Factor

Measuring immune responses to therapeutic protein products that possess Fc regions, such as mAb and Fc-fusion proteins, may be particularly difficult when RF is present in the matrix. RF is generally an IgM antibody that recognizes IgG, although other RF Ig specificities have been noted. Consequently, RF will bind Fc regions, making it appear that specific antibody to the therapeutic protein product exists. Several approaches for minimizing interference from RF have proven useful, including treatment with aspartame (Ramsland et al. 1999) and careful optimization of reagent concentrations so as to reduce background binding. When examining immune responses to Fc-fusion proteins in clinical settings where RF generates false-positive results during development, FDA recommends developing an assay specific for the non-Fc region of the proteins rather than against the intact biotherapeutics.

3. Monoclonal Antibodies

Technologies reducing the presence of non-human sequences in mAb, such as chimerization and humanization, have reduced but not eliminated ADA. In these cases, the immune responses are directed largely against the variable regions of the mAb (Harding et al. 2010; van Schouwenburg et al. 2014). The assays that can detect the reactivity against variable regions are considered more appropriate to evaluate the potential impact of antibodies against mAb-based therapeutics in subjects. If the Fc region is engineered or bound to another molecule, an assay that characterizes this response may be needed.

4. Conjugated Proteins

Antibody-drug conjugates (ADCs) are antibodies conjugated with small molecule drugs, so they represent a classic hapten-carrier molecule. Therefore, the immunogenicity assays should measure the responses to all components of the ADC therapeutic protein product, including the antibody, linker-drug, and new epitopes that may result from conjugation. When ADCs need to be labeled for immunogenicity assays, the conjugation should consider the potential for increased hydrophobicity of the labeled molecules because they may cause aggregation. The

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stability and solubility of these capture reagents should be adequately characterized (see section IV.A.3).

V. ASSAY DEVELOPMENT

Information specific to the development of respective assay types is provided in sections A through D below. These sections supplement the information provided in section IV that is relevant to all assay types.

A. Development of Screening Assay

Based on the multi-tiered approach discussed previously in section IV.A, the first assay to be employed for detection of ADA should be a highly sensitive screening assay that detects low- and high-affinity ADA. Approximately 5 to 10 individual samples may be used to estimate the cut-point early in assay development; however, this may need to be adjusted when treatment-naïve samples from the target population become available. A low but defined false-positive rate of approximately 5% is desirable for the initial screening assay because it maximizes detection of true positives. Subsequent assays can be employed to exclude false-positive results when determining the true incidence of immunogenicity.

B. Development of Confirmatory Assay

Because the screening assay is designed to broadly detect the presence of antibodies that bind product in serum samples with a defined false-positive rate of approximately 5%, FDA recommends that the sponsor develop assays to confirm the binding of antibodies that are specific to the therapeutic protein product. Implementation of a suitable confirmatory assay is important to prevent data on ADA false-positive subjects from confounding the analyses of the impact of ADA on safety and efficacy.

1. Selection of Format for Confirmatory Assay

It is expected that the selected confirmatory assay will have similar sensitivity to the screening assay, with the caveat that the assay false-positive rates are different, but have higher specificity and at least as good selectivity to identify any false-positive samples. The method and instrument platform selected may be similar to or different from those used for the screening assay. Frequently, both screening and confirmatory assays use the same method and instrument platform. In such cases, the sensitivity of each assay should be determined in mass units and confirmed using system suitability controls to ensure that the assay is sensitive to the presence of binding antibody. When using a binding competition assay, the concentration of competing product should be optimized to confirm the presence of antibodies throughout and above the range of the assay.

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2. Cut-Point of Confirmatory Assay

If a competitive inhibition format is selected, a recommended approach to determining the cut-point uses the data from the signal generated by antibody-negative treatment-naïve subject samples in the presence of the competitor, which is usually the therapeutic protein product. In this case, the amount of therapeutic protein product used to establish the cut-point should be the same as the amount of therapeutic protein product that will be used as a competitive inhibitor in the assay. However, this approach may not be appropriate when dealing with samples where pre-existing antibodies are present in the treatment-naïve population. In those cases, the sponsor should exclude true positives from the cut-point assessment. In rare cases when baseline negative samples are not available, sponsors may evaluate changes in titer or use an orthogonal method to confirm samples that screen positive.

C. Development of Titration Assay

In subjects that have pre-existing ADA, treatment-boosted ADA responses may be identified by post-treatment increases in titer. A cut-point for defining the treatment-boosted responses should be determined. For example, a boosted ADA response may be defined as a titer that is two dilution steps greater than the pre-treatment titer, when twofold dilutions are used to determine the titer. If titer is established by extrapolating the dilution curve to the assay cut-point, treatment-induced responses may be determined using estimates of assay variability.

D. Development of Neutralization Assay

In vitro neutralization assays indicate the potential of ADA to inhibit the therapeutic activity of the product. Such NAb can interfere with the clinical activity of a therapeutic protein product by preventing the product from reaching its target or by interfering with its pharmacologic activity such as receptor-ligand interactions. The testing method selected to assess neutralizing potential for ADA-positive samples should be based on the mechanism of action of the therapeutic protein product.³⁰ In selected cases, where there is a highly sensitive PD marker or an appropriately designed PK assay or both that generate data that inform clinical activity, it may be possible to use these in lieu of a NAb assay. This determination should be done in consultation with the Agency.

1. Selection of Format for Neutralization Assay

Two approaches have been used to measure NAb activity: cell-based bioassays and non-cell-based competitive ligand binding assays. Selection of the appropriate assay format depends on various factors (Wu et al. 2016). These factors include, but are not limited to, the mechanism of action of the therapeutic protein product and the selectivity, sensitivity, precision, and robustness of the assay. In general, FDA recommends that neutralization assays use a cell-based bioassay format. Depending on the therapeutic protein product's mechanism of action, there may be alternative strategies for assessing neutralizing activity. For example, ligand binding assays may be appropriate for antagonistic mAbs or receptor Fc fusion proteins that bind and inhibit the

³⁰ See footnote 13.

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target; however, alternative strategies to assess neutralizing activity should be discussed with the Agency before implementation.

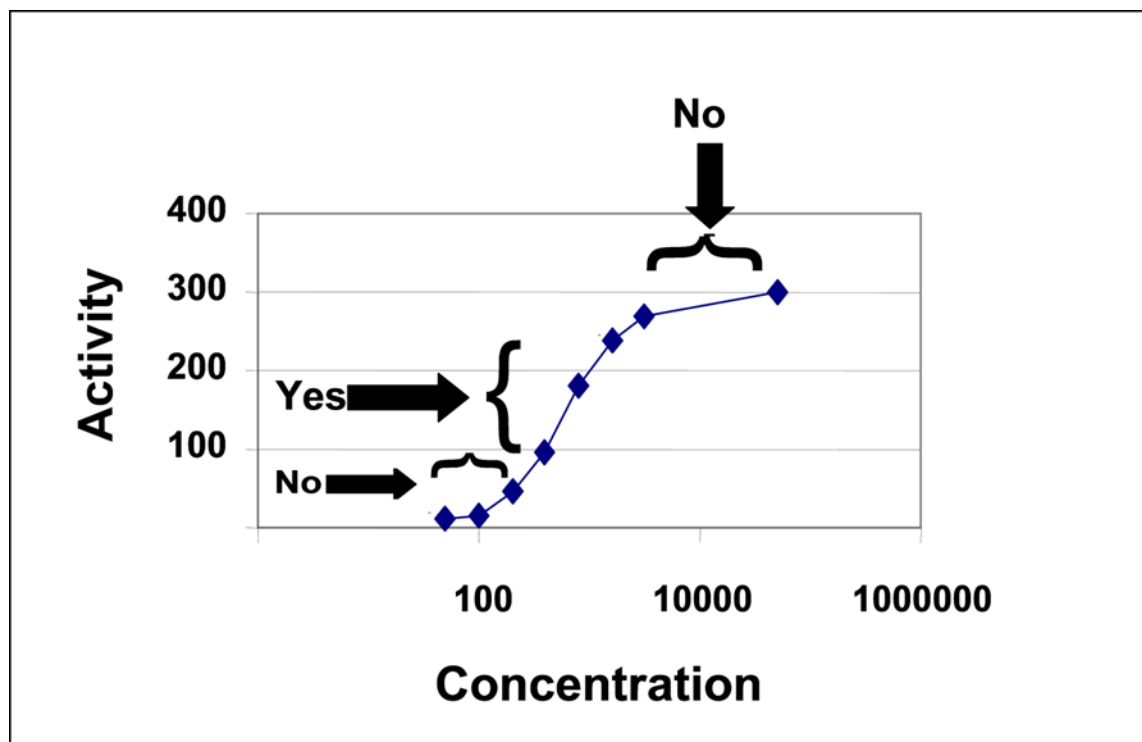
Different cellular responses may be measured in these bioassays, such as phosphorylation of intracellular substrates, calcium mobilization, proliferation, and cell death. In some cases, sponsors have developed cell lines to express relevant receptors or reporter constructs. When therapeutic protein products directly stimulate a cellular response, the direct effect of NAb on reducing bioactivity in the bioassay can be measured. When therapeutic protein products indirectly impact cellular activity (for example, by blocking a receptor-ligand interaction), the indirect effect of the NAb on restoring bioactivity in a bioassay can be measured. Some bioassays have significant variability and a limited dynamic range for their activity curves. Such problems can make development and validation of neutralization assays difficult.

There are cases when non-cell-based assay formats, such as ligand binding assays or enzyme activity assays, may be used (Wu et al. 2016). One such case is when sufficiently sensitive or selective cell-based bioassays cannot be developed. Another case is when the therapeutic protein product does not have a cell-based mechanism of action; for example, enzyme therapeutic protein products that do not require cellular uptake. Sponsors should discuss using ligand binding assays with FDA in such cases.

2. Activity Curve of Neutralization Assay

Generally, the neutralization bioassays use a single concentration of therapeutic protein product with a single dilution of antibody. Consequently, the sponsor should choose a therapeutic protein product concentration whose activity readout is sensitive to inhibition. Dosing cells in the lower part of the dose response curve may not allow for enough dynamic range in the response to meet neutralization thresholds. If the assay is performed at concentrations near the plateaus of the dose-response curve, marked “No” in Figure 1 below, it may not be possible to discern NAb-positive samples with low amounts of NAb. FDA recommends that the neutralization assay be performed at therapeutic protein product concentrations that are on the linear range of the curve, marked “Yes” in Figure 1.

Figure 1. Activity Curve for a Representative Therapeutic Protein Product



The x-axis (Concentration) indicates a concentration of the therapeutic protein product, and the y-axis (Activity) indicates resultant activity; for example, the concentration of cytokine secretion of a cell line upon stimulation with the therapeutic protein product. The curve demonstrates a steep response to a therapeutic protein product that plateaus at approximately 300. The “No” arrows indicate a concentration of a therapeutic protein product that may be inappropriate to use in a single-dose neutralization assay because it would represent a range of concentrations where the activity induced by the therapeutic protein product would be relatively insensitive to inhibition by NAb. The “Yes” arrow represents a range of concentrations on the linear part of the curve where the activity induced by the therapeutic protein product would be sensitive to neutralization by antibody.

3. *Considerations for Matrix Interference for Neutralization Assay*

The matrix can interfere with neutralization assays, particularly as matrix components may enhance or inhibit the activity of a therapeutic protein product in bioassays. For example, sera from subjects with particular diseases may contain elevated levels of one or more cytokines that might serve to activate cells in the bioassay. This could obscure the presence of NAb by increasing the response to the original stimulatory factor or therapeutic protein product. Therefore, the sponsor should understand matrix effects in these assays and choose a cell line that is specifically activated by the therapeutic protein product. Alternatively, the interfering factors can be inhibited or depleted by using a specific antibody or a cell line that specifically responds to drug treatment. Enriching the ADA from matrix samples may be appropriate for

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these types of situations. However, this approach may result in the loss of NAb and, consequently, will require careful examination and validation by the sponsor.

4. Cut-Point of Neutralization Assay

As with all assays, the cut-point should be determined based on the assay variability established using samples from treatment-naïve subjects. If neutralization assays are performed on samples that tested positive in screening and confirmatory assays, a 1% false-positive rate is usually acceptable. In the rare cases when the neutralization assay is used for screening, a 5% false-positive rate should be used (see section VI.B.2). If the degree of sample variation makes it difficult to assess NAb activity, other approaches may be considered, but should be discussed with FDA before implementation. Alternatively, exploring other assay formats that lead to less variability and provide a more accurate assignment of cut-point may be necessary. Most frequently fixed cut-points are established for NAb assays where, depending on the mechanism of action of the drug, a threshold percent inhibition or stimulation of signal is established, but floating cut-points may be used. See section IV.B for general information on assay cut-point.

5. Additional Considerations for Neutralization Assay

Because neutralization assays are most commonly performed only on samples that are confirmed to have antigen-specific ADA, confirmatory approaches are not usually necessary. However, because of the complexity of bioassays, in some cases confirmation of assay specificity may be useful in determining whether subjects have mounted a true NAb response. The sponsor should consider the following approaches:

- a. Unrelated inhibitory molecules may cause neutralizing activity, and sometimes it may be unclear whether the observed neutralizing activity is caused by neutralizing antibodies or by other inhibitory molecules. Test results from baseline pre-exposure samples may be informative. When there is concern that there is non-specific inhibition, antibody depletion assays should be performed to evaluate whether the neutralizing activity is truly caused by ADA and not caused by other inhibitory molecules.
- b. Cell lines may be responsive to multiple stimuli other than the therapeutic protein product under study. In such cases, the presence of NAb can be examined in the presence of the therapeutic protein product, which should be blocked by a specific NAb response, versus alternative stimuli, which should not be blocked by a specific NAb response.
- c. The matrix contains components such as soluble receptors or endogenous product counterparts that may yield false results in the neutralization assay. In such instances, adding test matrix samples directly to the bioassay in the absence of therapeutic protein product, or blocking the matrix factor, if known, is useful in understanding assay results.

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- d. The presence of onboard drug should also be considered when designing neutralization assays, particularly when drugs with a long half-life are used.

VI. ASSAY VALIDATION

Assay validation is a process of demonstrating, through specific laboratory investigations, that the performance characteristics of the ADA assay employed are suitable for its intended use. The extent of validation depends on the stage of product development and the risks of consequences of immunogenicity to subjects associated with the therapeutic protein product. For most products, a partial validation involving assessments of assay sensitivity, specificity, precision, cut-point, and drug tolerance — with less emphasis on robustness, reproducibility, and stability — may be adequate for the earlier stages of clinical development such as phase 1 and phase 2 studies. High-risk products may require full validation before any clinical studies. However, as stated in section VI.A, fully validated assays should be used for testing samples from pivotal and postmarketing studies.

Information specific to validation of respective assay types is provided in sections VI.B through VI.E. These sections supplement information relevant to all assay types provided in sections IV and VI.A.

A. General Considerations for Assay Validation

Samples derived from pivotal studies should be tested with fully validated assays. At the time of license application, the sponsor should provide data demonstrating that the assays are fully validated. Validation includes assessments that demonstrate that an assay used for measurement of ADA is suitable for the intended purpose. The fundamental parameters for validation include (1) cut-point, (2) sensitivity and drug tolerance, (3) specificity and selectivity, (4) precision, (5) reproducibility³¹ when relevant, (6) robustness of some assay features, and (7) in-use stability of critical reagents.

Determination of cut-point is a fundamental aspect of assay validation. Balanced study designs should be used for cut-point determination. If plate homogeneity of response is not demonstrated, alternative plate layouts should be used during cut-point determination. If treatment-naïve samples from the appropriate subject population are not available for the pre-study validation exercise, alternative samples may be used.³² Frequently, these are samples from commercial sources. When alternative samples are used to determine the cut-point in the validation exercise, the cut-point should be confirmed once samples from the appropriate population are available; for example, samples from treatment-naïve subjects that are collected, handled, and stored under study conditions. If the cut-point established using matrix samples

³¹ Reproducibility (also called cross-validation) is needed when more than one laboratory will be used to assess samples.

³² Treatment-naïve subjects could be healthy individuals or a patient population not exposed to the therapeutic protein product, depending on the stage of assay development or validation and on the availability of samples. Sponsors should provide justification for the appropriateness of the samples used.

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from the treatment-naïve population is significantly different from that obtained during assay validation, the cut-point should be amended. The cut-point established using the appropriate samples should be used to determine whether study samples are positive for ADA.

For validation of the fundamental assay parameters, FDA recommends that inter-assay precision be evaluated on different days and by different analysts using the same instrument platform and model, although different instruments should be used to include all sources of variability. This design results in at least six independent determinations for each sample. Intra-assay precision should be evaluated with a minimum of six independent preparations of the same sample per plate independently prepared by the same analyst. Alternatively, in assays with low throughput (for example, titer assays), when it may not be possible to run six independent preparations of the same sample on a plate, intra-assay precision should be evaluated with a minimum of three independent preparations of the same sample per plate and at least nine total independent preparations of the same samples. Samples should include negative controls and positive samples whose testing yields low, intermediate, and high values of the assay dynamic range. In general, the intra-assay and inter-assay precision as expressed by percent coefficient of variation (%CV) is expected to be lower than 20%. However, it may be higher in some assay formats such as cell-based assays. In cases where intra-assay or inter-assay precision has a %CV greater than 20%, sponsors should consider the need to refine the assay parameters to optimize the assay precision to the extent possible or provide justification to explain why higher %CV should be acceptable. For negative controls, a larger %CV is acceptable. The sponsor should evaluate inter-instrument and inter-operator precision when relevant.

Specific parameters may need to be validated depending on the method, technology, or instrument platform used for the assay. For example, surface plasmon resonance assays should be validated for surface stability upon regeneration, and criteria should be set for baseline performance of the chip. The sponsor should examine robustness during the development phase and determine whether aspects of assay robustness should be validated. For example, the efficiency and stability of labeled reagents and incubation times and temperature should be established.³³

When changes are made to a previously validated method, the sponsor should exercise judgment as to how much additional validation is needed. During a typical product development program, a defined ADA assay may undergo modifications. Occasionally, samples may need to be re-tested with the optimized validated assay. Therefore, provisions should be made to preserve sufficient sample volume under conditions that allow for re-testing until the assays have been completely validated and evaluated by the Agency.³⁴

³³ A reagent is considered *labeled* if it is conjugated or fused to a moiety that will aid in its capture or visualization; for example, conjugation to biotin, streptavidin, or a fluorochrome. An *unlabeled* reagent is a reagent (for example, a drug) that is not *labeled*.

³⁴ See the guidance for industry *Bioanalytical Method Validation* for different types and levels of validation. Also, see the USP General Chapters 1106 and 1106.1.

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B. Validation of Screening Assay

1. Sensitivity of Screening Assay

All the general considerations for assay validation discussed previously apply to validation of screening assay. As noted earlier, the sensitivity is particularly important in the initial screening assay because these results dictate the further analysis of the sample.

2. Cut-Point of Screening Assay

The cut-point should be determined statistically with an appropriate number of treatment-naïve samples, generally around 50, from the subject population. Each sample should be tested by at least two analysts on at least three different days for a total of at least six individual measurements. One approach that allows for high assurance of a 5% false-positive rate is to apply a 90% one-sided lower confidence interval for the 95th percentile of the negative control population (Shen et al. 2015). This will assure at least a 5% false-positive rate with a 90% confidence level. This approach improves the probability of the assay identifying all subjects who may develop antibodies. When using the approach published by Shen et al., the reportable value for each sample should be the average of the six measurements. The statistical method used to determine the cut-point should be based on the statistical distribution of the data. For example, the 95th percentile of the normal distribution is estimated by the mean plus 1.645 standard deviation. Other approaches may be used for estimating 95th percentile, including the use of median and median absolute deviation value instead of mean and standard deviation.

The mean response of negative control samples may be constant or may vary between assays, plates, or analysts. When the mean varies between assays, plates, or analysts but the variance around the mean is constant, a normalization factor can be statistically determined and applied in-study. This is known as a floating cut-point and is the most common type of cut-point used. For normally distributed data, when the mean is constant, a cut-point may be established during assay validation that can be applied to the assay in-study. This is known as a fixed cut-point. The use of a fixed cut-point is discouraged because it does not allow for the possibility that negative control means may vary in-study. When both the mean and variance vary, a cut-point may need to be established for each assay, plate, or analyst. This is known as a dynamic cut-point. However, this approach is frequently not practical because of the need to have more replicates of the negative control. When a dynamic cut-point is indicated, further assay development should be considered instead of using a dynamic cut-point.

C. Validation of Confirmatory Assay

Confirmatory assays should be fully validated in a manner similar to screening and neutralization assays. As a scientific matter, the studies to validate the assay will depend on the assay format and instrumentation chosen. If these assays are based on competition for antigen binding³⁵ by the antibodies in subject samples and the measurement is loss of response, it is critical to identify

³⁵ *Competition for antigen binding* refers to a competition assay where the ability of antigen-specific antibodies to bind to either labeled or plate-bound antigen is inhibited by unlabeled or soluble antigen.

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the degree of inhibition or depletion that will be used to ascribe positivity to a sample. In the past, fixed percentages of binding reduction were used, but these numbers were often arbitrary and are unlikely to be relevant for all assays. FDA recommends establishing a cut-point based on the assessment of the binding changes observed in negative control samples that are known to lack the antibodies when competing antigen is added. FDA also recommends that the sensitivity of the confirmatory assay be demonstrated using a low concentration of the positive control antibody.

One approach for the estimation of the confirmatory assay cut-point is to use an 80% to 90% one-sided lower confidence interval for the 99th percentile. Because the purpose of this assay is to eliminate false-positive samples arising as a result of non-specific binding, it is adequate to use a 1% false-positive rate for the calculation of the confirmatory cut-point. The use of tighter false-positive rates such as 0.1% is not recommended, but may be acceptable for larger studies. See section IV.B for general information on assay cut-point.

The confirmatory assay format is frequently a competition assay in which a competitor, usually an unlabeled therapeutic protein product,³⁶ is added to the reaction mixture to inhibit ADA binding to the capture reagent for the assay. For this assay format, the same concentration of unlabeled therapeutic protein product should be added to the negative control samples when determining the confirmatory cut-point.

D. Validation of Titration Assay

The principles of assay validation described in section VI.A apply in general to validation of titration assays. The cut-point of the titration assay may be the same as or different from that of the screening assay. For example, the United States Pharmacopeia recommends establishing a titration assay cut-point when the signal from the assay diluent or matrix causes higher results than the screening assay cut-point because of a blocking effect of serum or if samples at a dilution higher than the MRD do not generate consistently negative results, usually, when the screening cut-point falls on the lower plateau of the positive control dilution curve.³⁷ When a titration assay specific cut-point is used, it should be validated. When the titration assay is not used for screening, the cut-point may be established using a 0.1% false-positive rate. When the titration assay is used for screening (for example, when the subject population has a high incidence of pre-existing ADA), the cut-point should be established using a 5% false-positive rate.

E. Validation of Neutralization Assay

A minimum of 30 samples tested on at least 3 different days by at least two analysts should be used to determine the cut-point, using suitable statistical methods (see section V.D.4). The assessment of assay sensitivity can be affected by the kind of positive control that is used (for example, mAb or polyclonal antibody), how variable the assay is, and how the assay cut-

³⁶ See footnote 33.

³⁷ See the USP General Chapter 1106.

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point is determined. Nevertheless, it is important to evaluate assay sensitivity during the validation exercise.

The positive control for neutralization assays can be either monoclonal or affinity purified polyclonal antibodies. Further, if an affinity purified polyclonal positive control antibody preparation is used, it is likely that only a portion of the antibodies are neutralizing, which can make the assay appear less sensitive.

Sponsors should validate assay specificity for cell-based neutralization bioassays. As mentioned, for cells that may be responsive to stimuli other than the specific therapeutic protein product, the ability to demonstrate that NAb only inhibit the response to the therapeutic protein product and not the response to other stimuli is a good indication of assay specificity. In such studies, FDA recommends that the other stimuli be employed at a concentration that yields an outcome similar to that of the therapeutic protein product. The sponsor should also confirm the absence of alternative stimuli in subject serum (see sections IV.C through E).

Cell-based neutralization bioassays frequently have reduced precision when compared to ligand binding assays because biologic responses can be inherently more variable than carefully controlled binding studies. When assay precision is poor, the sponsor may consider performing more replicates for assessment of precision and assessment of subject responses than for the screening assay (see section IV.F).

When cells at the low, middle, and high range of the allowed passage numbers and the cell density and cell viability are used, additional assay performance parameters should be established. This is frequently done during assay development and may not be part of the validation exercise (see section IV.H).

VII. IMPLEMENTATION OF ASSAY TESTING

A. Obtaining Subject Samples

FDA recommends that sponsors obtain pre-treatment samples from all subjects. Because there is the potential for pre-existing antibodies or confounding components in the matrix, understanding the degree of reactivity before treatment is essential. The sponsor should obtain subsequent samples, with the timing depending on the frequency of dosing. Optimally, samples taken 7 to 14 days after the first exposure can help elucidate an early IgM response. Samples taken at 3 to 6 weeks after the first exposure are generally optimal for determining IgG responses. IgA responses may peak earlier than IgG responses, at around 2 to 3 weeks after antigen exposure (Schütz et al. 2013; Macpherson et al. 2008). For individuals receiving a single dose of a therapeutic protein product, these time frames may be adequate. However, for subjects receiving a therapeutic protein product at multiple times during the trial, the sponsor should obtain samples at appropriate intervals throughout the trial and obtain a sample approximately 30 days after the last exposure. For products with long half-lives, samples should be obtained approximately five half-lives after last exposure. When there is a high risk of serious consequences from ADAs, sponsors should plan to collect samples from subjects until ADAs return to baseline levels.

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Obtaining samples at a time when there will be minimal interference from the therapeutic protein product present in the matrix is essential. A sponsor should consider the therapeutic protein product's half-life and dosing regimen to help determine appropriate times for sampling. This is especially important for mAb products because these products can have half-lives of several weeks or more and, depending on the dosing regimen, the therapeutic mAb itself could remain present in the serum for months. Under circumstances when testing for IgE is needed, the timing of sample collection should be discussed with FDA.

If therapeutic protein product-free samples cannot be obtained during the treatment phase of the trial, the sponsor should take additional measures to ensure that the assay is sensitive in the presence of expected onboard drug; and samples should be obtained after an appropriate washout period, generally five half-lives. Obtaining samples to test for meaningful antibody response can also be complicated if the therapeutic protein product in question is itself an immune suppressant. In such instances, the sampling schedule should be adjusted in accordance with the immunosuppressant regimen, to the extent possible.

Samples to determine serum concentrations of the therapeutic protein product should be obtained at the same time as immunogenicity samples. Testing such samples can provide information on whether the therapeutic protein product in the samples is interfering with ADA testing and whether ADA is altering the therapeutic protein product's pharmacokinetics. It is important that study subjects be properly consented to allow for continued testing until ADAs reach baseline and samples are available to confirm or requalify assays as needed. It may also be useful to consent subjects to allow for sample use in assay development and control.

B. Concurrent Positive and Negative Quality Controls

If the sponsor completes the proper validation work and makes the cut-point determinations, the immunogenicity status of subjects should be straightforward to determine. However, positive control and quality control (QC) samples are critical and should be run concurrently with subject samples. We recommend that these samples span a level of positivity with QC samples having a known negative, low, and high signal in the assay. More important, the QC samples should be diluted in the matrix in which subject samples will be examined. For example, the QC sample should be diluted in the same anticoagulant as the subject samples. For the low-positive QC sample, we recommend that a concentration be selected that, upon statistical analysis, would lead to the rejection of an assay run 1% of the time. In this way, the sponsor ensures that the assay is performing as expected and that subject samples are correctly evaluated. If the assay is subject to a prozone effect, the concentration of high-positive QC samples should be set to monitor prozone effects.³⁸

FDA also recommends that these QC samples be obtained from humans or animals possessing antibodies that are detected by the secondary detecting reagent to ensure that negative results that might be observed are truly caused by lack of antigen reactivity but not caused by failure of the

³⁸ Prozone effects, also referred to as hook effects, are a reduction in signal that may occur because of the presence of a high concentration of a particular analyte or antibody and may cause false-negative results.

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secondary reagent. This issue is not a problem for bridging assays where labeled antigen is used for detection.

C. Confirmation of Cut-Point in the Target Population

Samples from different populations can have different background activity in ADA assays. Similarly, the background activity can change when samples used to determine the cut-point during assay validation were not obtained and handled in a manner that represents how samples will be obtained and handled in-study. Therefore, it is necessary to confirm that the cut-point determined during assay validation is suitable for the population being studied. A sufficient number of samples from the target population should be used, and justification for the number used should be provided. If sufficient numbers of samples are not available, agreement with the Agency should be sought for the number of samples to be used.

VIII. DOCUMENTATION

Currently the data relevant to the assessment of immunogenicity are dispersed throughout different locations of the eCTD. To facilitate the clinical development of therapeutic biologics, we recommend a life-cycle management approach to immunogenicity through the creation of an integrated immunogenicity summary report that sponsors begin populating early in therapeutic protein product development and update at regular intervals as the individual product clinical program progresses through IND stages into the BLA and even postapproval stages. We recommend that the document be arranged into distinct sections to be populated with stage-appropriate information as it becomes available, including (1) Immunogenicity Risk Assessment, (2) Tiered Bioanalytical Strategy and Assay Validation Summaries, (3) Clinical Study Design and Detailed Immunogenicity Sampling Plans, (4) Clinical Immunogenicity Data Analysis, and (5) Conclusions and Risk Evaluation and Mitigation Strategies (REMS).

For the BLA file, we recommend that the applicant provide brief summaries of the immunogenicity results in relevant places in eCTD section 2.7. Clinical Summary and the full report in section 5.3.5.3 Reports of Analysis of Data from More than One Study.³⁹ This Integrated Summary of Immunogenicity should provide the following:

- a. Immunogenicity Risk Assessment: This section should provide a concise immunogenicity risk assessment specific to the therapeutic protein product.⁴⁰ This section should include discussions on therapeutic protein product quality-related factors

³⁹ See the FDA website for further information on eCTD submissions, available at <https://www.fda.gov/Drugs/DevelopmentApprovalProcess/FormsSubmissionRequirements/ElectronicSubmissions/ucm153574.htm>. For more information about the agreed-upon common format for the preparation of a well-structured Efficacy section of the CTD for applications that will be submitted to regulatory authorities, see the ICH guidance for industry *M4E: The CTD — Efficacy*. For more information on how sponsors and applicants must organize the content they submit to the Agency electronically for all submission types under section 745A(a) of the Federal Food, Drug, and Cosmetic Act, see the guidance for industry (and the technical specification documents it incorporates by reference) *Providing Regulatory Submissions in Electronic Format — Certain Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications*.

⁴⁰ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*.

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and how these may impact the immunogenic potential of the therapeutic protein product; subject-related factors, including a discussion on how likely is the subject population and clinical indication to result in immunogenic responses to the therapeutic protein product; and a section on trial design-related factors, as well as a discussion of any strategies or clinical study conditions implemented to manage the immunogenic response to the therapeutic protein product.

- b. Tiered Strategy and Stage-Appropriate Bioanalytical Assays: This section should provide a summary of the immunogenicity assessment strategies used during each phase of the clinical program and a characterization for the various methods that were developed throughout the program. In addition, this section should provide links to the method development and validation reports for the pivotal clinical studies supporting the application.
- c. Clinical Study Design and Sampling Strategy: This section should include the immunogenicity sampling plan(s) for all clinical studies that had an immunogenicity assessment performed. This section should also include sampling time points for immunogenicity and pharmacokinetics of the therapeutic protein product, where applicable.
- d. Clinical Immunogenicity Data Analysis: This section should provide summary results of immunogenicity analyses for all clinical studies having an immunogenicity component, including the results of linear or non-linear correlation analyses between ADA status and titers with PK, PD, efficacy, and safety (adverse event) data. This section should include drug levels measured in the samples tested for ADA and should trace drug product lots used in the individual clinical studies. Discussion should examine the impact of any pre-existing antibodies or treatment-boosted or treatment-induced antibodies on pharmacokinetics, pharmacodynamics, efficacy, and safety of the therapeutic protein product.
- e. Conclusions and REMS, if applicable: This section should discuss how therapeutic protein product immunogenicity affects the safety and efficacy of the therapeutic protein product for the subject population. In addition, consideration should be given to how therapeutic protein product immunogenicity will be monitored in the postmarketing stage and how this will be incorporated into any planned risk evaluation and mitigation strategies. Lastly, a discussion should be provided regarding life-cycle management of approved immunogenicity assays, including an assay requalification schedule and assay transfer to contract testing laboratories for postmarketing surveillance.

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APPENDIX: MULTI-TIERED APPROACH TO ANTI-DRUG ANTIBODY TESTING

