Immunogenicity Testing Guidelines

The Culmination of a Decade of Progress

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Dr. Bowsher is a co-founder of the Ligand Binding Assay Bioanalytical Focus Group within AAPS, a group that dedicates its efforts to promoting harmonization and education for the development, validation and application of ligand binding assay methods for the bioanalysis of biotherapeutics, anti-drug antibodies and novel biomarkers. Dr. Bowsher has championed the missions of this focus group since its inception,and applies the same principles to his leadership of BioPharma Services at Millipore.

ABSTRACT

Unwanted immunogenicity, the major safety concern for biotherapeutics, is a topic of current interest and discussion in the field of drug development because of the recent emergence of biotechnology as a major source for new therapeutics. In this article, bioanalytical methods expert Dr. Ron Bowsher of Millipore Corporation describes the evolution of immunogenicity testing standards over the decade, culminating in the 2009 formal draft guidance issued by the United States Food and Drug Administration (U.S. FDA). Millipore's BioPharma Services provides complete immunogenicity testing services, and the listed capabilities (pages 9-10) address the recommendations outlined in the 2009 FDA draft guidance.

CONCERN	CLINICAL OUTCOME
Safety	○ ADA cause hypersensitivity reactions
	○ ADA neutralize activity of an endogenous equivalent resulting in a deficiency syndrome.
Efficacy (PD)	○ ↑ or ↓ biotherapeutic efficacy resulting from a change $T_{1/2}$ or biodistribution
PK	○ Altered PK due to a change in CL
	○ ADA presence dictates change in dosage level
None	○ Dispite ADA generation, there are no discernable clinical effects / sequelae

Table 1. Potential Clinical Immunogenicity Concerns as presented by S.L. Kirshner of the U.S. FDA, at the 2009 AAPS Immunogenicity Ligand Binding Assay (LBA) Training Course[5].
Abbreviations: PD = pharmacodynamics; PK = pharmacokinetics; CL = clearance.

INTRODUCTION

The immunogenic potential of a biotherapeutic is defined as its ability to provoke an immune response, either by humoral production of anti-drug antibodies (ADA) or through cellular-based immune responses[1-3]. For protein-based biotherapeutics, an immune response can range from the development of detectable but clinically insignificant ADA, to one that can impact drug safety and/or effectiveness[4]. Various categories of concerns and their potential clinical relevance are listed below in Table 1[5]. Because we have limited ability at this time to accurately predict the immunogenic potential of a biotherapeutic, immunogenicity testing is now integral to investigation of new biotherapeutics and follow-on biologicals.

Over the past decade, much progress has been made in gaining consensus in the overall approach for laboratory testing of ADA. The American Association of Pharmaceutical Scientists (AAPS) Ligand Binding Assay Bioanalytical Focus Group was formed in 2000, followed by formation of the Immunogenicity Working Group, under the leadership of Drs. Tony Mire-Sluis (United States Food and Drug Administration (U.S. FDA)) and Steven Swanson (Amgen). Moreover, reports of antibody-mediated pure red cell aplasia secondary to the administration of a recombinant erythropoietin had already spurred interest in neutralizing antibodies (NAb) and focused attention on immunogenicity testing[7]. A timeline of some key events shaping immunogenicity testing is depicted in Figure 1.

In 2004, Mire-Sluis and colleagues in the AAPS Immunogenicity Working Group introduced the tiered assay approach for ADA testing (Figure 2) and defined requirements for ADA assay development, articulating the important analytical performance characteristics, providing definitions, and offering a standardized approach for computing an assay's screening cut-point[8].

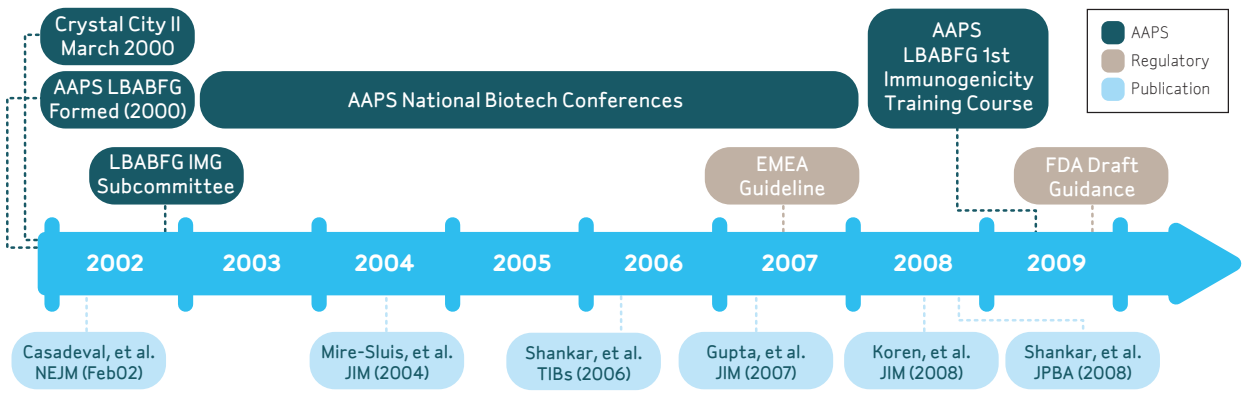

Figure 1. Timeline of key events that culminated in the draft European Medicines Agency (EMEA) guideline and FDA draft guidance, including publications that contributed to the convergence in ADA detection methodology. This timeline illustrates the involvement of both sponsors and regulatory agencies in developing a unified approach to immunogenicity testing.

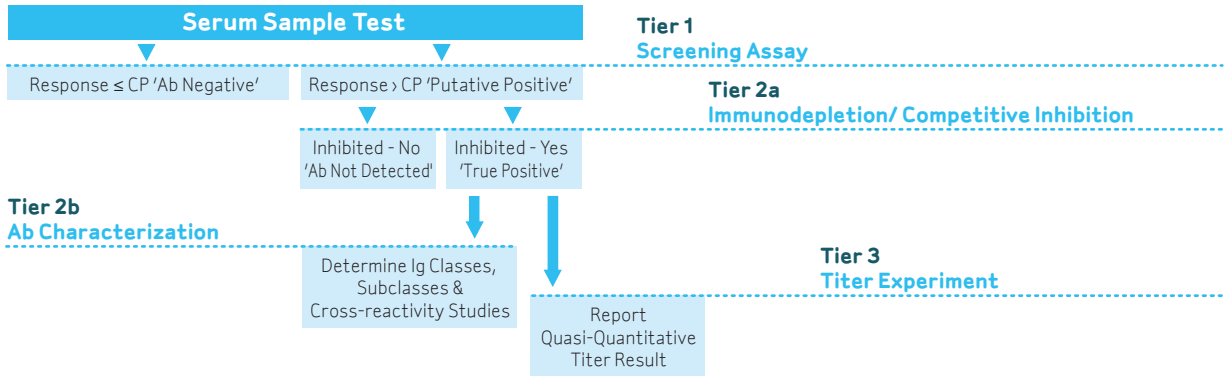

Figure 2. Tiered approach for screening, characterization and quasi-quantitative titer assessment of serum samples for the presence of ADA. Tier 1 refers to initial antibody detection with assay sensitivity of at least 250 – 500 ng/mL for a surrogate antibody. "CP" indicates cut-point. Tier 2a tests for competitive inhibition after addition of excess therapeutic. Depending on the risk-based plan, additional studies may be appropriate to characterize the ADA in terms of the types of antibodies and their binding characteristics (Tier 2B). The final step, Tier 3, involves serial titering of positive samples to provide a quasi-quantitative estimate of the concentration of antibody in the test sample[8].

In 2008, Shankar and co-workers published expanded details for ADA assays, including criteria for methods validation. The pre-study validation performance criteria addressed included 1.) screening cut-point, 2.) specificity (confirmatory) cut-point, 3.) sensitivity, 4.) selectivity/ interference, 5.) precision, 6.) robustness, 7.) stability, 8.) ruggedness[9]. The authors also made recommendations for in-study QC performance for run acceptance and presented a systematic stepwise approach for evaluating cut-point data and calculating the screening cut-point, shown in Figure 3. By emphasizing and delineating statistical methods for assay validation, the authors strove to eliminate subjectivity from and promote consistency of the validation process.

Other important immunogenicity publications in the past decade focused on establishment of valid cell-based assays to detect and characterize neutralizing antibodies[10], implications for regulatory agencies[11, 12], risk-based strategy[12, 13], and ADA testing in nonclinical safety studies[14].

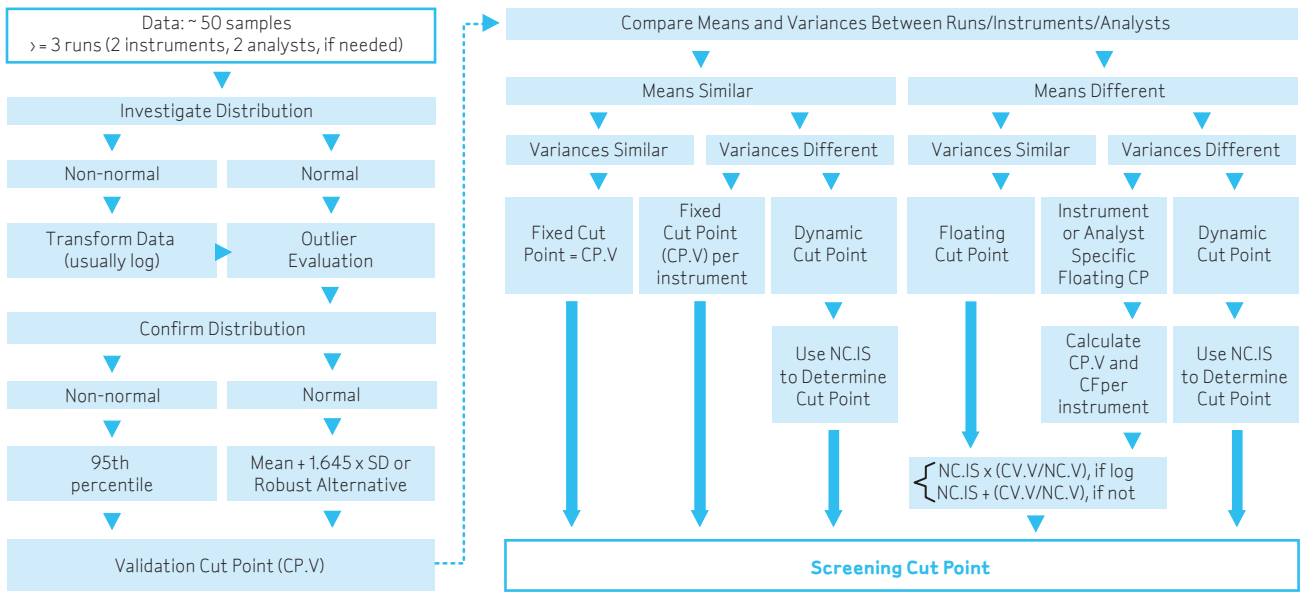

Figure 3. Systematic stepwise approach for data-driven determination of a screening cut-point as presented by Shankar and colleagues[9]. Key elements in the statistical analysis are data normality, outlier evaluation, need for transformation, and evaluation of run means and variances[9]. Outliers can result from nonspecific interactions or preexisting antibodies.
Abbreviations: NC.V = Neg. control from validation runs, NC.IS = Neg. control from in-study run.

RECENT DRAFT GUIDANCE: OVERVIEW AND HIGHLIGHTS

Early in December 2009, the U.S. FDA published a draft guidance to industry pertaining to the conduct of assay development for immunogenicity testing of therapeutic proteins[15]. This document followed the January 2007 publication by the EMEA Committee for Medicinal Products for Human Use (CHMP) Draft Guideline on the same topic[16]. These documents are complementary and quite consistent in their specific recommendations.

The new draft document provides guidance for ADA detection, confirmation, and assays for NAbs to support clinical investigation of protein therapeutics. Although the document is not yet finalized and acknowledges that ADA testing in preclinical species is not necessarily predictive of the human immune response, the guidance is a useful analytical framework that can aid in interpretation of toxicology and pharmacology data and may help reveal potential antibody-related toxicity.

The new FDA draft guidance supports an evolving assay approach with an expectation for preliminary validated assays by Phase I and full validation needed at the time of license application. The US FDA Draft Guidelines recommend an analytical approach to immunogenicity testing that addresses the following key considerations:

- **Sensitivity** – detect clinically relevant levels of ADA
- **Interference** from matrix and from circulating therapeutic – ensure assay is valid for relevant clinical samples
- **Physiological consequences** – both NAb-related and induced hypersensitivity responses
- **Risk-based application** – testing strategy is case-by-case and takes into account the risk to patients of mounting an immune response to a therapeutic protein

Consistent with previously published white papers and the EMEA draft guidance, the FDA recommends a multi-tiered assay approach as shown in figure 2, with the following additional criteria:

1. Tier 1 screening assay should:

- Exhibit 5% false positive rate to maximize detection of true positive samples
- Be able to detect all immunoglobulin classes and IgG subclasses
- Ensure that labeling of the detection reagent should not obscure important binding epitopes
- Involve careful selection of the assay buffer and blocking reagents used to prevent nonspecific binding to the solid surface
- Rule out matrix interference via selectivity experiments, in which different lots of matrix are analyzed after spiking with zero, low, and high concentrations of surrogate antibody
- When diluting samples to minimize matrix interference, evaluate minimal required dilution (MRD) from a panel of ten or more samples
- Should not dilute samples more than 1:100 so as not to compromise sensitivity

2. Tier 1 screening assay should be validated for sensitivity, specificity, and precision:

- **Sensitivity**
  - Assess sensitivity using a preparation of purified antibodies
  - Sensitivity = interpolated concentration at the predetermined cut-point response
  - Determine sensitivity in test sample matrix at MRD
  - Required sensitivity = 250 – 500 ng/mL
- **Specificity**
  - Critical for interpretation of immunogenicity results
  - If therapeutic is related to an endogenous protein, assess antibody cross-reactivity with both proteins
  - If therapeutic is in a family of homologous proteins, assess antibody cross-reactivity with all family members
- **Precision**
  - Measure intra-day precision: 6 replicates per plate
  - Measure inter-day precision: 3 replicates per day for 3 days
  - Measure inter-operator variability when appropriate

3. Tier 3 quasi-quantitative result should be reported as a titer value, as opposed to a result in mass units, following interpolation against a standard curve.

4. The assay cut-point needs to be determined systematically using a statistically valid approach (as in Figure 3), removing outliers to lessen analytical variance. To estimate the cut-point, 50-100 presumed negative samples should be screened multiple times across multiple assays, using a balanced statistical design. The cut-point may need to be re-established for different patient populations.

5. For the preparation of in-study cut-point assay controls, the FDA guidelines make the following recommendations:

- Purify surrogate ADA from animal sera and spike into the human sample matrix
- For direct-binding ADA, use a primate surrogate ADA to prepare QC samples
- Establish a negative control for pre-study validation and in-study sample analysis
- The positive controls should reflect the human immune response
- Prepare QC samples at low, mid and high values in the ADA assay
- The low QC is set statistically so its failure rate is approximately 1%

The new draft guidance provides some recommendations for collection of sera from patients for detection and characterization of ADA. First, pre-exposure samples should be obtained from all patients to provide baseline information. For detection of IgM, collect samples 7 to 14 days post-exposure. In contrast, serum samples should be collected 4 to 6 weeks post-exposure for detection of IgG ADA. Secondly, test samples need to be collected when there will be minimal interference from the therapeutic protein. If drug-free samples cannot be obtained during the study, consider sampling at approximately 5 half lives.

In its recommendation for assessments of NAbs, the U.S. FDA strongly recommends cell-based bioassays. Cell-based assays are believed to be more reflective of *in vivo* immunogenicity than competitive ligand-binding assays (LBAs), despite the higher variability and limited quantitative ranges. If a therapeutic protein possesses multiple domains, it may be appropriate to consider several NAb assays. Tier 2 confirmatory assays, involving competition or immunodepletion, are critical for NAb assays. The NAb cut-point should be determined statistically in a systematic manner similar to the Tier 1 screening assay. In addition, the sensitivity of NAb assays are estimated in a manner similar to screening assays and should be reported in terms of mass units (ng/mL).

MILLIPORE'S IMMUNOGENICITY SUPPORT SERVICES

Having participated in the evolution of immunogenicity testing standards over the decade, and having been personally involved in the development of these standards, we at Millipore's BioPharma Services - BioAnaLab Team are able to provide our customers with unique expertise and insight into creating effective, customized immunogenicity programs.

Following the FDA and EMEA current draft guidelines and AAPS white papers, we use a stepwise approach to assess the extent and nature of ADA responses elicited by therapeutic biomolecules. We use the tiered method of immunogenicity testing (Figure 2), using a variety of methods to measure responses, including ELISA, EIA, RIPA, SPR (Biacore™, ECL, DELFIA®) and cell-based assays.

Differentiating Capabilities

Millipore's BioPharma Services - BioAnaLab Team features particular capabilities that set our services apart from other providers. These capabilities include:

1. Affinity purification of surrogate antibodies and characterization of assay sensitivity
2. Antibody characterization and isotyping
3. Cell-based assays for detection of NAbs
4. Data-driven cut-point assignment / expertise in statistics

The following pages provide a quick reference for immunogenicity testing services offered by Millipore, and how they address the recommendations put forth by the recent FDA draft guidance.

For more details on Millipore's BioPharma Services - BioAnaLab Team and any information presented in this article, please contact:

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MILLIPORE'S IMMUNOGENICITY TESTING CAPABILITIES

- Development & validation of assays to detect ADA:
  - ELISA, RIA, IRMA, Meso Scale™
  - Direct binding and bridging formats
  - Biacore
- Custom generation of polyclonal and monoclonal antibodies
- Immunoaffinity purification of antibodies
- Protein / peptide conjugation
- Statistical data analysis & support
  - Approach outlined in Shankar et al. (2008)[9]
  - Data-driven cut-point assignments
- Class / subclass characterization of ADA
- Cell-based assays for NAb detection

MILLIPORE'S DATA-DRIVEN CUT-POINT ASSIGNMENT SERVICES

- Balanced experimental design
  - Evaluate normality and perform log transform if necessary
  - Biological vs. analytical outliers
  - Parametric vs. nonparametric
- Assess sources of variability from analysis of variance (ANOVA)
  - Restricted maximum likelihood (REML) = unbiased variance estimates
  - Biological variability, intra-assay and inter-assay variability
- Compare assay run means and homogeneity of variances
  - Fixed vs. floating cut-point
  - Evaluate correlation between negative control and mean of samples
  - Test assigned cut-point

MILLIPORE'S CRITERIA FOR VALIDATION OF ANALYTICAL METHODS

	Included in FDA's Draft Guidance
MRD assessment	✔
Selectivity (recovery)	✔
Specificity (competitive inhibition), including nonrelevant protein	✔
Sensitivity	✔
Assignment of screening CP	✔
Estimation of specificity CP	✔
Drug tolerance (drug interference)	✔
Precision (intra- & inter-assay)	✔
Robustness	✔
Stability	✔

MILLIPORE'S EXTENSIVE IMMUNOGENICITY EXPERIENCE

- Monoclonal antibody therapeutics
- Novel protein therapeutics / enzymes
- Peptides / PEGylated peptides
- Insulin / insulin analogues

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FOR MORE INFORMATION

about the new **Millipore's BioPharma Services – BioAnaLab** Team and its focus on large molecule services, please contact:

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