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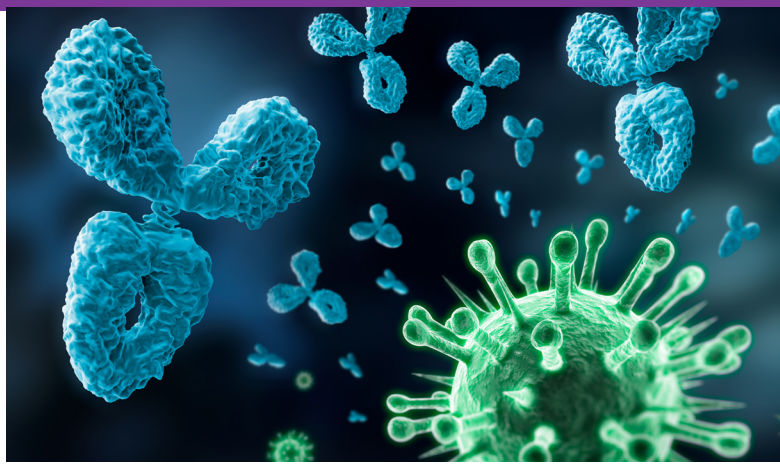
VALIDATION OF IMMUNOGENICITY ASSAYS TO SUPPORT NONCLINICAL AND CLINICAL STUDIES: DIFFERENT PURPOSES AND CHALLENGES

Regulatory guidelines focusing on immunogenicity assays for clinical studies are not all applicable to nonclinical requirements and their unique challenges. While we have a full understanding of regulatory requirements for clinical studies, the lack of regulatory documents addressing the particular needs of nonclinical immunogenicity studies has contributed to less consensus on appropriate immunogenicity testing strategies and validation parameters. This, by extension, has led to different approaches being adopted by the drug development industry. In the absence of applicable guidelines, a recent white paper from Laurén et al [1] describes a strategic approach to nonclinical immunogenicity assessment.

CHALLENGES AND GOALS — OVERVIEW



Nonclinical and clinical immunogenicity assessments each have their own challenges. For instance, achieving the appropriate level of drug tolerance in nonclinical studies may be difficult, and availability of critical reagents in early stages of nonclinical studies versus a long-term supply to support clinical phases of the drug development program may present a challenge.

The main goal of clinical immunogenicity assessment is to ensure patient safety, and provide the required information to support efficacy results by characterizing the immune response generated against the drug in terms of quality and quantity.

The purpose of nonclinical immunogenicity assessment of drugs destined for human use is primarily to support the understanding of toxicokinetic data. For example, we may assess the impact of administering high doses of the drug following single or multiple injections, as the impact of anti-drug antibodies (ADAs) can contribute to the planning of subsequent nonclinical toxicology studies, and ultimately clinical dosing decisions. In nonclinical studies, the drug may be foreign to the host as the biotherapeutics are developed to mimic human molecules, and they may be administered at much higher doses than will be given to humans. As a consequence, the immune response generated in nonclinical studies is often expected, and needs to be interpreted with caution.

In some cases, the polyclonal sera generated in rabbits as a positive control may be sufficient for nonclinical studies. However, the positive control may be changed to well-characterized monoclonal antibodies for the monitoring of assay performance to ensure the long-term supply for Phase II and III efficacy trials.

Sensitivity of the ADA method is sometimes an issue in clinical studies, especially when working with complex matrices such as those from oncology disease-type populations. Clinical studies also require additional levels of characterization of the immune responses, and the development of cell-based neutralizing antibody assays and domain binding specificity determinations, both of which can be complex and challenging, may be necessary.

This review highlights Altasciences' approach and experience in validating immunogenicity assays for nonclinical and clinical studies, and shares some of our approaches for each.

VALIDATION OF IMMUNOGENICITY ASSAYS FOR NONCLINICAL STUDIES

Altasciences offers nonclinical immunogenicity method qualification or validation based on the latest industry recommendations[2,3,4,5] as well as study-specific requirements. The choice between method qualification versus validation is mostly related to the stage of drug development, and whether the study is GLP or non-GLP. ADA method qualification will include basic parameters of nonclinical validation in smaller numbers of replicates and lots used for cut-point analysis.

Validation of a nonclinical immunogenicity assay at Altasciences is comprised minimally of the screening assay, with the option to include either the titration and/or the confirmation assay, depending on project needs. The validation is tailored to support the needs of a nonclinical study. Therefore, drug tolerance, cut-point analysis, sensitivity, precision, robustness, and stability are evaluated to detect immune responses that impact drug exposure and the interpretation of toxicology results. Fewer individual lots and a reduced number of data points are used to establish cut-points relative to clinical immunogenicity assessments, and assay parameters such as sensitivity and precision are evaluated using a reduced number of runs and requirements.

Drug Tolerance

One of the main challenges encountered in nonclinical bioanalytical immunogenicity assessment is the ability to ensure that the assay is designed to reach the appropriate level of drug tolerance. Drug tolerance of an assay may be defined as the ability of the assay to detect ADA in the presence of a defined concentration of drug. If the drug is present at higher concentration in the sample, it can hinder the detection of ADAs. As nonclinical immunogenicity assays are used to support toxicology studies, the concentration of drug in circulation is often still very high at ADA sample collection timepoints. Therefore, assay performance is critical to ensure that drug tolerance is achievable. Factors that may impact assay performance are the source of the positive control used and the design of the immunogenicity assay.

Positive Control

The positive control used can be generated against a single epitope of the biotherapeutic drug (monoclonal antibody) or against the entire drug molecule (polyclonal), from animals immunized with the entire biotherapeutic protein. Since the main function of the positive control is to mimic the immune response that may be generated against the full biotherapeutic protein, polyclonal sera are considered to be more representative of the expected anti-drug antibody response observed in animals or humans. These consist of various affinities and binding sites, and are therefore recommended whenever possible. In contrast, a monoclonal antibody is usually of high affinity and specific to one epitope, which may not be as representative of the immune response to the full biotherapeutic protein.

Regardless of the source of the positive control, they are used to estimate the relative assay sensitivity and drug tolerance of an assay to the drug. Multiple strategies are available and have been used by Altasciences to mitigate the impact of the drug on ADA detection. Our laboratories have evaluated drug tolerance using several types of assay formats covering a variety of biotherapeutic drug molecules.

Table 1 represents results of drug tolerance obtained for various therapeutic entities and demonstrates that typically, in order to reach a higher drug tolerance level, more complex methods are required. For example, an indirect ELISA method has provided the lowest level of drug tolerance; however, the addition of sample pre-treatment, such as acid dissociation, solid-phase extraction, and precipitation of the ADA:Drug complexes have resulted in drug tolerance levels up to 3500 µg/mL.

Table 1.

ASSAY TYPE	DRUG TYPE	DRUG TOLERANCE
Indirect ELISA	Peptide	2 µg/mL
Indirect ELISA	Oligonucleotide	6 µg/mL
Indirect ELISA with streptavidin magnetic beads	PEGylated protein	100 µg/mL
Bridging ECLIA with acid dissociation	Human angiotensin-converting enzyme 2	10 µg/mL
Bridging ECLIA with acid dissociation	Humanized monoclonal IgG4 antibody	1000 µg/mL
Solid-phase extraction with acid dissociation (SPEAD) ECLIA	Fc fusion protein	150 µg/mL
SPEAD ECLIA	Humanized IgG4 monoclonal antibody	2000 µg/mL
Acid pre-treatment with a SPEAD ECLIA	Humanized IgG4 antibody	2000 µg/mL
Acid pre-treatment with a SPEAD ECLIA	Humanized IgG4 monoclonal antibody	3500 µg/mL
Precipitation and acid dissociation ECLIA	Humanized IgG1 monoclonal antibody	750 µg/mL

ECLIA=electrochemiluminescence immunoassay; ELISA=enzyme-linked immunosorbent assay

Table 2 shows that the assay format can play a determining role in the achieved drug tolerance, and how it might be challenging to obtain the desired drug tolerance while working with a monoclonal anti-idiotypic antibody as a positive control. These are often of high affinity to the drug, resulting in multiple approaches being tested to achieve the appropriate drug tolerance for the study.

Table 2.

POSITIVE CONTROL	DRUG TYPE	ASSAY TYPE	DRUG TOLERANCE
Humanized IgG1 anti-Id-mouse monoclonal antibody	Monoclonal antibody	Bridging ECLIA	Not achieved
		Bridging ECLIA with acid dissociation	Not achieved
		Affinity capture elution (ACE) ECLIA	Not achieved
		High ionic strength dissociation (HISDA) ECLIA	Not achieved
		SPEAD ECLIA	10 µg/mL
		Precipitation and acid dissociation ECLIA	750 µg/mL

In the example below, the initial acid dissociation pre-treatment was not sufficient to provide the appropriate level of drug tolerance and therefore more complex strategies were tested:

The **affinity capture elution (ACE)[6] method** and **high ionic strength dissociation (HISDA)[7]** were tested, with no success. The ACE method is based on affinity capture of the ADA to the drug coated onto a solid-phase followed by removal of the excess free drug, release of the ADA through acid dissociation and transfer of the bound ADA, and subsequent detection using biotinylated drug. The HISDA method in this case uses $MgCl^{1}$ instead of acid to allow the dissociation of the ADA:Drug complexes.

The **solid-phase extraction with acid dissociation (SPEAD)[8]** was also tested, which consists of capturing the ADA via a biotinylated drug capture onto streptavidin plates to physically separate ADA and ADA:Drug complexes from the drug and the sample matrix. The acid dissociation step removes the ADA from the biotin-streptavidin complex, and detection is performed by simple direct electrochemiluminescence immunoassay (ECLIA). This method resulted in an improved drug tolerance level of 10 µg/mL. However, this level was much lower than the expected trough concentration of 400 µg/mL in the study samples.

Precipitating the ADA:Drug complexes from the free drug as a sample pre-treatment step, followed by an acidic treatment to dissociate the ADAs from the drug, was deemed successful in reaching the required drug tolerance (drug tolerance of 750 µg/mL obtained).

This case study successfully demonstrated how the use of monoclonal antibodies as surrogate positive control, combined with the longer half-life of many biotherapeutic drugs such as monoclonal antibodies, bi-specific antibodies, and antibody drug conjugates, can lead to challenging immunogenicity method development to achieve the appropriate drug tolerance levels to support toxicology studies. When there are high levels of circulating drug, one or multiple mitigation strategies are often required, and when the affinity of the positive control is very high (high affinity monoclonal anti-idiotypic antibody), it has to be considered in the method development strategy.

Planning for Nonclinical Immunogenicity Assessments

Early discussion between the bioanalytical scientists and sponsors is crucial for identification of possible challenges that may be encountered in assay development. As nonclinical immunogenicity assays are required at the early stage of a drug development program, critical reagents are not always readily available — for example, the positive control requires several months to obtain and, to avoid delays, needs to be generated well before initiation of the immunogenicity assay development. In some cases, the use of commercially available positive control may be an option.

Nonclinical studies are usually limited in data that would help support immunogenicity assay development, and address specific questions such as the expected concentrations of drug to anticipate at the appropriate immunogenicity sample collection timepoints. The bioanalytical scientists proactively request these data as soon as the toxicokinetic (TK) analysis is complete, to ensure that the drug tolerance level validated is appropriate to support the study needs.

VALIDATION OF IMMUNOGENICITY ASSAYS FOR CLINICAL STUDIES

Immunogenicity assays have to adapt as the drug development program progresses. Nonclinical assays may be qualified or validated with few requirements. However, immunogenicity assays used to support clinical studies need to be validated with well-defined, regulation-compliant requirements.

For instance, the requirement level of sensitivity for anti-drug antibody assays must be of 100 ng/mL as per the FDA's 2019 guidance on immunogenicity. Development effort must be invested to achieve these levels while dealing with different levels of interference.

The interference observed in immunogenicity assays, especially when disease-type populations are used, can present a significant challenge. Multiple factors can contribute to immunogenicity assay interference, such as:

- the drug itself
- rheumatoid factor (RF)
- the presence of soluble target or receptor
- an endogenous counterpart
- co-administered drug
- pre-existing antibodies
- other proteins such as lipid or hemoglobin

Over the years, useful strategies have proven to be successful in mitigating interference challenges. Access and availability of disease-type matrices to support method validation can also be limited; and despite the effort to obtain such matrices, the question of whether they represent study samples may also arise due to medications, strict inclusion and exclusion criteria of the clinical study protocol, and other considerations.

In some cases, clinical immunogenicity assessments require the characterization of ADA domain specificity of biotherapeutic drugs that have multiple domains, such as bispecific antibodies, FC fusion proteins, antibody-drug conjugates, or PEGylated proteins. While optional, the characterization of the binding specificity may be a regulatory requirement when it is linked to the clinical safety risk assessment. Characterization of the antibody specificity can also be important to perform when pre-existing antibody reactivity is observed in naïve individuals. It is important to understand its source, and the extent of investigation required will depend on the stage of the drug development program. While for nonclinical studies such investigations are often not required because the biotherapeutic drug is foreign to the host, it is critical when supporting clinical immunogenicity assessment.

Managing Variability

An immunogenicity assay was to be developed at Altasciences to support a clinical study for a phosphorothioate oligonucleotide drug that activates TLR9 through its un-methylated repeated CpG motifs. A high background signal was observed in many naïve human serum lots, which created a significant inter-lot variability that could have impacted the cut-point determination.

As part of our investigation, it was first important to determine whether this reactivity was due to non-antibody interfering components in the serum, or whether it was due to pre-existing antibodies, since a non-antibody interfering molecule could possibly be mitigated by assay optimization. We used magnetic beads coupled with protein A/G as a sample pre-treatment step to deplete the samples from all antibodies. Results demonstrated that the depletion completely inhibited the signal.

Following confirmation that the reactivity was mostly due to antibodies, we characterized the domain specificity of the antibodies and confirmed whether they were directed against the sequence only (without backbone modification) or to the phosphorothioate backbone itself (using a scramble sequence). Results demonstrated that most of the treatment naïve samples were reacting against the sequence itself, the full molecule, and the phosphorothioate backbone in a similar proportion. In contrast, the positive control that was raised against the full molecule showed a response against both the backbone and the sequence, and a higher response against the full molecule, which suggests a synergistic response when both the sequence and the backbone are combined.

Although oligonucleotides are typically non-immunogenic, this particular pre-existing reactivity is suspected to be directed against the repeated CpG motifs. The repeated CpG motifs are thought to be immunogenic in nature because they are a staple of bacterial DNA, which subjects would have previously mounted an immune response against due to exposure in daily life. Knowing the specificity of the pre-existing antibody response observed allows us to adopt the appropriate statistical model to determine the cut-point that takes this pre-existing reactivity into account when identifying post-dose, drug-induced immunogenicity.

Critical Reagent Validation

Critical reagents will directly impact assay performance, and as such have an important role in immunogenicity assays. Although critical reagent qualification is deemed important by regulatory agencies, the qualification requirements are not well described in the guidelines. Fortunately, industry recommendations can be found and are typically used[9].

A change in critical reagent can be considered minor or major, depending on the type of critical reagent (e.g., drug product, positive control, labeled reagents, matrix, or coated plates) and the nature of the change.

Minor changes may include such modifications as a new lot of the drug product with the same formulation and protein concentration, new matrix lots with no contributing interfering factors, or

new lots of labeled reagents with the same labeling ratio. Major changes can be due to a new drug product formulation, a new matrix lot with contributing interfering factors, or a new lot of positive control from a new bleed from the same animal. Major changes need to be addressed accordingly in order to avoid a significant impact on ongoing studies.

A new lot of critical reagent considered as a minor change can be qualified by assessing its performance through a precision run, and comparing the performance to the original lot. To address major changes, additional qualification assessments are required to ensure that the critical assay parameters, such as cut-points, drug tolerance, sensitivity, or selectivity, are not impacted. The strategy applied to qualify these critical reagents will need to be scientifically sound.

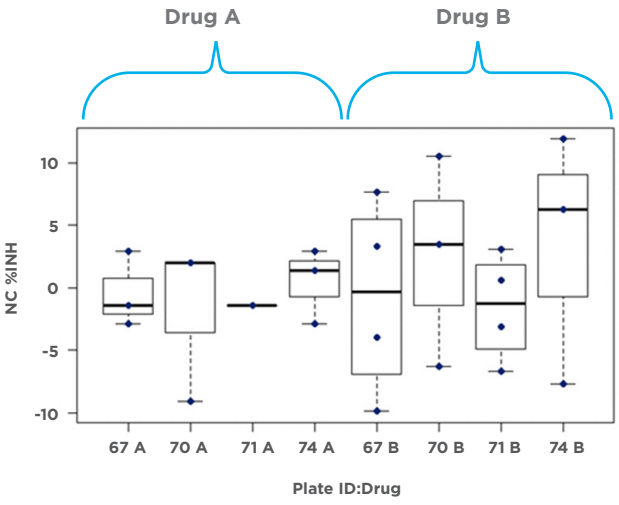
Critical Reagent Changes

For clinical studies, it is often difficult to evaluate the complete duration of the study as subject enrolment time is variable. Thus, the selection and availability of critical reagents for clinical studies need to be confirmed ahead of time. For example, as the clinical drug development program moved from Phase I to Phase III for one of our sponsors, the lot of the drug used changed. The critical reagent used in validation was therefore different from the one to be used in Phase III. Differences were observed in the formulation as well as the protein content, and adding an additional level of qualification to the reagent before using it to support the study was justified.

In this example, the drug product was used for capture and confirmation in an indirect ELISA assay. Precision runs were performed with both drug products (head to head) in the screening and confirmatory assay on different days, by different analysts, following an appropriate balanced design. Results between the two drug products were compared statistically by evaluating the variances and the means.

As shown in Figure 1, there was a statistically significant difference between the variances of the negative controls (NC; 4 data points) used in the confirmatory assay between both drug lots (Drug lot A and B). As the variance was significantly different between the two drug products although the intra-precision is within acceptance criteria (< 20.0% CV), it was determined that the initially validated confirmatory cut-point was not suitable for the new drug product lot and it was necessary to re-validate the assay to establish new cut-points that reflect the actual material used.

Figure 1.



Means statistically similar (p = 0.480)
Variances statistically different (p = 0.026)

This example highlights the importance of maintaining the same lots of critical reagents throughout the study whenever possible. We also must address the impact of changes on the current assay to ensure consistency in sample analysis, especially when the study is spread over several years.

Neutralizing Anti-Drug Antibodies (NAbs)

During drug development, cell-based assays are used for multiple purposes, including the determination of NAbs. The detection of NAbs is part of the characterization of the immune response in patients treated with a biotherapeutic drug. In these cell-based assays, the ability of NAbs to inhibit the biological action of the biotherapeutic drug is monitored, including the modulation of a biological process in the target cell. Therefore, the presence of the drug in study samples may interfere in the assay response read-out, and result in a loss of sensitivity to detect the NAb.

During development of a neutralization assay, drug tolerance and sensitivity should be considered as important factors to address in parallel. The drug tolerance evaluation should be performed with an accurate drug concentration estimated at the time of sampling, based on available pharmacokinetic (PK) data. Usually, the use of acid dissociation or other mitigation strategies could improve the drug tolerance of the cell-based assay, but can reduce the sensitivity of the assay due to harsh acid conditions used in the procedure which may lead to denaturation of the NAbs.

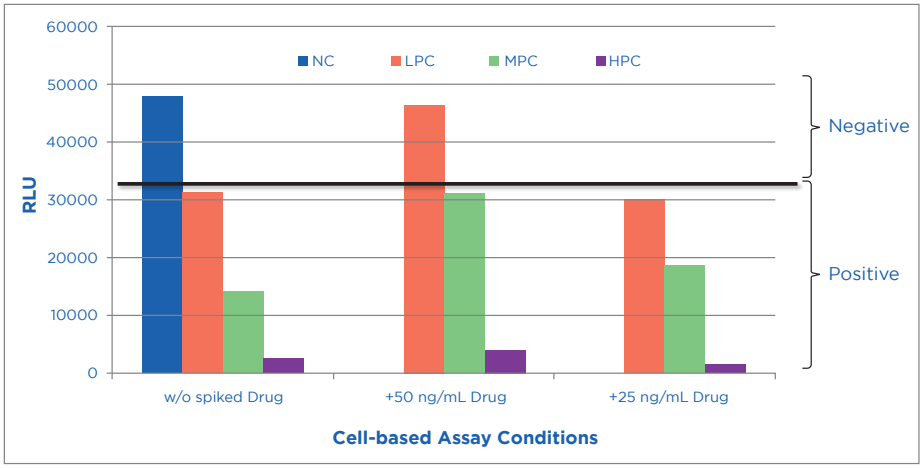
This was observed in a cell-based NAb assay that was developed by Altasciences for a peptide drug. In this example, drug tolerance was significantly low, leading to the use of several mitigation strategies to remove the drug from the samples, which either led to a decrease in sensitivity, or was proven not to be effective. The final assay format for that NAb cell-based assay was defined by adjusting the minimum required dilution (MRD) to a level that maintained an acceptable sensitivity with a minimum impact on the cell-based assay from the peptide.

We performed PK analysis of the drug at the time of the NAb sampling to ensure that an appropriate estimate of the drug concentration was present in regards to the required sensitivity. This information was crucial since small variations of the drug level in the sample could lead to loss of sensitivity and result in false negative interpretation of the results.

Sometimes, it may not be possible to reach the regulatory sensitivity and the appropriate drug tolerance level for cell-based assays. In such a case, after evaluating the impact on the clinical study, a compromise may be needed between sensitivity and drug.

In the study case shown in Figure 3, the drug tolerance failed at Low Positive Control (LPC) in presence of 50 ng/mL of drug in matrix, whereas in presence of a lower drug concentration, established from PK data, the drug tolerance was reached at the LPC.

Figure 3.



CONCLUSION

Altasciences' deep knowledge of fit-for-purpose nonclinical and clinical immunogenicity assessment allows efficacious acceleration of drug development. We routinely tailor assay development and undertake a validation that supports the 3Rs principle of reduction, and saves time, and resources.

We have nonclinical and early phase clinical expertise, with integrated solutions to ensure that as your drug development program accelerates from nonclinical to clinical phases, we achieve efficiencies through adaptation of the nonclinical ADA method to human serum. This is achieved by minimally changing the matrix, whenever possible, and extensively assessing the appropriate parameters required for the clinical study, such as sensitivity, specificity, cut-points, and selectivity in normal or disease matrix, and adjusting the assay accordingly. The same critical reagents are ideally secured in large amounts in order to maintain assay reproducibility throughout the clinical study and are re-qualified in the new assay format.

Our nonclinical and clinical scientific experts work closely together to maximize efficiencies, and share their knowledge and experience to deliver the best possible approaches to your immunogenicity assay requirements.

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ABOUT ALTASCIENCES

Altasciences is an integrated drug development solution company offering pharmaceutical and biotechnology companies a proven, flexible approach to preclinical and clinical pharmacology studies, including formulation, manufacturing, and analytical services. For over 25 years, Altasciences has been partnering with sponsors to help support educated, faster, and more complete early drug development decisions. Altasciences' integrated, full-service solutions include preclinical safety testing, clinical pharmacology and proof of concept, bioanalysis, program management, medical writing, biostatistics, clinical monitoring, and data management, all customizable to specific sponsor requirements. Altasciences helps sponsors get better drugs to the people who need them, faster.