White Paper

Cerba Research

Overcoming Bioanalytical Challenges in Anti-Drug Antibody (ADA) Detection for Antibody-Drug Conjugates (ADCs)

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Antibody-drug conjugates (ADCs) have changed the landscape of oncology by offering more targeted drug delivery, boosting treatment efficacy while reducing systemic toxicity. But their complex makeup brings unique challenges to bioanalytical testing, especially when it comes to detection of anti-drug antibodies (ADAs). Since immunogenicity can impact both the safety and effectiveness of ADCs, having reliable, well-characterized assays to detect ADA is crucial.

This whitepaper outlines some analytical challenges associated with detection of ADAs against ADCs and different approaches for overcoming them. Then, a case study is presented for an ADC drug product where pre-existing antibodies added a layer of complexity. Mitigation strategies for testing tiers and cut-point determinations are described, but also for unexpected challenges encountered during clinical sample analysis (presence of a hook effect and signal cross-talk). Finally, a different approach used for the determination of the NAb assay cut-point is described as the strategy used for the ADA assay was not adequate for the cell-based NAb assay format.

About the Author

Martin Roberge, Ph.D., Director, Development & Innovation, BioAnalytical Center of Excellence

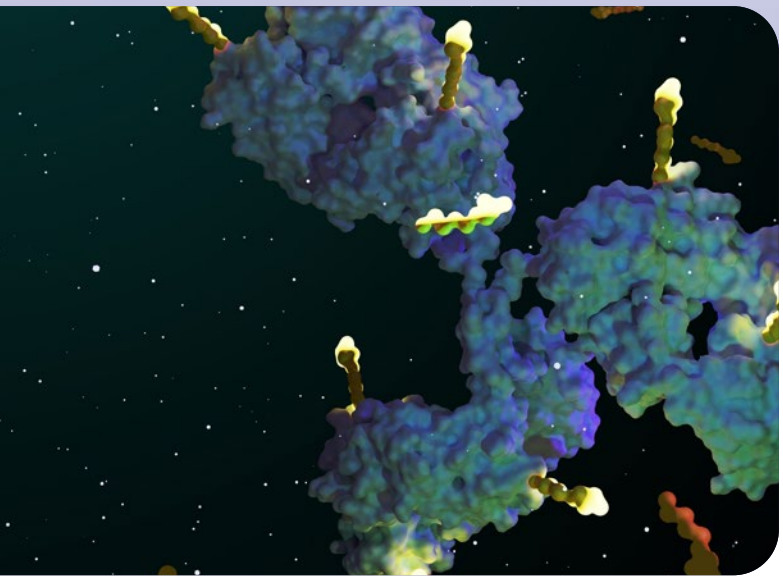
Martin Roberge, Ph.D., has over 26 years of experience in the life sciences industry, specializing in protein engineering, bioanalysis, and drug development. At Cerba Research, he leads method development and GLP/GCLP validation projects for large molecule drug products, supporting preclinical and clinical studies. His expertise includes immunogenicity assays (ADA, NAb), PK assays, biomarkers, and qPCR. Martin earned his Ph.D. in Biology from the University of Sherbrooke in Québec, Canada, and has held scientific roles at a start-up CRO in Montréal, Genencor International Inc., and completed a postdoctoral fellowship at Genentech Inc. in protein engineering

Introduction

ADCs are monoclonal antibodies (mAbs) or antibody fragments (e.g. scFv) that are attached via chemical linkers, with labile bonds, to biologically active, small drug molecules or toxins, combining the antibody's targeting specificity with cytotoxic effects of chemotherapeutics/toxins. ADCs are presently used in oncology (e.g. breast cancer, lymphomas, solid tumors), but their use is also expanding to other therapeutic areas (e.g. infectious diseases, neurological disorders and more).

Although ADCs show great clinical promise, their complexity makes them very susceptible to immunogenic responses. ADAs can affect the pharmacokinetic/pharmacodynamic properties of ADCs, but can also neutralize ADCs, accelerate their clearance, or trigger hypersensitivity reactions, causing severe adverse effects to patients. Monitoring of ADAs in patients treated with ADCs is therefore essential.

However, monitoring of these ADAs presents unique challenges due to their multidomain structure. Sensitive and specific immunogenicity assays that can distinguish between antibodies directed at different ADC components are required.



Key Challenges in ADA Detection for ADCs

1. Choosing the Right Assay Format

Available assays for ADA detection:

- **Bridging ECL:** A widely used format where the ADA binds to a capture reagent and a detection reagent, creating a complex that forms the "bridge". This sensitive format combines electrochemistry and chemiluminescence with low background interference.
- **Sandwich ELISA:** Generally used in cases where the bridging format cannot be used. ADAs can be coated directly on assay plates or captured with the drug which is coated on the assay plates; they are then detected using an enzyme labeled anti-antibody reagent. This format can be prone to drug interference and is species-specific depending on the detection reagent, unlike the bridging assay format which can detect ADA from different species (useful for preclinical and clinical studies).

2. Reagents for ADA Assays

Positive control antibody(ies)

A positive control antibody is required to validate ADA assays. The recommendation is to use purified positive control antibodies against the ADC as a whole and/or against its individual components (antibody portion or toxin portion). This surrogate positive control antibody can be custom made or commercially available depending on the ADC. Furthermore, specific positive control antibodies against each domain of the ADC are also required for the domain specificity assays.

Drug product conjugates (for bridging assay format)

Biotinylation and Sulfo-TAG labeling must be performed for bridging ADA assays, whereby the therapeutic drug is conjugated to biotin to be used as the capture reagent, and the drug is also conjugated to a Sulfo-TAG to be used as the detection reagent. Conjugate stability must be confirmed during validation and/or sample analysis to ensure accurate sample analysis results.

Meticulous labeling of the ADC or its components is required to preserve antigenicity and avoid altering epitope recognition.

Best practices

- Optimize challenge ratio for labeling.
- Confirm that the label does not interfere with ADA binding.
- Characterize conjugates (concentration, incorporation ratio)
- Ensure consistent reagent production across labelings.

3. Assay Interference by High Drug Levels

Drug interference is a significant challenge in ADA detection, especially when circulating drug levels are high during the early treatment phase. When the residual drug competes with assay reagents, ADA detection is masked. Various approaches can be used to increase drug tolerance of ADA assays:

- Acid dissociation
- Solid phase extraction with acid dissociation (SPEAD)
- Affinity capture and elution (ACE)
- Biotin-drug extraction with acid dissociation (BEAD)
- Precipitation and acid dissociation (PandA)

4. Case Study

The ADC drug product consisted of an antibody fragment (ScFv) linked to a bacterial toxin protein. Since most people have been in contact with the bacterial toxin of this ADC, there is a high incidence of pre-existing antibodies against the ADC. The chosen ADA assay format was a standard MSD-based bridging assay, using a biotynalted ADC as the capture reagent and a Sulfo-TAG-conjugated ADC as the detection reagent.

Determination of Anti-Drug Antibody Assay Validation Cut-Points

During method development and optimization, it quickly became apparent that most samples from naïve donors (~90%) contained pre-existing antibodies at highly variable levels (Figure 1).

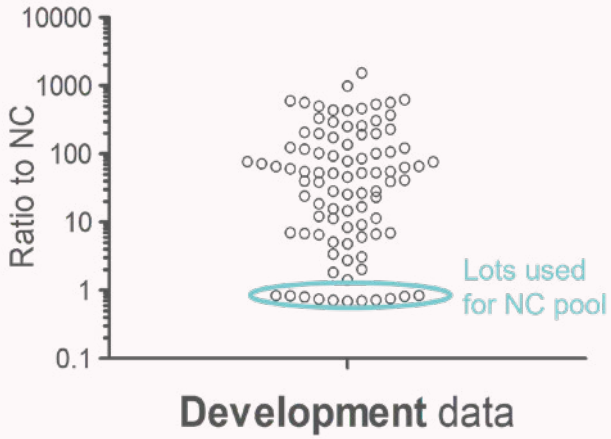


Figure 1: Pre existing anti-ADC antibodies in naïve donors

The scarce samples with low signals were pooled to prepare a negative pool which could be used as a negative control and also to prepare positive control samples. The method initially included screening, confirmatory, domain characterization and titer tiers according to current regulatory requirements. Because most subjects had prior exposure to the bacterial toxin, it was expected that most pre-existing antibodies would bind the toxin domain.

As a result, most clinical samples were anticipated to test and confirm positive for the presence of ADAs; the confirmatory tier in the testing cascade was therefore considered superfluous and was removed. Data interpretation would instead focus on the increase in titer values to determine whether the treatment enhanced the production of anti-ADC antibodies.

Cut-point determinations require a large number of samples expected to be ADA-negative; given the prevalence of pre-existing antibodies, the challenge of obtaining a sufficient number of negative samples emerged. Given the resources and time requirement, screening of many samples to find at least 50 negative was not considered for the ADA assay.

The use of antibody depletion (e.g. protein A/G) to produce "negative" samples was tested during method development, but was not effective enough to sufficiently deplete pre-existing antibodies. Therefore, surrogate negative samples were generated by introducing an excess of ADC to the samples during the procedures.

This strategy was effective in lowering the amount of pre-existing antibody-generated signal in most of the samples, which allowed the determination of screening, titer and domain characterization cut-points (Figure 2, Figure 3, Figure 4).

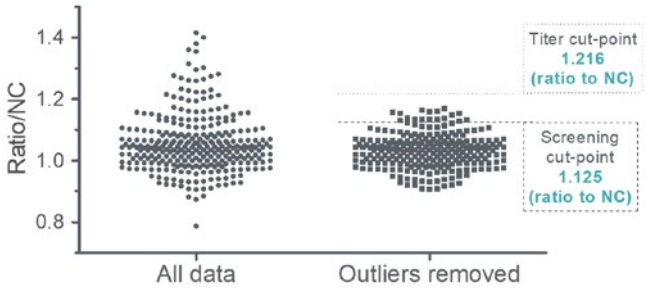


Figure 2: Screening and titer cut-point determinations

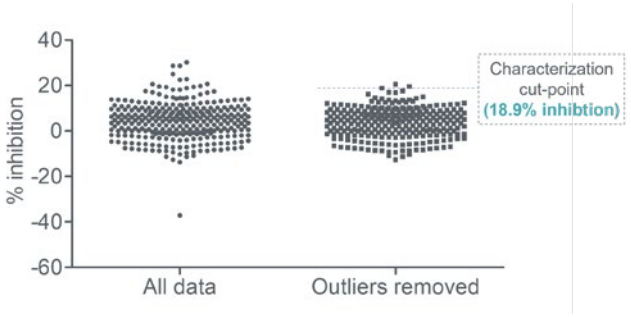


Figure 3: Characterization cut-point (toxin binding) determination

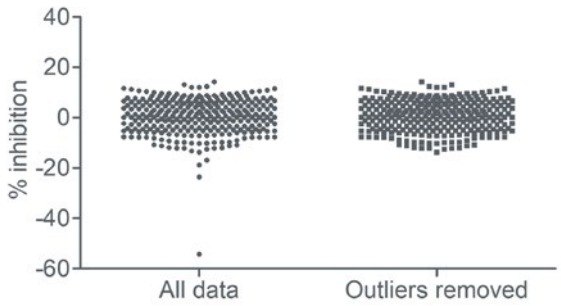


Figure 4: Characterization cut-point (single chain antibody binding) determination

Analysis of Clinical Study Samples

Analysis of the phase 3 clinical study samples using the validated assay confirmed that ~90% of human samples collected before initiating the ADC treatment contained pre-existing antibodies (Table…), similar to what was observed during assay development with commercial matrices. Moreover, most of the subjects developed treatment-boosted antibodies against the ADC as shown by the increase in titers (figure x).

In hindsight, the screening assay tier was possibly not required for this study since the titer assay seemed sufficient to identify samples with treatment-boosted anti-ADC antibodies. On the other hand, the screening assay signal was useful for the selection of sample dilutions applied in the titer assay tier. The positive samples were subsequently evaluated in the characterization tiers, and the results demonstrated that most pre-existing (baseline samples) and treatment-boosted antibodies were specific to the toxin portion of the ADC (Figure 5).

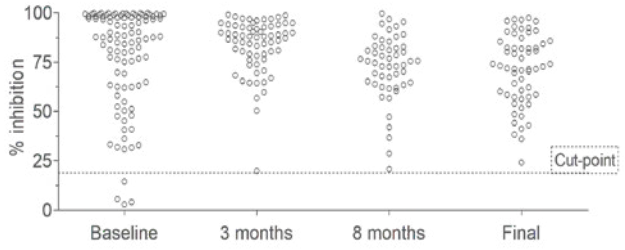


Figure 5: Boosting of anti-toxin ADAs

For a minority of subjects, ADC-induced antibodies directed against the antibody portion of the ADC were detected (Figure 6).

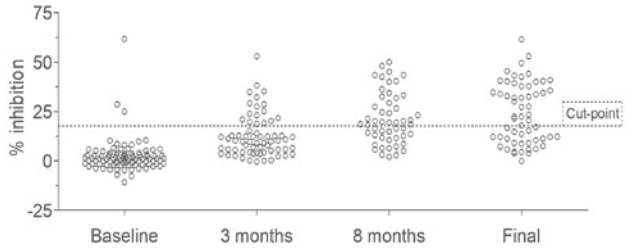


Figure 6: Boosting of anti-single chain ADAs

Mitigation of Hook Effect

During the analysis of the clinical study samples, unexpected results were obtained in the toxin domain characterization assay tier where some very highly negative %inhibition values were obtained (down to -2500%!). These were caused by an increase in signal (instead of a decrease in signal) in the presence of the competitor toxin (Table 1).

ECL without competitor Biomarker	ECL with competitor	%inhibition
2000	200	90%
2000	20 000	**-900%**

Table 1: Cause of highly negative %inhibitionstoxin ADAs

Further investigation of the data revealed that a hook effect was present at very high titers (Figure 7).

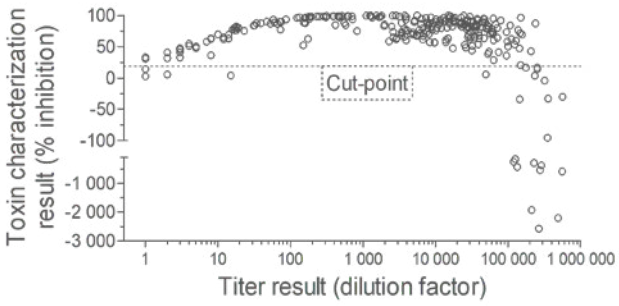


Figure 7: Hook effect when detecting anti-toxin ADAs

To mitigate the possibility of false-negative results, a total of 20 samples with very high titer values with negative %inhibitions in the toxin domain characterization assay were retested after a 1/1000 dilution to bring the assay signal outside of the hook effect zone. The results of all 20 study samples went from negative to positive, indicating the presence of ADA specific to the toxin domain (Figure 8).

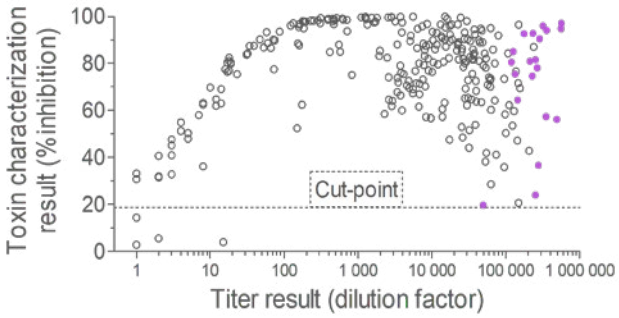


Figure 8: Mitigation of hook effect when detecting anti-toxin ADAs

Mitigation of Signal Cross-Talk

Another challenge occurred during sample analysis which was also due to the presence of elevated ADA levels and high assay signals. Multiple run failures were observed because the signal of the negative control samples was higher than the validated threshold. The first hypothesis was that high signals in some wells of the MSD plate was overflowing in neighboring wells.

Although there was a low correlation between NC signals and signals from neighboring wells, the increase in NC signals correlated much more with the total signal of the MSD plate sector. As MSD plates are read in 6 distinct sectors of 4 wells by 4 wells, the presence of a very high total signal in a sector could increase the signal of each well in a sector by up to ~100 ECL counts. This signal cross-talk could affect the result of samples with low assay signal. For instance, it could result in an increase in the NC signal above the validated threshold, causing run failure; this cross-talk could also affect study sample results:

- False-positive results could occur in the screening assay; an increase of the signal from a negative sample above the screening cut-point would result in a positive result.
- False-negative results could occur in the domain characterization assays; an increase of the signal of a sample with competitor could result in a negative result in the domain characterization assay.

To ensure that study sample results were accurately reported, all study samples with low signals that were initially tested in a plate sector with a high signal were retested in sectors with low signals. The retest results indicated that:

- Out of 21 positive study samples from the screening assay, 3 were negative when retested
- Out of 20 negative study samples from the toxin characterization assay, all 20 were positive when retested
- Out of 80 negative study samples from the antibody characterization assay, 22 were positive when retested