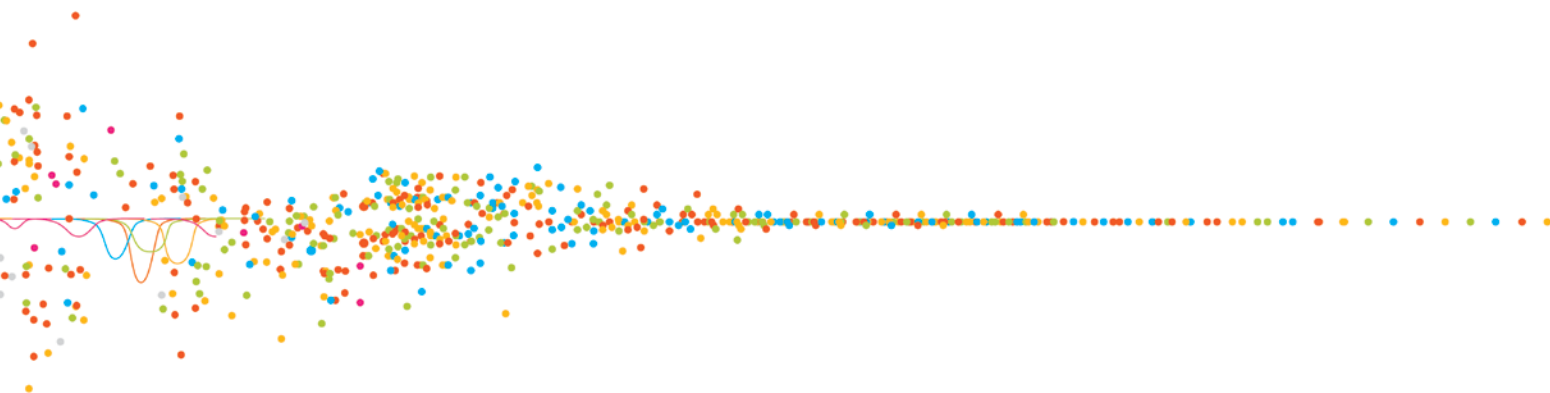


# Top Four Analysis Considerations for Antibody Drug Conjugates

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Chemotherapy is a mainstay of a standardized treatment regimen for cancer. However, the nonspecific targeting of healthy cells as well as tumor cells by cytotoxic small molecule drugs often results in intolerable side effects. These side effects compromise the efficacy of the treatment regimen and dramatically decrease the quality of life for cancer patients. Antibody drug conjugates (ADCs) are a newer class of chemotherapeutics comprised of monoclonal antibodies that selectively bind to tumor-associated antigens and have an associated cytotoxic small-molecule payload. The payload is attached to the antibody using enzyme cleavable linkers. Much effort has been made to identify highly tumor-specific monoclonal antibodies (mAbs), cytotoxic payloads with maximum efficacy, and linkers that are stable in circulation but allow for rapid cleavage to release the cell killing drugs following intracellular uptake of the ADCs. More than 30 targets have been investigated and more than 20 ADCs are now in various phases of clinical development, including Trastuzumab-DM1 (Roche), SGN-35 (Seattle Genetics), HuN901-DM1 (ImmunoGen), CR011-vcMMAE (Celidex Therapeutics), SAR3419 (Sanofi-Aventis), CMC-544 (Pfizer), and BIIB015 (Biogen Idec). Given the high complexity of ADCs resulting from the addition of the drug payload to already complex antibodies, the development and validation of analytical methods for ADC characterization, formulation analysis, and bioanalysis present significant challenges. A comprehensive review of bioanalytical assays for ADCs was published by Jean Philippe Stephan *et al.* In the current discussion, attention is focused on the development of bioanalytical assays for ADCs from a preclinical perspective.

### 1. Assay Formats for Pharmacokinetic Methods

The ADC is a heterogeneous mixture containing a cocktail of monoclonal antibodies with different drug payloads. Because of this heterogeneous nature, ligand binding assays (LBAs) are generally used for ADC bioanalysis. A variety of platforms are used including, colorimetric ELISA, CD-formatted Gyrolab, and electrochemiluminescence-based MSD. Total antibody assays can be used to quantify total antibody with or without the cytotoxic drug conjugated to it. Targeted tumor antigens, anti-idiotype monoclonal antibodies, anti-human IgG (Fab')<sub>2</sub>, and anti-human IgG (Fc) antibodies can be used as capture reagents. Anti-human IgG (Fab')<sub>2</sub>-HRP or anti-human IgG (Fc)-HRP, or biotinylated anti-human antibodies with streptavidin-HRP, can then be used for detection (Figure 1).

To eliminate non-specific binding, monkey adsorbed anti-human IgG antibodies are commonly used for nonhuman primate studies. Depending on the type of linkers, the position of drug conjugation may be located on (Fab')<sub>2</sub>, or the hinge region of the carrier antibodies. Increasing stoichiometry of drug conjugation may affect the binding of ADCs for capture, or detection reagents, and significantly affect the assay performance. Moreover, some assay formats are sensitive to the drug load even though the binding sites are not directly blocked. It has been reported that different assay formats yield different pharmacokinetic (PK) profiles and markedly affect the calculation of critical PK parameters such as clearance and drug exposure.<sup>3</sup> It appears that most of the assay formats are drug-load sensitive, but the generic human IgG assay using anti-human IgG (Fc) for capture and detection is an exception to this observation.

Figure 1. Example of total antibody assays.

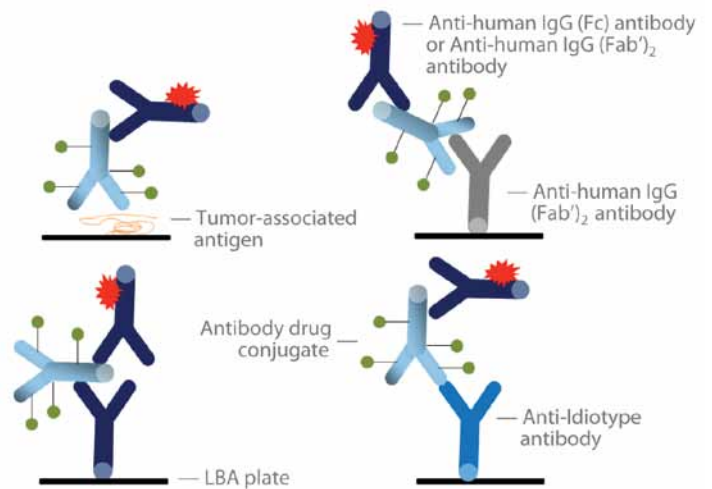


Figure 2. Example of conjugated antibody assays.

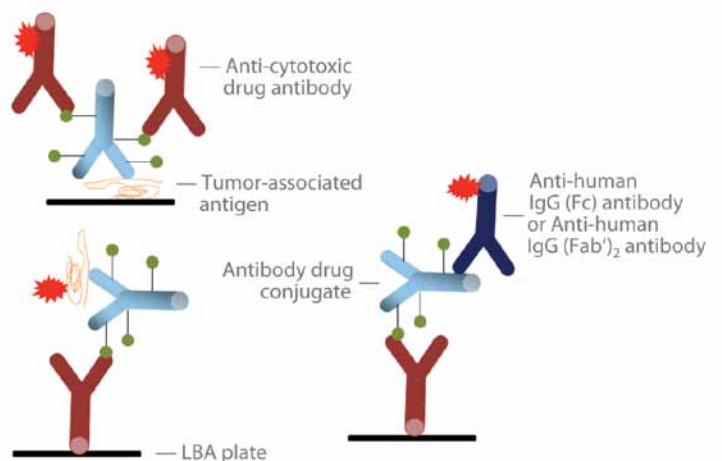
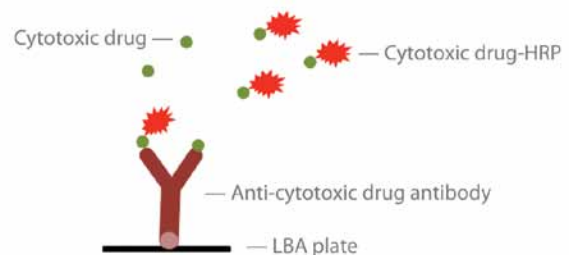


Figure 3. Competitive LBA for free drug.



For conjugated antibody LBAs, the anti-cytotoxic drug antibodies are used as capture or detection reagents and then paired with the capture and detection reagents outlined above to measure the antibody which conjugates at least one drug (Figure 2). The merit of conjugated antibody assays is their ability to quantify the possible progressive loss of drug from the ADCs in circulation. However, extra caution should be taken with the format using anti-drug antibodies for detection because the drug load of ADCs might change *in vivo*, compared with the ADC reference material used for the assays.

A successful design of an ADC is a combination of high drug-linker stability in circulation with efficient intracellular cytotoxic drug release. Significant achievements have been made in the past years to develop more stable linkers.<sup>4</sup> However, the nonspecific release of drug from the carrier antibody in circulation is still a crucial factor in determining the half-life of ADCs. To measure the drug moieties that have been released from the carrier antibody (i.e., free drug), a competitive LBA format could be used with anti-drug antibodies coated for capture and a constant concentration of HRP-drug as the reporter (Figure 3).

Because the clearance of free drug released from an ADC is much faster than the clearance of the ADC itself, the free drug might not be detectable. To solve this problem, one could measure the remaining drug that is bound to the ADC. This measurement can be achieved through the use of cathepsin B digestion to release the drug previously bound to the carrier antibody, followed by quantification of the free drug either via a competitive LBA assay or LC-MS.

Ideally, the assay methods for nonclinical PK bioanalysis should be developed during the early stages of ADC development and characterization. Evaluating the assays could be done with the recovery of enriched or purified drug antibody ratio (DAR) fractions compared with the average DAR standard to ensure that the different assay formats recover drug equally. If this analysis is not possible, detailed information of the ADC's mechanism of action, targeted tumor antigen, type of linker, drug antibody ratio, cytotoxic drug, etc., are necessary for the bioanalytical method design. In addition to LBA methods, hydrophilic interaction liquid chromatography (HILIC), HPLC, and LC-MS are being used to quantify ADCs. These methods, however, are beyond the scope of the current discussion and are reported elsewhere.<sup>1,3,4</sup>

## 2. Immunogenicity of ADC

Although the same methods used for determining the immunogenicity of general therapeutic antibodies can be used to determine the immunogenicity of ADCs, further characterization of anti-ADC antibodies for the targeting antibody, the linker, and the drug components are required to address the specificity of positive samples. The complexity of the ADC structure raises additional questions not previously encountered in the analysis of monoclonal antibody therapeutics or small molecule drugs. For instance, does an antibody response against the linker or the cytotoxic drug affect ADC internalization? Alternatively, do only neutralizing antibodies against the complementarity determining region (CDR) of the targeting antibody reduce the efficacy of the ADC?

## 3. Matrix Selection

Antibodies are commonly recognized as stable; therefore, most bioanalytical assays for therapeutic monoclonal antibodies are established in serum. This blanket approach, however, is not appropriate for the bioanalysis of ADCs where the conjugation of small molecule drug to the antibody via a linker creates a molecule whose overall stability depends upon the least stable of the three components. To this end, plasma is suggested as being the preferred matrix used for PK, and immunogenicity sample analysis because the inhibition of the clotting cascade in plasma results in much less proteolysis than in serum. Moreover, protease inhibitors could be added during sample collection to further stabilize the ADCs in plasma.

## 4. Critical Reagents

The LBA has been the primary analysis platform used for ADC bioanalysis because of its many advantages, including the ability to measure the test article in matrix without further sample extraction, its high-throughput nature, the broad dynamic range and high sensitivity that can be achieved, and the requirement of minimal sample volume. However, the availability of critical reagents is a key component to the development of highly specific and sensitive LBA methods. Much time and effort is usually taken to create anti-idiotypic antibodies against the carrier antibody or the cytotoxic drug. Because of the low immunogenic nature of cytotoxic drugs, conjugation with keyhole limpet hemocyanin (KLH) or an adjuvant may be required for antibody creation. The tight timelines for most IND-enabling studies necessitate early planning during the design of bioanalytical assays and must factor in the timelines for generation of critical reagents. These critical reagents are either prepared in-house or subcontracted to third parties, and at least six months lead time is typically required. Antibody screening, assay format testing, and reagent purification are all steps that are part of the reagent generation process that will add even more time, cost, and risk to projects. In some instances, the anti-carrier antibodies and targeted antigens may be commercially available. In these cases, it is critical to secure sufficient quantities of the lot so that the reagent inventory can cover the entire study. Communication and establishing a good relationship with the reagent vendors are important and should be a key consideration. If different reagent lots required to be used throughout a study, an appropriate way of assessing and bridging the different reagent lots must be established and implemented.

In conclusion, ADC technology has become increasingly prevalent, it is imperative that new and reliable methods are developed to better characterize different ADCs both *in vitro* and *in vivo*. Because of the complexity of ADCs, many LBA formats are available for the same bioanalytical purpose. Therefore, caution has to be taken during assay development and validation to ensure that the selected method is the best fit for a given compound. The critical reagents are crucial factors for LBAs; therefore, sufficient lead time and effort must be factored into meeting project milestones.

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