The administration of protein therapeutics to animals or humans carries the risk of eliciting an immune response, culminating in the production of antitherapeutic antibodies (ATAs). The consequences of ATA development range in severity and include changes in the PK, safety or efficacy profiles [1,2]. In nonclinical studies, the presence of ATAs can affect exposure and confound study interpretation, or in rare cases result in severe immune complex-mediated toxicity [3]. In clinical studies, ATAs can decrease efficacy by neutralizing the biotherapeutics’ activity or clearing it from circulation, or cause adverse events such as hypersensitivity or infusion reactions [1,2]. For these reasons, industry and regulatory agencies have issued recommendations for immunogenicity testing during various stages of drug development and for the validation of bioanalytical methods for ATA detection [4–10]. According to the ICH S6(R1) guideline for nonclinical safety evaluation of biotechnology-derived pharmaceuticals, ATA testing should be performed in nonclinical studies when there is evidence of altered PD activity, unexpected changes in exposure in the absence of a PD marker or evidence of immune-mediated reactions [8,10]. Immunogenicity testing is also an important component of clinical development, and typically follows a tiered approach. All clinical samples are first analyzed in the screening and specificity immunoassays to detect and confirm all antibodies capable of binding to the biotherapeutic. Confirmed positive samples may be tested in subsequent characterization assays to determine attributes including neutralizing capability, epitope, concentration/titer and isotype/subclass [6,11].

Antibody–drug conjugates (ADCs) are a novel class of biotherapeutic, combining the targeted delivery capability of a monoclonal antibody (mAb) with the potent cytotoxic activity of a small molecule [12–14]. The mAb may be conjugated to a variety of highly potent cytotoxic entities, including tubulin polymerization inhibitors (auristatins and maytansinoids) or DNA-damaging agents (calicheamicins and doxorubicins) [12]. Various linkers (cleavable or noncleavable) and conjugation chemistries may be employed for attachment to the mAb, most commonly through amino groups of lysines or the sulfhydryl groups of the interchain cysteines [13,14]. As they contain a protein component, ADCs could potentially induce an immune response upon administration. Antibody responses against ADCs may be directed against the mAb, the linker or the cytotoxic drug portion of the biotherapeutic [15]. The development of a mature immune response is enhanced by the stimulation of B-cells by T-cells, through the recognition of peptide fragments in the context of MHC class II molecules (MHCII), as well as by signals from co-stimulatory molecules and cytokines [16]. Internalization of the ADC by antigen-presenting cells and B-cells, and subsequent processing of the protein portion into peptides, results in the presentation of ADC peptides on MHCII. Recognition of the peptide–MHCII complex by T-cells may lead to the development of a specific and potent immune response.

**Background:** Immunogenicity testing is an important component of clinical development for large-molecule biotherapeutics. New complex types of large molecules, such as antibody–drug conjugates (ADCs), require careful evaluation of the testing strategy and bioanalytical assays used to monitor the development of antitherapeutic antibodies. **Results:** An electrochemiluminescence-based immunoassay for the detection and epitope characterization of anti-ADC antibodies was validated. Using this assay format, antibodies directed against the monoclonal antibody and linker–drug components of the ADC were successfully detected in a multiple-dose rat toxicity study. **Conclusion:** Immunogenicity assays incorporating epitope determination may provide additional information about the characteristics of induced antitherapeutic antibodies, including the magnitude and timing of the various types of antibody responses.
Key Terms

Antitherapeutic antibodies: Antibodies produced against a therapeutic after administration. Detection and characterization of antitherapeutic antibodies in subject study samples may be accomplished using a variety of bioanalytical assay formats and methods.

Immunogenicity: Potential to induce an immune response in humans or animals. During drug development, immunogenicity is assessed primarily through the measurement of antitherapeutic antibodies.

Epitope characterization: Determination of the portion of a biotherapeutic an antitherapeutic antibody is directed against.

Electrochemiluminescence-based assay: Method based on the detection of binding events using labels that emit light when stimulated electrochemically (application of electric current in the presence of a co-reactant).

Assay cut point: Statistically determined threshold value used in screening and epitope characterization assays to differentiate between negative and reactive samples.

Depletion cut point: Statistically determined threshold value used in a specificity assay to differentiate between nonspecific reactive samples (reported negative) and confirmed positive samples (reported positive).

complex by the T-cell receptor results in the proliferation and maturation of B-cells into antibody-secreting plasma cells. For antibody responses against ADCs, the mAb-derived peptides have the potential to bind to MHCI and stimulate T-cells, resulting in the activation of B-cells that secrete antibody against either the mAb or the linker–drug component. However, ADC antigen presentation may, theoretically, be diminished by the cytotoxic effects of the ADC on any cell upon internalization.

Immunogenicity assessment strategies employ a risk-based approach that combines the likelihood of immunogenicity (based on the biotherapeutic’s characteristics, the treatment regimen and the target population) with the potential consequences of ATA development [6, 11, 17–19]. ADCs are primarily used in oncology indications, for which the target population may be immunocompromised and therefore less likely to mount a robust immune response. ADCs and unconjugated mAbs may share some immunogenicity risk aspects, including a common partial structure and similar potential impact on PK and efficacy. However, there are a few key differences between ADCs and unconjugated antibodies in terms of immunogenic risk. Although the development of a robust T-cell-dependent immune response against an ADC is predominantly dependent upon the mAb sequence (e.g., human- or animal-derived), there is evidence to suggest that the type of linker/conjugation chemistry may also contribute to immunogenicity [20]. The epitope of the ADC targeted by an ATA response (i.e., the mAb or the linker–drug) may not influence the degree of impact on PK. However, anti-linker–drug antibodies may promote the formation of large crosslinked immune complexes due to the presence of multiple linker–drug entities on each ADC, which may pose a unique safety risk or neutralize the ADC if the complex size inhibits cell internalization required for ADC activity. Once internalized, the complexes should be catabolized within the lysosome and not affect cytotoxic drug activity. Anti-mAb antibodies, on the other hand, are more likely to neutralize the ADC if they bind to the complementarity-determining region of the mAb. Given these potential differences, it may be useful to incorporate epitope characterization into the ATA testing strategy. Importantly, both types of anti-ADC antibody responses may be associated with an increased safety concern. In addition to the adverse events commonly observed with ADC dosing, the uptake of cytotoxic drug by nontarget cells during immune complex clearance may cause additional toxicity. In light of these considerations and the limited clinical data available, the overall risk associated with ADC immunogenicity may be intermediate between an unconjugated mAb (low risk) and a biotherapeutic protein with an endogenous counterpart (high risk). Therefore, implementing an immunogenicity testing strategy incorporating appropriate assays capable of detecting ATAs against all ADC components is recommended for clinical development.

The complex and heterogeneous structure of ADCs presents new challenges for the development of analytical methods for their physicochemical characterization, QC, in vitro potency analysis, and PK and immunogenicity evaluations [21–23]. Currently, the most commonly used platforms for ATA detection are ELISAs and electrochemiluminescence (ECL)-based assays, utilizing a competition format to confirm specificity. These methods require conjugation of the biotherapeutic to labels for capture and/or detection, which may be problematic for an ADC because label conjugation sites may already be occupied by the linker–drug and additional labeling may compromise the ADC's stability. Therefore, careful assay design and consideration of assay platform are required for optimal detection of ATAs against all components of the ADC. In this manuscript, we describe the development, optimization and validation of an immunoassay to detect anti-ADC antibodies and determine their epitope specificity. The assay was used to monitor ATA development and characterize the various types of ATA responses observed in a nonclinical toxicity study.

Experimental

Reagents

The rabbit polyclonal anti-mAb positive control antibody was raised by immunizing rabbits with the ADC (Amgen Inc., CA, USA). The resulting antiserum was affinity-purified using the ADC covalently bound to cyanogen bromide-activated Sepharose 4B (GE Healthcare, WI, USA), followed by immunoabsorption against human immunoglobulin bound to Sepharose 4B. The hybridoma for the murine monoclonal anticytotoxic drug antibody (positive control in the immunogenicity assay and reagent in the conjugated antibody concentration assay) was obtained from Immunogen, Inc. (MA, USA), and the antibody was purified at Amgen Inc. using a Protein A column (MabSelect, GE Life Sciences) followed by ion exchange on an SP Sepharose HP column (GE Life Sciences). Bovine serum albumin (BSA)-linker–drug was produced at
Amgen Inc. using the same lysine conjugation procedure used for the ADC. Labeled reagents in the bridging ECL-based assay were generated by conjugating capture (ADC) or detection (ADC, mAb, BSA–linker–drug) reagents to biotin (Sulfo-N-hydroxysuccinimide (NHS)-LC-Biotin; Thermo Scientific, MA, USA) or ruthenium (Sulfo-TAG-NHS ester; Meso Scale Discovery [MSD], MD, USA) according to the manufacturers’ recommendations.

Reagents used for measuring conjugated antibody concentrations included the murine anticytotoxic drug antibody (Immunogen/Amgen, Inc.) and the murine anti-idiotype mAb (Amgen, Inc.) labeled with ruthenium (Sulfo-TAG-NHS ester, MSD) according to the manufacturers recommendations.

### ECL-based bridging assay (anti-ADC detection & epitope characterization)

#### Analytical procedure

Serum samples and controls were diluted 1:40 in 300 mM acetic acid to enable antibody complex dissociation prior to analysis. In the screening assay, acid-treated serum samples (50 µl/sample) were incubated at ambient temperature overnight on an orbital shaker in a neutralization buffer containing 1 M Tris, pH 9.5 (18 µl/sample) and 100 µl/sample of 1.0 µg/ml biotinylated ADC (B-ADC) and 1.0 µg/ml ruthenylated ADC (Ru-ADC) in 5× milk diluent/block (0.5% nonfat dry milk in a borate buffer; KPL, Inc., MD, USA). In the specificity assay, the same neutralization mixture was used except it also contained 100 µg/ml unlabeled ADC. In the epitope characterization assay (competition method), samples were incubated in the same screening assay neutralization mixture with the addition of 100 µg/ml of unla-beled mAb or BSA–linker–drug. In the epitope characterization assay (detection method), samples (50 µl/sample) were incubated in a neutralization buffer containing 1 M Tris, pH 9.5 (18 µl/sample) and 100 µl/sample of 1.0 µg/ml B-ADC and either 1.0 µg/ml ruthenylated mAb or 1.0 µg/ml ruthenylated BSA–linker–drug (Ru-BSA–linker–drug). The sample mixture was added to a blocked (5× milk diluent/block, KPL, Inc.) and washed (1× wash solution, imidazole-buffered saline with Tween 20, KPL, Inc.) MSD 6000 avidin high-bind microtiter plate. Following a 2-h incubation at ambient temperature on an orbital shaker, the plate wells were washed using 1× wash solution to remove any unbound complexes and 2× read buffer T (MSD) was added to each well. Using the MSD 6000 plate reader, an electrical current was placed across the plate-associated electrodes, resulting in an ECL signal. The mean ECL of each sample or control was divided by the mean ECL of the appropriate negative control (untreated or ADC-treated), to obtain the S/N.

### Validation

The **assay cut point** (ACP) and **depletion cut point** (DCP) were determined using 46 drug-naive donor serum samples from the appropriate study population (rat for the nonclinical toxicity studies). Both untreated and ADC-treated samples were tested within a statistically balanced design of experiments consisting of the following assay variables: two MSD avidin high-bind plate lots, two MSD 6000 instruments and two read buffer T lots. For ACP determination, S/N values from all untreated donor samples were statistically evaluated to establish the upper bound of a one-sided reference interval (95th percentile) for the distribution of S/N values. The equation used was:

\[
U_{95} = \text{LS-mean} + TINV(.95, \text{df}) \times \text{SQRT} (\text{variance total} + \text{variance LS-mean})
\]

**Equation 1**

where ‘U95’ refers to the upper 95% prediction limit; ‘LS-mean’ refers to the least squares mean of the untreated donor sample S/N values; ‘TINV’ is the inverse of the t-statistic used to calculate the prediction limits; ‘df’ is an estimate of the total degrees of freedom; ‘variance total’ is the total variance of each of the assay values; and ‘variance LS-mean’ is the variance of the LS-mean, which is estimated by using the square of the standard error of the LS-mean. In each case, no donor samples were excluded from the analysis, and there was no transformation necessary as the data were normally distributed.

For DCP determination, a ‘%T/U’ was calculated using the following formula:

\[
\%T/U = (\text{S/N of treated}/\text{S/N of untreated}) \times 100
\]

**Equation 2**

where for each donor sample the S/N value of the ADC-treated (‘T’) sample is divided by the S/N value of its corresponding untreated (‘U’) sample and then multiplied by 100.

The DCP is calculated using the equation:

\[
\text{DCP} = 100\% - L_{99.9} \text{ of } \%T/U
\]

**Equation 3**
where $L_{99.9}$ is the lower bound of a one-sided 99.9% prediction interval for the distribution of %T/U values. The equation used to calculate the $L_{99.9}$ was:

$$L_{99.9} = \text{LS-mean} - \text{TINV}(99.9, \text{DF}) \times \text{SQRT} (\text{variance total} + \text{variance LS-mean})$$

**Equation 4**

One donor sample was excluded from the analysis due to a high %CV between replicates, and there was no transformation necessary as the data were normally distributed.

The concentration of excess unlabeled ADC within the neutralization buffer used in the specificity assay was determined by testing a range of ADC concentrations and selecting the concentration capable of depleting (producing percentage S/N depletion greater than the DCP) samples containing high and low levels of ATA, while simultaneously having minimal impact on the assay background.

**Toxicity study design & ATA impact analysis**

A 1-month intravenous injection toxicity study consisting of five weekly doses (days 1, 8, 15, 22 and 29) of an ADC was conducted in Sprague-Dawley rats. The study consisted of four dose groups (placebo, low, medium and high ADC dose) with 42 animals in each group (equal number of males and females). Twenty-two animals per group were designated to undergo a 20-week recovery phase following dose administration. Serum samples were collected for ATA testing at study day 30 (dosing phase) and study days 64, 92, 120, 148 and 172 (recovery phase). Plasma samples for TK analysis were collected from a subset of the animals at various time points. To evaluate the impact of ATAs on TK, ADC concentration measurements (conjugated antibody) for days 23 to 172 are shown. Conjugated antibody concentrations were determined using an ECL-based sandwich immunoassay. Standards and QCs were prepared by spiking the ADC into normal Sprague-Dawley rat plasma (sodium citrate) pool (Bioreclamation, Inc., NY, USA). MSD 6000 standard microplates were passively adsorbed with murine monoclonal anticytotoxic drug antibody (antibody specific for the cytotoxin moiety of the ADC) and then blocked with Blocker™ BLOTTO (Thermo Scientific)/Tween 20 buffer after discarding excess capture antibody. The plate wells were washed with 1x wash solution (imidazole-buffered saline with Tween 20, KPL, Inc.), and standards, QCs, matrix blank and plasma samples were added after being pretreated in Blocker™ BLOTTO/Tween 20 buffer. Following an incubation step at room temperature and another wash step, ruthenylated murine anti-idiotype mAb (specific for the antibody portion of the ADC; Amgen, Inc.) was added to bind captured conjugated antibody. Following a final incubation and wash step, 1x read buffer T (MSD) was added to allow detection of bound ruthenylated murine anti-idiotype mAb. Using the MSD 6000 plate reader, an electrical current was placed across the plate-associated electrodes, resulting in an ECL signal that was proportional to the amount of conjugated antibody bound by the capture reagent. The inter-assay accuracy (%bias) and precision (%CV) for the method was -7 to -3% and 3 to 7%, respectively. The dynamic range was 20.0 ng/ml (LLOQ) to 1000 ng/ml (ULOQ). Assay acceptance criteria were %bias: ±15 to 15% for calibration standards; -20 to 20% for QCs. Standards, QCs and samples were run in duplicate wells, and the acceptable %CV of response between wells was ±15%. Ninety-eight percent of all assays performed to generate the ADC concentration results met acceptance criteria.

**Results & discussion**

**Platform/format & testing strategy selection**

Various platform technologies and assay formats are available for detection of ATAs [9,24,25]. Technology options include label-free platforms (Biacore™, Octet®) and label-dependent platforms (MSD, Alphalisa®, Gyrolab™), while format options include direct assays, bridging assays and novel methods such as SPE with acid dissociation and affinity-capture elution. The selection of an appropriate method for an anti-ADC assay is particularly important and should take into consideration the complex structure of the molecule, the requirement to detect antibodies against all components and the potential need to determine the epitope. An acid-dissociation bridging ECL-based assay (MSD 6000 instrument) was selected for the development of an anti-ADC detection method because it was expected to have superior sensitivity and drug tolerance. A disadvantage of this method is that it requires conjugation of the biotherapeutic to assay labels (i.e., biotin and ruthenium) for capture and detection. The mAb component of the ADC has already been modified by the conjugation to the cytotoxic drug. Additional conjugation to the assay labels
**Figure 1. Bridging anti-antibody–drug conjugate screening and specificity assay.** (A) In the screening assay, capture and detection using labeled whole ADC reagents (biotinylated and ruthenylated) enables the detection of antibodies against any portion of the ADC (monoclonal antibody or linker–drug). (B) In the specificity assay, incubation with excess unlabeled ADC competes away binding of anti-ADC to the labeled assay reagents, resulting in reduction of signal and confirming specificity of the response.

ADC: Antibody–drug conjugate; Ru: Ruthenium.

**Figure 2. Two potential epitope characterization strategies.** (A) In the epitope competition method, biotinylated and ruthenylated whole ADC is used for capture and detection in the presence of excess unlabeled epitopes (mAb or BSA–linker–drug). (B) In the epitope detection method, biotinylated whole ADC is used for capture and ruthenylated epitope (mAb or BSA–linker–drug) is used for detection.

ADC: Antibody–drug conjugate; BSA: Bovine serum albumin; mAb: Monoclonal antibody; Ru: Ruthenium.
may increase hydrophobicity and propensity for aggregation, potentially compromising ADC stability or immunological reactivity. For this reason, labeled ADC assay reagents (biotinylated and Ru-ADC) were thoroughly characterized to ensure stability (size exclusion chromatography) and immunological reactivity. Upon completion of validation experiments, this method was determined to produce acceptable assay parameters, and therefore alternative method technologies or formats were not pursued. For the immunogenicity testing strategy, a three-tiered approach was selected. In the screening tier of the assay, the biotinylated- and Ru-ADC are used for capture and detection of ATAs directed against any portion of the ADC (mAb or linker–drug; Figure 1). Samples that test above the ACP are further analyzed in the specificity assay, in the presence of excess unlabeled ADC. Samples that display a reduction in ADC-treated signal compared with the untreated signal are reported as positive for anti-ADC antibodies and further tested to determine the epitope.

Table 1. Evaluation of two different epitope characterization strategies.

<table>
<thead>
<tr>
<th>Anti-cytotoxic drug (minor epitope; ng/ml)</th>
<th>Anti-mAb (major epitope; µg/ml)</th>
<th>Molar excess</th>
<th>Anti-ADC</th>
<th>Anti-linker–drug epitope characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Screening and specificity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S/N</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>0</td>
<td>1.99</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>33</td>
<td>17.43</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>83</td>
<td>42.12</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>167</td>
<td>96.61</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>333</td>
<td>176.25</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>667</td>
<td>463.75</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>0</td>
<td>1.61</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>32.61</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>50</td>
<td>121.06</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>100</td>
<td>297.12</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>200</td>
<td>690.72</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>400</td>
<td>2175.42</td>
<td>100</td>
</tr>
</tbody>
</table>

*Samples containing 300 ng/ml of anticytotoxic drug or 500 ng/ml of anti-mAb antibody (minor epitopes) and increasing concentrations (0–200 µg/ml) of anti-mAb or anticytotoxic drug antibody (major epitopes) were tested within the screening/specificity assay and using two epitope characterization strategies (competition and detection, see Figure 2). The epitope competition strategy produced false-negative results for antibodies against the minor epitope when in the presence of ≥20 molar excess of antibodies against either major epitope. The epitope detection strategy produced accurate results up to 333 and 400 molar excess of the major epitope (anti-mAb or anticytotoxic drug, respectively).

‡False negative.

ADC: Antibody–drug conjugate; mAb: Monoclonal antibody.
epitopes. As expected, in the screening and specificity assays, each sample correctly produced a final result of anti-ADC positive (Table 1). In the epitope competition method, incubation with excess unlabeled linker–drug (‘minor’ epitope), when anticytotoxic drug was present alone, did cause reduction in S/N, producing a positive result as expected. However, when antibodies to both epitopes were present, significant reduction in S/N was not observed, producing false-negative results for the presence of anti-linker–drug antibodies (Table 1). Similar false-negative results for the presence of anti-mAb antibodies were obtained in the reverse scenario, with simulated samples containing a low concentration of anti-mAb and a large excess of anticytotoxic drug. In contrast, the epitope detection method was able to detect (produce a S/N above the ACP) antibodies against the minor epitope, despite the presence of a large molar excess of antibodies against the major epitope (up to ~333 molar excess of anti-mAb and up to ~400 molar excess of anticytotoxic drug). At higher molar excess of antibodies against the major epitope, the capacity of the capture reagent (B-ADC) is saturated, producing false-negative results even in the detection method. These experiments demonstrated that the epitope detection strategy is superior at characterizing polyclonal anti-ADC responses, and therefore this method was incorporated into the immunogenicity testing strategy.

Positive control characterization

In order to demonstrate the ability of the assay to detect all types of anti-ADC responses, the inclusion of positive controls capable of selectively binding to either of the two regions of the ADC (mAb or cytotoxic drug) is optimal. A monoclonal positive control antibody that recognizes the cytotoxic drug portion of the ADC was available (anticytotoxic drug). A polyclonal antibody that recognizes the mAb was generated by immunizing rabbits with the ADC and purifying the resulting antiserum against an ADC affinity column. The specificity of these reagents was assessed in the screening and epitope characterization assays. Both positive control antibodies produced increasing S/N values with increasing concentration in the screening assay (using Ru-ADC for detection), demonstrating that the screening assay is capable of detecting responses against all portions of the ADC (Figure 3). In the anti-mAb epitope characterization assay (using Ru-mAb), high S/N values were observed for the polyclonal antibody, indicating that it contains antibodies against the mAb portion of the ADC. For the monoclonal anticytotoxic drug antibody, concentrations as high as 1000 µg/ml did not produce S/N values above background levels. In the anti-linker–drug epitope characterization assay (using ruthenylated BSA–linker–drug), the anticytotoxic drug antibody demonstrated high responses as expected. Interestingly, high concentrations (630 µg/ml) of the polyclonal antibody showed no binding to ruthenylated-BSA–linker–drug, indicating that this reagent does not contain any anticytotoxic drug antibodies, despite being generated using the whole ADC as the immunogen. The availability of these two positive control reagents with different

![Figure 3. Characterization of positive control reagents. (A) The anti-mAb antibody demonstrated specificity for the Ru-labeled ADC and mAb reagents in the screening and epitope characterization assays, but did not show reactivity with the ruthenylated-BSA–linker–drug even at high anti-mAb concentrations (up to 630 µg/ml). (B) The anticytotoxic drug antibody demonstrated specificity for the Ru-labeled ADC and BSA–linker–drug reagents in the screening and epitope characterization assays, but did not bind to ruthenylated-mAb even at high anticytotoxic drug antibody concentrations (up to 1000 µg/ml). ADC: Antibody–drug conjugate; BSA: Bovine serum albumin; EC: Epitope characterization; mAb: Monoclonal antibody; Ru: Ruthenium.](image-url)
specificities enabled verification of assay performance and its ability to distinguish between the two types of anti-ADC responses.

- **Method validation**

Method validation followed the recommendation of relevant published White Papers and Regulatory Guidance [4–7,9]. Parameters evaluated during validation included assay and depletion cut points, matrix effect, precision, hook effect, sensitivity and drug tolerance.

To establish the ACPs used to designate reactive samples in the screening assay and positive samples in the epitope characterization assays, 46 serum donors were analyzed in each assay using a statistically balanced design incorporating multiple instruments and reagent lots. The ACPs were determined by calculating the upper bound of a one-sided 95% reference interval for the distribution of S/N values. In each assay, at least 95% of unspiked donors tested below the assay-specific cut point (true negatives; Figure 4A). The depletion cut point, used to designate positive samples in the specificity assay, was based on the upper bound of the 99.9% reference interval for the distribution of %S/N reduction values generated by the same serum samples (ADC-treated compared with untreated; Figure 4B). To evaluate matrix effect, donor samples were spiked with the anti-mAb (500 ng/ml) or anticytotoxic drug (300 ng/ml) positive control. In the screening and epitope characterization assays, all ATA-spiked samples produced S/N values above the assay-specific cut point (true positives; Figure 4A). In the specificity assay, all ATA-spiked samples produced a drug-treated %S/N reduction greater than the DCP (Figure 4B).

**Figure 4. Cut points and matrix effect.** Serum samples from 46 donors (unspiked or spiked with 500 ng/ml of anti-mAb antibody or 300 ng/ml of anticytotoxic drug antibody) were analyzed in (A) the anti-ADC screening and anti-mAb and anti-linker–drug epitope characterization assays and (B) the anti-ADC specificity assay. The unspiked donor S/N results were used to generate the assay cut points (95th percentile) and depletion cut point (99.9th percentile). All antitherapeutic antibody-spiked donors produced S/N values above the assay-specific cut points, and all drug-treated antitherapeutic antibody-spiked donors displayed S/N reduction above the specificity assay depletion cut point.

ACP: Assay cut point; ADC: Antibody–drug conjugate; DCP: Depletion cut point; EC: Epitope characterization; mAb: Monoclonal antibody.
To evaluate the assay sensitivities and range, standard curves were prepared using the two positive controls and evaluated in each assay. Assay sensitivity was determined by interpolating the average positive control concentration at the assay-specific cut points from multiple standard curves analyzed during the validation. For the anti-mAb positive control, the assay sensitivities in the screening and epitope characterization assays were 85 and 105 ng/ml, respectively (Figure 5A). A hook effect was observed in both assays at 315 µg/ml of anti-mAb (data not shown). For the anti-cytotoxic drug positive control, the screening assay sensitivity was 40 ng/ml (Figure 5B) and a hook effect was observed at 250 µg/ml. The anti-cytotoxic drug epitope characterization assay sensitivity was 60 ng/ml, and a hook effect was observed at 500 µg/ml of anticytotoxic drug antibody. The presence of a hook effect indicates that the assay reagents may become saturated above that level of ATA. Despite the presence of a hook effect (i.e., a reduced S/N value at higher concentrations), the S/N of high concentration ATA-spiked samples never fell below the ACP, suggesting that the hook effect is unlikely to cause false negatives in this assay.

The most common limitation of immunogenicity assays is the interference of drug present in the study samples with ATA detection. Drug-tolerance limits were evaluated in order to determine the ADC concentration at which the assay is no longer able to accurately detect the presence of anti-ADC antibodies. Samples containing anti-mAb or anticytotoxic drug positive control at 2 µg/ml were spiked with increasing concentrations (0–500 µg/ml) of the ADC and analyzed in each assay. Drug-tolerance limits were based on the highest ADC concentration at which the sample tests reactive or positive. In the screening and epitope characterization assays, both the anti-mAb and anticytotoxic drug antibodies produced a drug-tolerance limit of 250 µg/ml (125 molar excess) of ADC (Figure 6). The high drug-tolerance limits may be attributed to the acid pretreatment of samples, which serves to dissociate immune complexes and improve labeled drug competition with unlabeled drug that is present in the sample [26].

Finally, precision was assessed by calculating the intra-assay and inter-assay %CV of the negative and positive control samples analyzed throughout the validation. The screening intra- and inter-assay precision for all assay controls in the screening/specificity and epitope characterization assays did not exceed 20% (Table 2).

### I-month rat toxicology study

An acid-dissociation bridging ECL assay was used to support immunogenicity testing in a rat toxicity study. Five weekly intravenous injections were administered at four dose levels (placebo, low, medium and high) to 42 animals in each dose group. Twenty-two animals per group (with the exception of the high-dose group) were assessed during the 20-week recovery phase. Serum samples collected at the end of the dosing phase (day 30) and during the recovery phase (days 64, 92, 120, 148 and 172) were tested in the anti-ADC screening and specificity assays. Confirmed positive samples were further tested in the anti-mAb and anti-linker–drug epitope characterization assays.
Among all animals dosed with the ADC, 27% tested positive for anti-ADC antibodies at the end of the dosing phase, and 53% tested positive following discontinuation of treatment (TABLE 3). The lower rate of ATA positivity during the dosing phase may have been caused by drug interference in the assay, which diminished at later time points during drug washout. At higher dose levels (medium- and high-dose groups), an increased incidence of ATAs was observed. The high rate of antibody development and response magnitude indicates that the ADC was successfully presented by antigen-presenting cells, despite the presence of a cytotoxin within the Table 2. Assay precision: negative and positive controls†.

<table>
<thead>
<tr>
<th></th>
<th>Screening assay</th>
<th>Epitope characterization assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-ADC</td>
<td>Anti-mAb</td>
</tr>
<tr>
<td>Intra-assay precision (%)CV mean and range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>3% (1–7%) (37 plates)</td>
<td>5% (1–20%) (16 plates)</td>
</tr>
<tr>
<td>Anti-mAb LPC</td>
<td>3% (1–6%) (18 plates)</td>
<td>5% (2–19%) (15 plates)</td>
</tr>
<tr>
<td>Anti-cytotoxic drug LPC</td>
<td>3% (2–4%) (7 plates)</td>
<td>NA</td>
</tr>
<tr>
<td>Inter-assay precision (%)CV</td>
<td></td>
<td>8.0% (16 plates)</td>
</tr>
<tr>
<td>Negative control</td>
<td>7.7% (37 plates)</td>
<td>8.3% (15 plates)</td>
</tr>
<tr>
<td>Anti-mAb LPC</td>
<td>5.5% (18 plates)</td>
<td>NA</td>
</tr>
<tr>
<td>Anti-cytotoxic drug LPC</td>
<td>5.6% (7 plates)</td>
<td>NA</td>
</tr>
</tbody>
</table>

† Quadruplicates of each assay control were run within each plate during validation and analyzed to determine intra-assay precision. Inter-assay precision was determined by observing the precision of the mean control response (electrochemiluminescence signal for negative control and S/N for positive controls) across multiple reagents and days (number of observations indicated in parentheses).

ACP: Assay cut point; ADC: Antibody–drug conjugate; EC: Epitope characterization; mAb: Monoclonal antibody.

Figure 6. Drug tolerance. The anti-mAb and anticytotoxic drug positive control antibodies were diluted to 2 µg/ml in serum, spiked with increasing concentrations of the ADC (0 to 500 µg/ml) and analyzed in the screening or epitope characterization assays to determine the highest ADC concentration that the positive sample can contain and still produce a reactive or positive result (S/N above the assay-specific cut point). For the anti-mAb and anti-linker–drug screening and epitope characterization assays, the drug-tolerance limit was determined to be 250 µg/ml of ADC for both assays and positive controls.

ACP: Assay cut point; ADC: Antibody–drug conjugate; EC: Epitope characterization; mAb: Monoclonal antibody.
antigen. Antigen presentation was most likely not impaired because the cytotoxin primarily acts on rapidly dividing cells.

The analysis of the epitope of anti-ADC antibodies focused on the low and medium dose groups, which had recovery phase samples available. Although all types of immune responses and kinetics were observed, certain trends in the data were apparent. For animals that tested positive for ATAs during the recovery phase, immune responses were in all cases directed against the mAb epitope, while 71% (low-dose group) and 81% (medium-dose group) of animals also tested positive for antibodies directed against the linker–drug portion of the ADC (Figure 7A). ATA responses directed solely against the linker–drug were not detected. Interestingly, the anti-ADC and anti-mAb S/N values were higher than the anti-linker–drug values (Figure 7B), suggesting that antibodies against the mAb epitope were produced at higher levels or had higher affinities. The timing of peak response was also different between anti-mAb and anti-linker–drug responses, with the majority of anti-mAb responses reaching maximum concentration earlier in the study (days 64–120) than anti-linker–drug responses (day 172; Figure 7C). Within the same animal, the anti-linker–drug peak response occurred after the anti-mAb peak in the majority of animals (data not shown). Given the low magnitude of the anti-linker–drug responses throughout the study, however, it is unclear whether this observed shift in the timing of peak response is biologically meaningful.

Although it is possible that these observations of the characteristics of the anti-ADC responses are due to differences between the two epitope characterization assays (sensitivity and drug tolerance), validation experiments indicated that assay sensitivities were similar. The drug-tolerance limits were different (lower for the anti-linker–drug epitope characterization assay at low ATA levels), but serum drug concentrations measured during the recovery phase were not high enough to interfere with either of the epitope characterization assays. Therefore, it is unlikely that the observed trends in response levels (lower for anticytotoxic drug) and peak response times (later for anticytotoxic drug) are purely assay artifacts, and it is possible that the anti-mAb response is regulating the development of the anti-linker–drug response. One potential mechanism is that the robust anti-mAb response (e.g., the presence of immune complexes and complement) facilitates epitope spreading to linker–drug epitopes [27,28]. Accordingly, the anti-linker–drug response is delayed relative to the initial anti-mAb response. The anti-mAb response has had an opportunity to develop into a mature (high affinity) response during the course of this study, whereas the anti-linker–drug response develops later and has therefore not been affinity-matured.

To investigate the impact of anti-ADC antibodies on ADC exposure, ADC concentration profiles (conjugated antibody, day 23–172) from antibody-positive and antibody-negative animals were compared. In both the low- and medium-dose groups, anti-ADC-positive animals displayed enhanced ADC clearance (Figure 8), and the most dramatic effect was observed in animals with high levels of ATA. The mechanism of enhanced drug clearance is proposed to involve the formation of crosslinked drug–ATA immune complexes, whose rapid elimination may be mediated by the complement pathway or Fcγ receptors [29]. The difference in the effect on the TK profile of anti-mAb versus anti-linker–drug responses could not be evaluated because responses directed solely against the linker–drug were not observed. Although ATAs clearly impacted TK, the presence of antibodies did not appear to cause any additional toxicities in antibody-positive animals (data not shown).

### Conclusion

An acid-dissociation bridging ECL-based assay for the detection and epitope characterization of anti-ADC antibodies was optimized and validated. To enable the determination of epitope for all types of anti-ADC antibodies, even when the response is polyclonal and predominantly directed against one epitope, the detection method was found to be superior to the competition method. The assay demonstrated high sensitivity and drug tolerance along with the ability to differentiate

<table>
<thead>
<tr>
<th>Dose</th>
<th>Anti-ADC positive animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dosing</td>
</tr>
<tr>
<td>Low</td>
<td>7% (3/42)</td>
</tr>
<tr>
<td>Medium</td>
<td>39% (16/41)</td>
</tr>
<tr>
<td>High</td>
<td>39% (12/31)</td>
</tr>
<tr>
<td>Total</td>
<td>27% (31/114)</td>
</tr>
</tbody>
</table>

A large increase in anti-ADC incidence was observed between the low- and mid/high-dose groups. There was also an overall apparent increase in anti-ADC incidence between the dosing and recovery phases (most likely due to drug interference with antitherapeutic antibody detection during the dosing phase). Note: recovery samples were not available from animals in the high-dose group due to animal mortality.
between antibodies directed against the mAb or linker–drug components of the ADC. In a multiple dose rat toxicity study, all types of anti-ADC antibodies were detected. In most cases, antibody responses were determined to contain both anti-mAb and anti-linker–drug components. However, the anti-mAb responses were generally higher in concentration and detected earlier than the anti-linker–drug responses. The presence of ATAs was associated with enhanced clearance, but did not appear to affect the ADC’s toxicity profile.

**Future perspective**

Understanding the effects of an immune response against a biotherapeutic is valuable for the interpretation of nonclinical studies and an important component of safety monitoring in clinical
studies. Complex biotherapeutics containing multiple domains, such as ADCs, present challenges for the development of bioanalytical methods for immunogenicity assessment. These assays should be capable of detecting antibodies directed against all domains. Epitope characterization may be helpful in understanding the immune response elicited by these novel large molecules and inform the design of future biotherapeutics.

Financial & competing interests disclosure
Support for this study was provided by Amgen, Inc. All authors are employees and shareholders of the study sponsor. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.
Executive summary

**Platform/format & testing strategy selection**
- In light of the complex and potentially heterogeneous nature of antibody–drug conjugate (ADC) molecules, the development of bioanalytical methods for the assessment of ADC immunogenicity requires careful consideration of assay format and thorough characterization of assay reagents.

**Epitope characterization strategy evaluation**
- Epitope characterization using the detection method (labeled epitope used to detect the antitherapeutic antibodies) is less likely to produce false-negative results than epitope characterization using the competition method, especially with polyclonal responses directed primarily against one epitope.

**Positive control characterization**
- If feasible, inclusion of separate positive controls directed against either the monoclonal antibody (mAb) or cytotoxic drug components is recommended to confirm the ability of the assay to detect all types of anti-ADC responses.

**1-month rat toxicology study**
- A sensitive electrochemiluminescence-based bridging assay was implemented for the detection and characterization of anti-ADC antibodies in a rat toxicity study.
- Antibodies specific for each component of the ADC (mAb and linker–drug) were detected, and most responses were directed against both components.
- Anti-mAb responses were observed to be higher in signal-to-noise ratio (concentration) and reached maximum levels earlier than the anti-linker–drug responses.
- Enhanced ADC clearance was detected in animals testing positive for anti-ADC antibodies.

### References
Papers of special note have been highlighted as:
- of interest
- of considerable interest


**Presents the complexities and challenges associated with antibody–drug conjugate analytical method development.**


**Describes the considerations for the optimal selection of technology and platform for anti-therapeutic antibody methods.**


