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Comparison of competitive ligand-binding assay and bioassay formats for the measurement of neutralizing antibodies to protein therapeutics

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ABSTRACT

Administration of biological therapeutic proteins can lead to unwanted immunogenicity in recipients of these products. The assessment and characterization of such immune reactions can be helpful to better understand their clinical relevance and how they relate to patient safety and therefore, have become an integral part of a product development program for biological therapeutics. Testing for anti-drug antibodies (ADA) to biological/biotechnology-derived therapeutic proteins generally follows a tiered approach. Samples are initially screened for binding antibodies; presumptive positives are then confirmed in a confirmatory assay; subsequently, confirmed-positive samples may be further characterized by titration and with a neutralizing antibody (NAb) assay. Regulatory guidances on immunogenicity state that assessing the neutralizing capacity of antibodies should preferably be done using functional bioassays, while recognizing that competitive ligand-binding (CLB) assays may be substituted when neutralizing bioassays are inadequate or not feasible. This manuscript describes case studies from four companies in which CLB assays and functional bioassays were compared for their ability to detect neutralizing ADA against a variety of biotechnology-derived therapeutic proteins. Our findings indicate that CLB assays are comparable to bioassays for the detection of NAbs, in some cases offering better detection sensitivity, lower variability, and less matrix interference.

1. Introduction

Testing schemes for evaluation of the immunogenicity of therapeutic proteins generally utilize a tiered approach in which samples are initially screened for binding antibodies, subsequently confirmed for specificity, and often characterized as to the relative amount of antibody present (titer). Assessment of the neutralizing ability of ADA may also be performed [1–3]. NAbs rendering the drug biologically inactive have the potential to reduce clinical efficacy. If neutralizing antibodies generated to a therapeutic protein can cross-react to an endogenous protein, rendering both the therapeutic and the endogenous protein inactive, the patient may be faced with a greater risk for developing clinically relevant sequelae. If the endogenous protein to which Nabs develop does not have biologically redundant counterparts, the risk of clinical sequelae is augmented [4].

The type and extent of the immunogenicity monitoring program followed during product development should be based upon an immunogenicity risk assessment of the therapeutic. The risk assessment strategy should be driven by the risk of a particular therapeutic protein eliciting an immune response and a consideration of the potential consequences due to that immune response [5–7]. The immunogenicity assessment strategy may need to be revised during the lifetime of the program based upon incurred immunogenicity and clinical data. Depending on the assessed level of immunogenicity risk, specific considerations can be made as to what type of immunogenicity assays (binding and NAb) should be implemented and when [8,1].

The recent EMA guidance on immunogenicity [9] states that assessing the neutralizing capacity of antibodies is preferably done using functional bioassays, but that CLB assays may be substituted when neutralizing bioassays are not feasible or available. Based on the therapeutic protein drug's mode of action and its immunogenicity risk, a bioassay may be deemed more appropriate for a given stage of the drug development program [8,1]. However, in some instances a CLB approach may be found to be more suitable for NAb detection. This is particularly true if direct binding of ther-

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apeutic to its target is sufficient to evoke all biological processes that define its mechanism of action (MOA). However, if other functional aspects of the therapeutic may be affected by ADA/NAb that do not directly interfere with target binding, then the CLB assay format may not be adequate. Thus, a comprehensive understanding of the drug mode of action can be critical to adequately inform researchers when choosing the most appropriate assay format.

There are a number of factors to consider when choosing between a cell-based bioassay format and a CLB assay format. For instance, finding appropriate cell lines that yield adequate signal to noise responses and appropriate specificity can be challenging. In addition, bioassays tend to be more sensitive to matrix interference, tend to be less drug tolerant, less precise, less sensitive, and more time-consuming than CLB assays. Bioassays also may require the validation of the cell system as well as the method used for the measurement of response. Sponsors generally have timeline constraints that preclude assessment of multiple assay platforms, and therefore must choose one assay platform over another. Although one platform may appear to be the most scientifically relevant, based upon the MOA of the therapeutic and the product's risk assessment, the particular format chosen may not adequately detect clinically relevant antibodies. Thus a Sponsor may initially develop a method and then, as clinical data is accumulated and evaluated, switch to another assay platform that provides a more relevant or robust NAb measurement.

The available literature comparing CLB assays and cell-based bioassays for the assessment of neutralizing antibodies is limited or compares a cell-based binding assay with a functional assay [10]. Hybrid approaches utilizing cell surface protein expression for detecting antibody binding activity through binding inhibition have been reported previously [11,12] and are not included in the scope of this manuscript. This report presents case studies from four companies in which cell-based bioassays and CLB assays were compared for the detection of NAbs to various types of therapeutic proteins. Due to the fact that most companies do not develop and utilize two assays, in particular for analysis of clinical samples, no clinical NAb data comparison is provided. The case studies described herein demonstrate that CLB assays may not only provide acceptable assay characteristics for the detection of NAbs, but may also offer considerable operational advantages.

2. Case studies

2.1. Case study 1: neutralizing assays for a monoclonal anti-CD40 antibody

CP-870,893 is an agonistic human IgG2 monoclonal antibody that binds to the cell surface molecule CD40. Agonism of CD40 has been linked to anti-tumor activity in both nonclinical and clinical studies [13–16]. During nonclinical development of CP-870,893, both bioassay and CLB assay approaches were evaluated to assess the neutralizing activity of ADA in a cynomolgus monkey study.

2.1.1. Bioassay: CD40 induction of CD54 expression

A validated cell-based bioassay for detecting neutralizing anti-CP-870,893 antibodies in human serum and modified for use in monkey plasma was previously described [17]. Briefly, the B-lymphoblast cell line Daudi was treated with multiple concentrations of CP-870,893 to establish a reference curve and a concentration of 50 ng/mL was added to all samples for approximately 2 days in the presence of 10% monkey plasma. Daudi cells treated with CP-870,893 results in increased CD54 (ICAM-1) cell surface expression, which was measured by flow cytometry. Neutralizing antibodies prevent CP-870,893 from binding the target and result in a partial or full reduction in signal compared to plasma from drug naïve monkeys. Data was reported as the amount of drug neutralized by a fixed amount of sample, as derived from a CP-870,893 standard curve.

The neutralizing anti-CP-870,893 antibody assay in monkey plasma was deemed fit for use based on similar CP-870,893 concentration-response curves and neutralization responses using anti-idiotypic (anti-ID) and primate anti-human antibodies (data not shown). A floating cut point was determined by multiplying the negative control (NC) for each run by a constant normalization factor of 0.88. The normalization factor was calculated using 51 individual drug naïve monkey plasma samples and the following formula: (mean signal)/(mean signal + 2 × standard deviations). Normal monkey plasma spiked with a neutralizing anti-CP-870,893 monoclonal antibody (mAb) was included as a positive control (PC). Based on this anti-ID mAb PC, the sensitivity of the assay was 0.5 μg/mL. Specificity of the positive control was shown by the addition of a different mAb (same isotype with different target), which did not demonstrate neutralization in the assay. Drug interference was not assessed since only samples that tested below the limit of quantitation (BLQ, 0.008 μg/mL) in the pharmacokinetic (PK) assay were assessed for neutralizing anti-drug antibodies. A summary of the validation parameters tested for the monkey assay can be found in Table 1.

2.1.2. CLB assay

The CLB assay for detecting neutralizing anti-CP-870,893 antibodies in monkey plasma has been previously described [17]. Briefly, in the CLB assay, 0.2 μg/mL CP-870,893 and plasma samples diluted 1:10 are added to 96-well plates precoated with a purified recombinant human CD40 target and incubated for 1 h. After washing, drug bound to CD40 was detected using a mouse anti-human-IgG-Biotin, Streptavidin-HRP, tetramethylbenzidine (TMB) substrate, and spectrophotometer. Neutralizing antibodies prevent CP-870,893 from binding the target and result in a decreased optical density (OD) compared to plasma from drug naïve monkeys. During validation, a fixed cut point was established using 43 individual plasma samples from drug naïve animals. The percent of control OD signal was calculated using the following formula: ((individual sample OD/negative control pool OD) × 100). The mean of the individual percent control values minus 2 standard deviations was used to establish a fixed cut point of 91.59%. Samples with a percent control less than the cut point were defined as positive for neutralizing antibodies. Normal monkey plasma spiked with a neutralizing anti-CP-870,893 antibody was included as a PC, which was not the same anti-ID clone used in the bioassay. Assay sensitivity was determined by spiking pooled plasma with anti-ID mAb PC at concentrations ranging from 0.05 to 10 μg/mL. The last concentration tested to fall below the cut point (i.e., positive NAb response) was 0.2 μg/mL. Precision was determined using the anti-ID mAb at low and high concentrations. Ten sets of replicates (3 replicates per set) were tested on a single day for intra-assay precision. The low and high PCs were tested on 5 independent days to determine inter-assay precision. Precision of the low and high PCs was <7% CV and <13% CV for intra and inter-assay precision respectively. As with the bioassay, drug interference was not assessed because only samples with no detectable drug were tested. A summary of CLB assay validation parameters can also be found in Table 1.

2.1.3. Nonclinical study interpretation

The impact of assay format on sample analyses and data interpretation was assessed using monkey samples from a 3-month toxicity study with 0.03, 0.1, and 1.0 mg/kg CP-870,893 followed by a 1 month recovery period. Twenty-five samples positive for binding ADA and BLQ in the PK assay were identified for analysis by both methodologies. Results obtained using both methodologies were in good concordance ($R^2 = 0.94$) with 24 samples tested

Table 1
Case study 1, summary of assay parameters.

Assay parameter	CLB assay	Bioassay
Endpoint	Inhibition of binding to purified receptor	Inhibition of CD54 upregulation
Minimum Required Dilution (MRD)	10% plasma	10% plasma
Specificity	Not determined	No interference from unrelated antibodies was detected
Sensitivity (μg/mL)[a]	0.2	0.5[b]
Drug tolerance	Not determined	Not determined
Intra-assay precision	≤6.9	Not determined (≤5.3)[c]
Inter-assay precision	≤12.3	Not determined (≤8.6)[c]
Signal to noise[d]	48	6.9

[a] Sensitivity was calculated using different a different PC for each assay.
[b] Sensitivity of 0.5 μg/mL was also observed in human serum [17].
[c] Precision of the neutralizing bioassay in human serum [17].
[d] Signal to noise was calculated as: signal of drug in serum at MRD/serum at MRD.

positive in both. One individual testing positive in the bioassay was negative in the CLB assay.

2.2. Case study 2: neutralizing assays for AER 001

AER 001 is a recombinant protein produced by mutating two amino acids in human interleukin-4 (IL-4) to yield a molecule that is an antagonist to the binding of IL-4 to the IL-4 receptor alpha (IL4Rα) subunit and subsequently blocks signaling of both IL-4 and interleukin-13 (IL-13) [18]. Immunogenicity risk assessment indicates that AER 001 is a low risk, homologous to IL-4 and being a small protein. Any NAb that develop against AER 001 however could cross-react with endogenous IL-4. IL-4 is known to stimulate T helper type 2 (Th2) cell proliferation but is not essential for development of the immune system; other cytokines such as IL-13 have a similar biological function [19]. AER 001 is currently in development for the treatment of patients with uncontrolled asthma. Both cell-based and CLB based assay approaches were evaluated to assess neutralizing activity of ADAs in human serum matrix during the drug development program.

2.2.1. Bioassay – TF-1 cell proliferation assay

The stimulation of the proliferation of TF-1 cells with either IL-4 or IL-13 can be used to assess the functional antagonistic activity of AER 001. The biological functions of IL-4 and IL-13 are mediated by the binding of the cytokines to the high-affinity type I or II IL-4 receptor complex on the cell surface. IL-4 interacts with the high-affinity IL-4Rα subunit, allows binding to the common gamma chain (IL-2Rγ) to create the type I IL-4 receptor complex. On the other hand IL-13 binds to IL-13 receptor alpha1 (IL-13Rα1) with high affinity, inducing heterodimerization with IL-4Rα to form a complex identical to the type II IL-4 receptor complex. AER 001 inhibits the affects of both IL-4 and IL-13 through its ability to bind to IL-4Rα, and thus inhibiting the growth of TF-1 cells in response to either IL-4 or IL-13 stimulation [19]. Because any NAb that inhibits AER 001 antagonistic activity might also neutralize IL-4 activity, the proliferative response of TF-1 cells to IL-13 was used for assessing the merits of a cell-based assay in detection of any NAb activity towards AER 001. The amount of proliferation of TF-1 cells was determined by the emitted fluorescence of reduced AlamarBlue (Trek Diagnostics Systems, Cleveland, OH) generated by live cells.

Since both human IL-4 and AER 001 have similar binding affinity to the IL-4Rα subunit, any polyclonal anti-IL-4 NAb will block AER 001 activity, thus an affinity purified goat anti-human IL-4 IgG (R&D Systems) was used as a PC. The PC has comparable neutralizing activity on both IL-4 and AER 001 binding to IL-4Rα subunit by Biacore® analysis (data not shown). A purified rabbit polyclonal IgG (prepared in-house) was used as a NC. NAb activity was tested in the presence of 50 and 150 ng/mL of AER 001. At 50 ng/mL of AER 001, IL-13 stimulated TF-1 cells experienced approximately 60% inhibition of proliferation, while at 150 ng/mL of AER 001, the inhibition was approximately 78%. PC at 160 μg/mL was diluted in assay medium and incubated with the specified amount of AER 001. The mixture was added to TF-1 cells stimulated with 5 ng/mL of IL-13. The proliferation of TF-1 cells was measured 3 days later. Nab activity of PC was observed with sensitivity at 2 and 6 μg/mL for 50 ng/mL and 150 ng/mL of AER 001, respectively. Some nonspecific inhibitory activity was also observed with the rabbit IgG negative control starting at concentrations of 30 μg/mL.

When PC was spiked into 20, 50 and 100% of pooled normal human serum (pNHS) and serially diluted in the same matrix before incubation with AER 001, NAb activity could not be detected for PC in samples containing 50 and 100% pNHS, and the sensitivity was also greatly reduced in samples of PC containing 20% pNHS. The proliferative response of IL-13 stimulated TF-1 cells diminished as the overall amount of human serum increased in the assay sample. Thus, it was determined that samples containing 10% pNHS represented the matrix tolerance limit of the assay. While the IL-13 stimulated TF-1 cell proliferation assay seems ideally formatted for characterization of NAb activity in AER 001 clinical samples containing ADA, the bioassay shows a significant limitation in sensitivity due to assay sample matrix.

2.2.2. CLB assay

The mechanism of action for AER 001 is binding to IL-4Rα which blocks the signaling response on Th2 cells [18]. A CLB assay targeting IL-4Rα binding was developed for the detection of AER 001 neutralizing activity in ADA-containing samples. Recombinant human IL-4 receptor/Fc chimera (R&D Systems) was immobilized on an immunoplate. In this assay, biotin-labeled AER 001 (prepared in-house) binds to the human IL-4 receptor and this binding is monitored utilizing streptavidin-labeled peroxidase and TMB as substrate for color development. The optical density corresponds to the amount of biotin-labeled AER 001 bound to the IL-4 receptor/Fc chimera. ADA-containing serum samples with neutralizing activity inhibit the binding of biotin-labeled AER 001 to the IL-4 receptor resulting in a decreased OD level.

During method development, biotin-labeled AER 001 was shown to have the same binding activity to IL-4 receptor/Fc as unlabeled AER 001 by Biacore® analysis (data not shown). An affinity purified goat anti-human IL-4 polyclonal antibody (R&D System) was used as a PC and neat pNHS was used as a NC. Briefly, NC and PCs in pNHS were preincubated with 10 ng/mL of biotin-labeled AER 001 (final serum content at 50%) for an hour at room temperature, then transferred onto an IL-4 receptor-coated plate (coated at 1 μg/mL). This CLB assay was validated in human serum matrix focusing on validation parameters that included evaluation of cut point, precision, sensitivity, matrix effect, specificity and drug tolerance.

The assay cut point was defined as the assay response (OD) below which a sample is identified as reactive for NAb in human serum. A floating cut point was selected as optimal. The floating

Table 2
Case study 2, summary of assay parameters.

Assay parameter	CLB assay	Bioassay
Endpoint	Inhibition of binding to purified receptor	Inhibition of TF-1 cell proliferation
MRD	100% serum (no dilution required)	10% serum
Sensitivity (μg/mL)	0.6–0.7	2–6
Specificity	No interference from unrelated antibodies	Endogenous IgG interference
Drug tolerance	2 ng/mL of free drug with PC at 1000 ng/mL	Not determined
Intra-assay precision	PC1: ≤24.07% PC2: ≤15.45%	Not determined
Inter-assay precision	PC1: 2.8% PC2: 14.5%	Not determined
Signal to noise[a]	73.9	1.7

[a] Signal to noise for CLB was calculated as: signal of drug in serum at MRD/serum at MRD. For bioassay, signal to noise was calculated as: cellular proliferation in response to IL-13 stimulation in serum at MRD/cell responses in serum at MRD.

cut point is determined by multiplying the NC geometric mean for each run by a constant normalization factor that was determined to be 0.518 by statistical analysis of the cut point data generated from 30 lots of normal individual human sera and the NC pool sample.

During evaluation of assay precision, six duplicate sets of PC at 1000 ng/mL (PC1) and five duplicate sets of PC at 600 ng/mL (PC2) in pNHS were each tested in six individual runs. The intra-assay %CV for PC1 was ≤24.1%; PC2 had intra-assay %CV ≤15.4%. The inter-assay precision is calculated based on the percent of mean PC response to mean NC response from each run, %CV for PC1 was at 2.8 and 14.5% for PC2. While PC1 was consistently classified as "positive" relative to the assay cut point on all six runs, PC2 was classified as "negative" in two of the six runs. This outcome is consistent with the identified method sensitivity of ~700 ng/mL of PC.

Ten lots of pNHS either unspiked or spiked with PC1 were assayed and no matrix effect on NAb detection was observed. Ten lots of unspiked pNHS were NAb negative, and all PC1 spiked pNHS were identified as NAb positive relative to assay cut point. Addition of 100 μg/mL of human IgG1 (Sigma) to either PC1 or PC2 in a single lot of pNHS does not change the classification status for either PC1 or PC2. These results suggest that the assay is not susceptible to interference by endogenous nonspecific IgG1.

Drug tolerance of the CLB assay was investigated by testing either NC or PC (added at final concentrations of 300, 700 and 1000 ng/mL) in the presence of 2, 5, 10 and 50 ng/mL of AER 001. NC can tolerate up to 5 ng/mL of drug and still have OD response above the assay cut point. The presence of drug above 2 ng/mL interfered with detection of the PC at concentrations less than 1000 ng/mL. The presence of drug causes interference of NAb detection that is non-monotonic with free drug concentration, i.e., OD response of PC increases and then decreases as amount of drug increased. In order to make an accurate assessment of NAb activity with this CLB assay it is highly desirable to have a clinical protocol that can provide patient serum samples collected after a complete drug washout. The drug level in patient samples can be measured by PK assay with LLOQ of 0.05 ng/mL of AER 001.

A comparison of both methods is summarized in Table 2. Due to difficulties associated with reproducibility and robustness as well as matrix effects leading to low assay sensitivity, the TF-1 cell-based assay in its current format was not validated. Efforts at optimizing the current TF-1 cell-based assay were not initiated at this stage of the clinical development program for AER 001. The cost, time and potential for success associated with screening, selection and optimization of a cell-based bioassay for Nab assessment is not warranted when a CLB method that has been shown to be appropriate for its intended use and is scientifically sound is available. The development and use of a cell-based bioassay to complement the CLB method will be reconsidered pending the results of Nab testing in an upcoming Phase II study. The validated CLB method will be used for current Phase IIB clinical studies as part of the immunogenicity assessment program.

2.3. Case study 3: anti-ranibizumab NAb assays

Ranibizumab (LUCENTIS®, Genentech, Inc., South San Francisco, CA), is a recombinant humanized monoclonal antibody fragment (Fab) that binds to VEGF-A and inhibits its biological activity. It was approved by the US Food and Drug Administration (FDA) in 2006 for ophthalmic intravitreal injection to treat neovascular age-related macular degeneration (AMD) and for retinal vein occlusion (RVO), in 2010.

We applied a multi-tier strategy for immunogenicity testing of ranibizumab-treated patients, based on published recommendations [20]. In this context, testing for neutralizing antibodies was performed on clinical samples already confirmed positive for anti-ranibizumab antibodies. We initially attempted to develop a sensitive and accurate cell-based assay for detecting anti-ranibizumab NAbs in clinical samples. However, the cell-based assay did not provide the desired sensitivity. Thus, a competitive ligand-binding immunoassay based on electrochemiluminescence (ECL)-technology was subsequently developed and successfully accomplished that goal.

2.3.1. Bioassay – HUVEC proliferation-inhibition assay

In choosing the format for a cell-based NAb assay for ranibizumab, an important point to consider was the range of pharmacological effects induced by VEGF. This pleiotropic cytokine promotes local angiogenesis, increases vascular permeability, stimulates tissue factor expression, induces endothelial cell migration, induces vasodilation, stimulates release of other cytokines, and acts as a cell-survival factor. We evaluated one of these properties in the bioassay: VEGF-induced proliferation of human umbilical vein endothelial cells (HUVEC).

In the HUVEC proliferation-inhibition bioassay, ranibizumab, VEGF, and serum samples (or controls), were mixed with HUVEC cells, and this mixture was incubated at 37 °C in a humidified CO_2 chamber. The cell monolayer was then incubated with alamar Blue dye (Trek Diagnostics Systems, Cleveland, OH), and fluorescence was measured after a 6 h-period. A serum sample containing any NAb activity that can neutralize ranibizumab would restore VEGF stimulated HUVEC proliferation.

Other growth factors that influence and promote endothelial cell proliferation and function, such as angiopoietin, fibroblast growth factor, tumor necrosis factor-α, apelin, and insulin-like growth factors [21,22], may have contributed to the undesirable baseline cell proliferation observed. In addition, variable levels of these factors in sera from individual patients could have affected HUVEC proliferation and impacted assay reproducibility when testing clinical samples.

In the cell-based assay, the in-well concentration of added VEGF was 10 ng/mL, whereas the VEGF EC_{50} for this assay, in the absence of ranibizumab and anti-ranibizumab, was approximately 20–30 ng/mL. These VEGF concentrations are well-above

Table 3
Case study 3, summary of assay parameters.

Assay parameter	CLB assay	Bioassay
Endpoint	Inhibition of VEGF-A binding to drug	Inhibition of proliferation
MRD	No dilution required	1:5 dilution in assay medium
Sensitivity (ng/mL)	~200	~1000
Specificity	No interference from endogenous VEGF-A	Endogenous VEGF-A interference
Drug tolerance (ng/mL)	50	Not determined
Intra-assay precision	Not determined	Not determined
Inter-assay precision	<25 CV%	Not Determined
Signal to noise[a]	~8:1	~3:1

[a] For the CLB assay, signal to noise was estimated as: maximal signal (uninhibited by NAb) at MRD/minimal signal (inhibited by NAb) at MRD. For the bioassay, signal to noise was estimated as: maximal signal with VEGF in serum (no drug) at MRD/minimal signal with VEGF and drug in serum at MRD.

physiological levels of serum VEGF reported in the literature. This observation led us to conclude that VEGF-removal from samples would not provide sufficient improvement in assay performance profile to justify adding this step to the procedure. Furthermore, this offline sample manipulation could potentially introduce microbial contaminants which would compromise the growth and viability of HUVEC cells. We found that as little as 25 ng/mL of ranibizumab was sufficient to considerably inhibit VEGF activity in the system, including endogenous activity. It is therefore unlikely that endogenous VEGF would have caused significant interference in the assay if it had been used to test clinical samples from clinical trials. The best sensitivity accomplished with the cell-based assay was approximately 1000 ng/mL (see Table 3), estimated using purified cynomolgus anti-ranibizumab-CDR enriched antibody spiked into pooled normal human serum.

2.3.2. CLB assay

The ligand-binding immunoassay was based on the premise that binding of ruthenium-labeled ranibizumab to biotinylated-VEGF would be blocked by the presence of NAbs.

The assay requires a pretreatment step to remove all endogenous VEGF present in serum samples. This step prevents competition between endogenous VEGF and biotinylated-VEGF for binding to ruthenium-labeled ranibizumab. It may also potentially remove all ranibizumab bound to VEGF in the samples, while not reducing the NAb concentration, since these would not bind ranibizumab bound to VEGF. VEGF-depleted samples are then transferred into tubes containing ruthenium-labeled ranibizumab. Following an overnight incubation, an aliquot of each sample was transferred into a plate containing biotinylated-VEGF. After a 2 h incubation time, streptavidin coated beads were added, followed by incubation for 2 h at room temperature. Samples were then analyzed for ECL signal. In the absence of NAbs, ruthenium-labeled ranibizumab and biotinylated-VEGF bind to each other, and the immune complex is captured; triggered by the application of an electrical current, the ruthenium label bound to ranibizumab emits an ECL signal, which was measured using a Bioveris Analyzer (BioVeris, Inc., Gaithersburg, MD; at the time of writing of this manuscript, BioVeris instrumentation is no longer commercially available). The signal intensity is directly proportional to the amount of complexes captured. Anti-ranibizumab NAbs compete for the binding of biotinylated-VEGF to ranibizumab; the competitive inhibition results in a reduction of ECL signal, relative to samples that do not have NAbs.

As in the cell-based assay, purified cynomolgus anti-ranibizumab-CDR enriched antibody was spiked into pooled normal human serum pool was used to assess assay relative sensitivity. The assay was able to detect as little as 200 ng/mL of anti-ranibizumab antibody. Serum from the same animal was diluted into pooled normal human serum to generate the assay's low positive and high positive controls.

Drug interference for the CLB assay was evaluated during the assay development and qualification process. Due to the fact that ranibizumab is administered intraocularly, a high level of drug tolerance was not needed. Ranibizumab was spiked into the assay's low positive and high positive controls at a final concentration of 50 ng/mL, which is approximately 10-fold higher than the highest observed concentration in patient sera to date. The assay was still capable of detecting neutralizing activity in both control samples. The VEGF-removal step in the assay is likely to remove all ranibizumab bound to VEGF in the samples, as well. This may contribute to the drug tolerance observed for this assay.

Serum VEGF levels are not expected to increase dramatically following ranibizumab treatment. Nevertheless, the CLB assay was designed to overcome interference caused by potential accumulation of high levels of serum VEGF. Accordingly, we demonstrated that the assay could tolerate up to 100 ng/mL of circulating VEGF. This amount of added VEGF did not cause false-positive results, and did not cause the assay's low positive control sample to test negative.

A comparison of the two methods is shown in Table 3. The cell-based assay procedure was relatively simple, and only required three-steps to be completed. HUVEC cells viability did not appear to be negatively impacted by the presence of pooled human serum at up to 20% concentration. The CLB assay, on the other hand, required multiple steps, including pre-removal of endogenous VEGF. However, it could be run with neat serum, and it is more amenable to automation. The cell-based assay is a 4-day procedure, and as a bioassay requires sterile assay conditions; this is not the case with the CLB assay, which requires 2 days and non-sterile conditions.

The maximal signal to noise ratio we attained in the bioassay was approximately 3:1 compared to approximately 8:1 in the CLB assay. Attempts to raise this signal to noise ratio by enhancing cell proliferation using higher concentrations of VEGF were unsuccessful – the amount of cell proliferation in this system was higher, but a proportionally higher concentration of ranibizumab was needed to effectively neutralize VEGF, in turn necessitating a higher amount of NAb, decreasing assay sensitivity. The assay baseline-signal was also difficult to reduce, most likely due to the fact that HUVEC cells were able to undergo low-level proliferation in the absence of added VEGF. In summary, to support Genentech's immunogenicity testing strategy for characterization of potentially NAb responses to ranibizumab, a cell-based assay was initially evaluated. However, this format was found to be considerably less sensitive than the screening antibody assay, and thus deemed inappropriate. An ECL-based CLB assay with better sensitivity was then developed and qualified.

2.4. Case study 4: neutralizing assays for therapeutic monoclonal antibody to TNFα

A study was performed to compare two assay methodologies that detect NAbs to a therapeutic mAb that inhibits TNFα. A cell-

Table 4
Case study 4, summary of assay parameters.

Assay parameter	CLB assay	Bioassay
Assay endpoint	Inhibition of TNFα binding to drug	Increased cell viability
MRD	5% human serum	5% human serum and plasma
Sensitivity (ng/mL)	192	150
Specificity	No interference from unrelated antibodies detected	No interference from unrelated antibodies detected
Drug tolerance (μg/mL)	0.49	1.43
Intra-assay precision	<20%	<5%
Inter-assay precision	<20%	<15%
Signal to noise[a]	~80	3.8

[a] For the CLB assay signal to noise was calculated as: maximal uninhibited signal/minimal inhibited signal. For the bioassay signal to noise was calculated as: signal of drug + TNF in serum at MRD/TNF in serum at MRD.

based assay and a non-cell-based CLB assay were developed to detect NAbs to the anti-TNFα therapeutic mAb in serum.

2.4.1. Bioassay

In the cell-based bioassay, a cell line was chosen based on its sensitivity to TNFα and the assay endpoint is a measure of cell viability (using Cell Titer Glo®, Promega Corporation). TNFα causes lower cell viability, and the presence of the therapeutic antibody in the assay prevents TNFα from triggering cell death. Consequently, the addition of a sample containing NAbs reverses this effect by inhibiting the activity of the therapeutic mAb, leading to lowered cell viability. Serum samples were incubated with TNFα and the anti-TNFα therapeutic antibody. The mixtures were then incubated overnight at 37 °C with TNFα-sensitive WEHI cells. In the presence of NAbs, the cells underwent TNFα-induced cell death while, in the absence of NAbs the cells remained viable. This assay takes 1.5 days (not including maintenance of cells before the performance of bioanalysis).

2.4.2. CLB assay

The CLB assay utilizes an ECL detection technology (BioVeris corporation), in which the ruthenium-labeled therapeutic is first incubated with a sample and then added to biotinylated-TNFα coupled to a streptavidin-solid support. Samples containing NAbs inhibit binding of the ruthenium-labeled therapeutic to the biotinylated TNFα in the CLB assay, thus reducing assay signal. It should be noted that the BioVeris ECL technology is no longer available. Both the bioassay and the CLB assay classify samples with ADAs as NAb positive when the percent change in signal from baseline is above an established assay cut point.

Critical assay performance characteristics were compared (Table 4) including sensitivity in serum, drug tolerance, intra-assay precision, and inter-assay precision. The sensitivities of the assays were similar using the same control NAb in serum (150 ng/mL and 192 ng/mL for the cell based and CLB assay, respectively). Under comparable conditions, the drug tolerances for both assays were within an order of magnitude of each other, with the cell-based bioassay slightly more drug tolerant than the CLB assay (1.43 μg/mL vs. 490 ng/mL, respectively). It is important to note that the corresponding pharmacokinetic assay for the anti-TNFα antibody had a detection limit of 39.05 ng/mL. Therefore, it is expected that samples with drug levels that could interfere with either assay could be identified and reported. Both assays had acceptable intra- and inter-assay precision with coefficients of variation below 20%.

Dose response curves from a panel of 17 mouse monoclonal ADAs (Table 5) were utilized to compare the detectability of NAbs in diluent using the two assay formats. The detection limits of 15 anti-drug antibodies, interpolated using the cut points from both assays, were within a log of each other. The cell-based bioassay was >3-fold less sensitive to four ADAs, while the CLB assay was >3-fold less sensitive to three ADAs. There was not a clear relationship between binding affinity (K_d, measured using biacore) and the neutralization capacity of each antibody. However, the ADA with the lowest K_d corresponded to the lowest detection limits in both NAb

Table 5
The performance of both assays was compared using a panel of monoclonal ADAs in diluent at concentrations between 40 μg/mL and 20 ng/mL. Detection limits were interpolated at the individual assay cut points.

Monoclonal ADAs	Biacore K_d (nM)	CLB assay Detection limit (ng/mL)	Bioassay Detection limit (ng/mL)
1	1.6	<20	<20
2	3.0	240[b]	1,900
3	3.0	690	1,700
4	3.5	325	300
5	3.6	870	1,050
6	3.8	16,940	21,000
7	4.4	405	800
8	4.7	550	450
9	4.9	405[b]	4,000
10	5.5	500[b]	3,500
11	6.6	3,155[b]	No inhibition[c]
12	8.5	2,685[b]	12,000
13	10.3	1,695	250[a]
14	10.8	870	550
15	11.8	34,590	4,500[a]
16	12.7	17,740	4,500[a]
17	14.3	No inhibition	No inhibition

[a] Detection limits of the cell-based bioassay are > 3-fold of the detection limits of the CLB assay.
[b] Detection limits of the CLB assay are >3-fold of the cell-based bioassay.
[c] Inhibition was not observed in the cell-based bioassay but was observed in the CLB assay.

assays and the ADA with the highest binding affinity corresponded to the highest detection limits in both NAb assays.

This case study demonstrates an example where cell-based and CLB assays perform equivalently in regards to critical assay performance characteristics. An advantage of the cell-based bioassay is that it does not require conjugated reagents to measure a response. It is conceivable that the biotin-TNFα and the ruthenium-labeled therapeutic mAb could partially alter epitopes recognized by the three ADA with higher detection limits in the CLB assay. However, with four additional monoclonal ADAs, the bioassay did not appear to be as sensitive as the CLB assay. Under comparable conditions, one weak NAb was detected by the CLB assay while no inhibition by this monoclonal was observed in the cell-based bioassay. Reduced matrix interference is another advantage of the CLB assay format. While both assays have the same MRD, a baseline sample unexpectedly resulted in reduced cell viability due to an unknown matrix factor, rendering the sample unevaluable in the cell-based bioassay. On the other hand, the performance of the CLB assay was not impacted by this matrix (data not shown). The CLB assay offers operational advantages over the cell-based assay format with a relatively rapid assay time for the CLB assay (4 h vs. 1.5 days for the cell-based assay). Additionally, significant levels of resources are needed to maintain the cells, notwithstanding those allocated to bioanalysis and the added complexity of the cell-based bioassay can lend to more opportunity for operator error.

Overall the non-cell-based bioassay and the cell-based assay provided similar results across 8 out of 17 ADAs. Although drug development programs routinely include the assessment of NAbs using a cell-based bioassay, the assay format is considerably more complex than CLB assay methods. In this case, a competitive ligand-binding assay has shown similar, but not identical, results to the cell-based approach.

3. Discussion

The EMA immunogenicity guidance document [9] states that assessment/characterization of neutralizing antibodies usually requires the use of bioassays. The case studies illustrated here using an agonistic monoclonal antibody, an antagonistic recombinant protein to IL-4 and IL-13, a humanized Fab fragment, and an antagonistic monoclonal antibody, indicated that CLB assays may, in a variety of contexts, be a suitable platform for NAb testing. In case study 1, for instance, where nonclinical samples were compared between the two assays, the results were very similar with one discrepant result in which a sample was positive in bioassay but negative in CLB assay. Similarly, in case study 4 where a panel of 17 monoclonal antibodies was assessed the results were very similar between the two assays although one monoclonal was not detected in the bioassay but was positive in the CLB assay. Both of these case studies had very similar sensitivities between the two assay platforms.

In other cases, CLB assays may be more sensitive and less prone to matrix interference than their cell-based counterparts. In case studies 2 and 3, for instance, the CLB assay was approximately five-times more sensitive than the bioassay. In case study 2, limitations of the bioassay robustness, reproducibility and sensitivity clearly demonstrated that the CLB assay platform was superior for the detection of NAbs against AER 001. Similarly, the HUVEC cell-based bioassay described in case study 3 was insufficiently sensitive to detect anti-ranibizumab NAbs. Therefore, the CLB assay method was selected as the most appropriate option for NAb assessment. In both instances, experimental evidence clearly supported the selection of the CLB assay method over the bioassay approach.

In all four case studies, the CLB assay format had comparable or better sensitivity than the bioassay, comparable or better precision, and less matrix interference. Drug tolerance was only compared in one case study and in this case study the bioassay demonstrated slightly better drug tolerance. In both assay formats, drug in patient samples can result in false negatives in the assays. Assays that use less drug as part of the assay will be more sensitive to having false negatives due to the presence of drug in samples. Data presented here also suggests that CLB assays often provide similar information to bioassays and in some cases the necessary characterization of immunogenicity can only be accomplished by CLB assay formats.

Characterizing the neutralizing capacity of ADA responses can be instrumental in understanding the effect of the ADA on drug safety and efficacy. Therefore, it is critical that the most appropriate and reliable methods be utilized to measure neutralization. The perceived preference for bioassays often makes them a logical first approach in the development of a NAb assay. However, as illustrated here, bioassays may not be sufficiently sensitive, and in some cases may not effectively measure drug neutralization, and may lack adequate robustness. In those cases, it would be reasonable to explore other appropriate drug-function related methods, including CLB assay formats, to ensure that the neutralizing capacity of ADA response is adequately assessed. In summary, a risk-based strategy, a scientific rationale informed by drug mode of action and sufficient datasets from each platform are critical components for selecting a NAb assay format.

Additional case studies, particularly those with clinical (disease state) data, would further our understanding of the utility and suitability of one format over the other. Based upon the data presented herein, however, it is clear that CLB assays should be considered viable options for NAb assessments of biotherapeutics.

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