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Clinical immunogenicity specificity assessments: A platform evaluation

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ABSTRACT

Immunogenicity assessment is an integral part of the evaluation of the safety and efficacy for protein therapeutics during drug development, and is required by the regulatory authorities. A tiered strategy is typically utilized to assess immunogenicity and is often comprised of a screening method, a confirmation/specificity step and a characterization step. To ensure methods with appropriate sensitivity are utilized, the threshold for screening assays is set to minimize false negatives resulting in a certain rate of false positivity. The confirmatory step is critical for determining assay specificity and eliminating false positives identified in the screening assay. Using a widely implemented technology and bridging assay format commonly used for immunogenicity assessments, unacceptably poor specificity was observed for the confirmatory/specificity step for a subset of monoclonal antibodies in our group. Therefore, we believe that this challenge will be relevant to others in the field. In this paper, we will describe our challenges with one of these antibodies, monoclonal antibody therapeutic X (rhuMAb X). This paper presents extensive evaluation of two technology platforms and various conditions to evaluate and provide solutions to improving the assay specificity in the immunogenicity assessment of antibody therapeutics.

1. Introduction

Protein therapeutics, such as monoclonal antibodies, show promising results in treating complex diseases. However, repeated administration of protein therapeutics can induce an immune response which leads to the production of anti-therapeutic antibodies (ATAs). Although in most cases these ATA responses are benign, they can lead to potentially serious side effects [1–4] and altered pharmacokinetics (PK) [5] and pharmacodynamics (PD) profiles [6]. Therefore, immunogenicity evaluation is an important part of protein therapeutic development and is routinely required in both nonclinical and clinical studies [7].

Immunogenicity assessment is typically done using a "tiered" strategy, generally including an ATA screening assay, a specificity confirmation step, and in some cases a characterization step. Samples are first evaluated in a screening assay, for which an assay threshold or cutpoint has been set based on the variability of samples from a drug naïve target patient population. Screen positive samples are further evaluated in a confirmatory assay to verify whether the signal observed in the screening assay is a result of a specific response to the protein therapeutic treatment. Confirmed positive samples are then put into downstream methods for sequential characterization based on the comprehensive consideration of immunogenicity risk assessment and mechanism of action for the protein therapeutic [8].

Screening assays are usually designed to have high sensitivity, moderate selectivity and are run in a high throughput mode. To minimize false negatives in screening assays, the assay cutpoint is set such that some false positives would be detected. ATA screening assays follow the overall guidance for ATA evaluations described elsewhere [7,9,10]. Currently the guidelines recommend a 5% untreated positive rate (UTPR) for screening assays.

Samples that screen positive are typically further characterized for their specificity in a confirmatory assay. This is critical to verify that the screen positive signals are indeed a consequence of saturable and specific responses to the protein therapeutic treatment. While the confirmatory assay is also developed to be a very sensitive immunoassay, it is primarily designed for optimal selectivity and ability to differentiate between a "true" versus a "false" positive result. One strategy for designing a confirmatory assay involves treating samples with excess level of soluble protein therapeutic and comparing the results with samples without treatment. This process is often referred as competitive inhibition or immunodepletion. The magnitude of signal inhibition needed to deem a sample positive for therapeutic-specific ATA must be experimentally determined. A signal generated from a nonspecific binding is likely to be less inhibited by soluble protein therapeutic than a specific signal from antibodies directed against the protein therapeutic [11]. An effective confirmatory assay should have a statistically sig-

Abbreviations: ATA, anti-therapeutic antibody; CDR, complementarity determining region; DIG, digoxigenin; ECLA, electrochemiluminescence assay; ECLU, electrochemiluminescent unit; ELISA, enzyme-linked immunosorbent assay; MSD, Meso Scale Discovery; IgG, immunoglobulin gamma; rhuMAb, recombinant human monoclonal antibody; Ru, ruthenium; S/N, signal to noise ratio; UTPR, untreated positive rate.

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nificant drop of the positive control (PC) signal but a minimum drop of the negative control (NC) signal in the presence of soluble protein therapeutic, so that the decision threshold, or confirmatory cutpoint, for the therapeutic-specific ATA positives can be clearly defined and applied for data interpretation.

There are many platforms available for ATA measurement, including enzyme-linked immunosorbent assay (ELISA), radioimmunoprecipitation assay, electrochemiluminescence assay (ECLA) and label free technologies such as surface plasmon resonance [12,13]. Each technology has its own limitations and capabilities which have been discussed previously [9,14]. Here we will share our experiences with two of these platforms: the ELISA and ECLA platforms

Stepwise ELISA was one of the earliest platforms used for immunogenicity detection [15]. A typical stepwise ELISA ATA assay is a heterogeneous assay with sequential reagent addition and wash steps. The assay readout can be color absorbance, fluorescent or chemiluminescent. Stepwise ELISA ATA assays generally possess good assay sensitivity but poor drug tolerance based on our experience. Hence stepwise ELISA is a less desirable ATA assay platform for protein therapeutics with long half lives since various levels of protein therapeutic may be present in samples, and analyzing samples using an assay with poor drug tolerance can mislead ATA data interpretation.

Electrochemiluminescence technologies are based on a process by which light is generated when a low voltage is applied to an electrode, triggering a cyclical oxidation–reduction reaction of ruthenium metal ion. Initially, there were two different platforms using this technology—a bead-based format called the BioVeris technology (BioVeris Corp., Gaithersburg, MD) and the plate-based version of the ECLA technology called MSD for Meso Scale Discovery (Gaithersburg, MD). The BioVeris technology was introduced to the biopharmaceutical industry first. It was adapted by many biopharmaceutical companies as the technology of choice for ATA detection due to the superior sensitivity and drug tolerance of this technology. The good sensitivity and drug tolerance of the BioVeris format were attributed to its homogeneous format and small ruthenium/biotin tag combination as well as limited wash steps compared to the stepwise ELISA. However, the BioVeris technology has been discontinued and is no longer available; therefore an alternative ATA platform to BioVeris was needed for ATA analysis.

The MSD platform has been chosen by many groups as a replacement for the BioVeris platform [16]. This is due to the similarity between the two technologies therefore ease of conversion from the BioVeris to the MSD platform. Although we have successfully used MSD platform for various projects, we encountered an unexpected problem during development of a clinical confirmatory ATA assay for a monoclonal antibody therapeutic X (rhuMAb X). As mentioned above, the confirmatory assay is developed to show selectivity of the positive results from the screening assay. In this assay addition of excess soluble protein therapeutic to the samples containing specific ATAs before evaluation in the assay, should result in a signal inhibition compared with samples without specific antibodies to the therapeutic. The signal inhibition is due to specific antibodies binding to added soluble drug and therefore unavailable for detection in the assay. During rhuMAb X clinical confirmatory assay development, we observed a severe signal inhibition of the NC in the presence of soluble rhuMAb X, resulting in a limited signal separation between the NC and low PC. This was very surprising as in the absence of a PC addition of soluble rhuMAb X should not have a significant effect on the assay signal.

Although rhuMAb X was the first molecule we had observed with this NC competitive inhibition problem, we have since observed this problem with several other monoclonal antibody therapeutics. Therefore our experience is not an isolated case and others developing antibody therapeutics may be facing similar problems. This paper will present our challenges and strategies in developing rhuMAb X clinical confirmatory ATA assay. We have discussed our attempts to understand the substantial signal inhibition of NC in our confirmatory assay, its significance and our efforts to overcome this problem using various assay conditions on two different assay platforms.

2. Materials and methods

2.1. Reagents

rhuMAb X is a fully humanized monoclonal antibody therapeutic generated at Genentech. (South San Francisco, CA). Individual serum samples from the target patient population and pooled normal human sera were purchased from Bioreclamation (Hicksville, NY) and BioChemed (Winchester, VA). Immunosorp high-binding plates for the ELISA method were acquired from Nunc (Rochester, NY); Costar polypropylene round-bottom plates were purchased from Corning Life Sciences (Lowell, MA); pre-blocked high binding neutravidin (NA) coated plates were purchased from Pierce (Rockford, IL). 1C8, a complimentary determining region (CDR) specific monoclonal antibody raised against rhuMAb X, was generated internally at Genentech. illustra™ Nap-10 columns were purchased from GE Healthcare (Buckinghamshire, UK). Bovine serum albumin (BSA) was purchased from Equitech-Bio Inc. (Kerrville, TX); CHAPS was from Research Organics (Cleveland, OH); fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT); fish gelatin and *N*-Lauroylsarcosine sodium salt (sarcosine) were from Sigma–Aldrich (St. Louis, MO); ProClin 300 was from Supelco (Bellfonte, PA).

2.2. Biotin, digoxigenin, and ruthenium conjugation of rhuMAb X

rhuMAb X was buffer exchanged into phosphate buffered saline (PBS) prior to conjugation using an illustra™ Nap-10 column. Experimental details followed the manufacturers' instructions (GE Healthcare).

Buffer exchanged rhuMAb X was conjugated with ruthenium (Ru) at challenge ratios of 10:1 and 5:1 (Ru:rhuMAb X) using sulfo-TAG (MSD, Gaithersburg, MD), with biotin at a challenge ratio of 10:1 and 5:1 (biotin:rhuMAb X) using EZ-Link SulfoNHS-LC-Biotin (Pierce, IL), and with DIG at a challenge ratio of 10:1, 5:1 and 2.5:1 (DIG:rhuMAb X) using 3-amino-3-deoxydigoxigenin hemisuccinamide, succinimidyl ester (Invitrogen, Carlsbad, CA). All conjugations were prepared according to the manufacturer's instructions followed by a buffer exchange step into formulation buffer (20 mM histidine acetate, 240 mM trehalose, 0.02% Tween-20, pH 5.5). The concentration of each conjugate was determined using a BCA protein assay kit (Pierce, IL).

2.3. MSD based bridging clinical ATA assay

2.3.1. Screening assay

Equal concentrations of biotin (5:1) and ruthenium (10:1) conjugated rhuMAb X were pre-mixed at 1 μg/mL and added to polypropylene round-bottom plates (50 μL/well). A minimum dilution of 1/25 was used for all samples and controls prepared in assay buffer F (Table 1). Samples or controls (50 μL/well) were then added to the plate(s) and allowed to incubate for 16–22 h at room temperature (RT) with gentle agitation in the dark. The next day, streptavidin (SA)-coated MSD plate(s) were blocked with 100 μl per well of blocking buffer (PBS/0.5% BSA/0.05% Tween-20/0.05% ProClin 300, pH 7.4) for 1 h at RT, then washed three times with washing buffer (PBS/0.05% Tween-20, pH 7.4). Samples/conjugate mix (50 μL/well) were transferred to the blocked SA-coated MSD

Table 1
Buffers tested for the optimization of homogenous bridging ELISA and ECLA MSD assays.

Buffer	Main components
A	1× PBS/0.5% BSA/0.05% Tween-20/0.05% ProClin 300, pH 7.4
B	Buffer A plus 0.25% CHAPS, 5 mM EDTA and 0.35 M NaCl, pH 8.9
C	Buffer A plus 10%FBS and 0.15 M NaCl, pH 7.4
D	Buffer A plus 10%FBS and 0.30 M NaCl, pH 7.4
E	Buffer A plus 20%FBS and 0.15 M NaCl, pH 7.4
F	Buffer A plus 0.5% sarcosine, pH 7.4
G	Buffer A plus 1% sarcosine, pH 7.4
H	Buffer A with 3% BSA instead of 0.5% BSA, pH 7.4
I	Buffer A plus 1% Fish gelatin, pH 7.4
J	Buffer A plus 10% FBS, pH 7.4
K	Buffer B plus 1% Fish gelatin, pH 8.9

plate(s) and incubated for 1 h at RT with gentle agitation in the dark. Plates were washed three times prior to addition of 150 μL per well of 1× Read Buffer T (MSD) and were analyzed using the MSD Imager (Sector Imager 6000 reader).

2.3.2. Confirmatory assay

A panel of untreated individual serum samples and controls (NC, high and low PCs) were pre-incubated with either 100 μg/mL of soluble rhuMAb X or sample diluent prior to minimum dilution into assay buffer F (Table 1) for analysis. Diluted samples and controls were incubated overnight with the mixture of 1 μg/mL of biotin and ruthenium conjugated rhuMAb X; other assay procedures are the same as the screening assay described above.

2.4. Homogenous bridging ELISA clinical ATA assay

2.4.1. Screening assay

Equal concentrations of biotin- (10:1) and DIG-conjugated (10:1) rhuMAb X were pre-mixed (master mix) at 1.25 μg/mL and added to polypropylene round-bottom plates (75 μL/well). A minimum dilution of 1/10 was used for all sample and control preparations in assay buffer B (Table 1) at pH 8.0. Samples or controls (75 μL/well) were then added to the plate(s) and allowed to incubate for 16–22 h at room temperature (RT) with gentle agitation. Next day, samples/conjugate mix (100 μL/well) were transferred to a pre-blocked high binding neutravidin (NA) coated plate and incubate at RT for 2 h with gentle agitation. Plates were washed three times with washing buffer prior to addition of 100 μL per well of 1/6000 diluted horseradish peroxidase (HRP) conjugated mouse-anti-DIG antibody (0.8 mg/mL stock, Jackson ImmunoResearch, West Grove, PA) and incubated at RT for an hour with gentle agitation. Plates were washed three times with wash buffer and 100 μL of tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well, and incubated at RT for 15–20 min. The reaction was stopped by addition of 100 μL per well of 1 M phosphoric acid and absorbance was measured at 450 nm on a plate reader (Molecular Devices, Sunnyvale, CA) (Fig. 1).

2.4.2. Confirmatory assay

A panel of untreated individual serum samples and controls (NC, high and low PCs) were pre-incubated with either 100 μg/mL of soluble rhuMAb X or sample diluent prior to minimum dilution into assay buffer B (Table 1) at pH 8.0 for analysis. Diluted samples and controls were incubated overnight with the mixture of 1.25 μg/mL of biotin and DIG conjugated rhuMAb X; other assay procedures are the same as the screening assay described above.

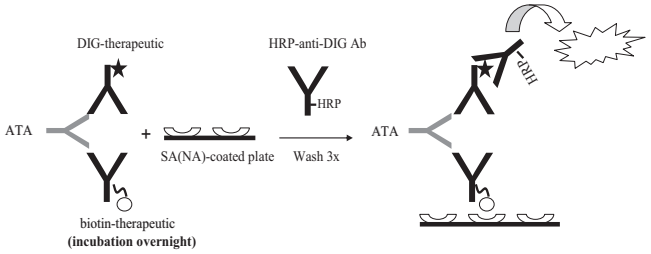

Fig. 1. Homogeneous bridging ELISA:ATA bridges biotin- and DIG-conjugated antibody therapeutics. This immune complex is then captured on a SA or NA coated plate. An HRP conjugated anti-DIG antibody is used as the detection reagent. Colorimetric signals are generated upon addition of an HRP substrate.

2.5. Assay parameters evaluated during ATA assay development

The assay cutpoint is the decision threshold above which samples are deemed positives. A panel of untreated individual samples was used to establish the assay cutpoint multiplication factor, and it is calculated using the following equation:

$$\text{Cutpoint multiplication factor} = \frac{\text{Average signals of drug naïve population} + 1.645 * \text{S.D. of population}}{\text{Mean of NC on plate}}$$

The 1.645 multiplier was chosen to generate a targeted 5% UTPR as recommended in the Mire-Sluis paper (2004). This is a conservative approach designed to minimize the false negative results in the screening assay.

The cutpoint of each run was calculated by multiplying the mean of the NC of the plate with the cutpoint multiplication factor. Sample with signals equal to or above the cutpoint were deemed positive; otherwise they were considered negative.

The relative sensitivity of the assay is defined as the concentration of PC where the signal is equal to the assay cutpoint.

The assay drug tolerance was tested by pre-incubating rhuMAb X (at various levels) with a constant concentration of PC in matrix, and then analyzed by performing the minimum sample dilution and evaluation in the assay. The highest drug concentration where the signal generated by a PC is above the assay cutpoint is defined as the drug tolerance of the assay.

The confirmatory cutpoint is calculated based on percent signal inhibition of a panel of untreated individual serum samples, pre-incubated with either 100 μg/mL of un-conjugated rhuMAb X or sample diluent prior to minimum dilution into assay buffer. It is calculated using following equations:

$$\%\,\text{Signal inhibition} = \frac{\text{Signal}_{(0\,\text{rhuMAb X})} - \text{Signal}_{(100\,\mu\text{g/mL rhuMAb X})}}{\text{Signal}_{(0\,\text{rhuMAb X})} * 100}$$

$$\text{Confirmatory cutpoint} = \text{Mean of the \% signal inhibition} + 2.326 * \text{S.D. of the population}$$

where 2.326 was chosen to generate a targeted 1% UTPR.

Other assay parameters, such as matrix effect, hook effect, and target inference, were evaluated during assay development according to recommendations described elsewhere [10] and will not be discussed in this paper.

3. Results

Clinical screening and confirmatory ATA assays for rhuMAb X were developed on the MSD platform. Initially, we adapted our clinical assay conditions from the optimized conditions for the non-clinical version of the assay (data not shown). Using this approach the screening assay proved to be adequate for sample analysis

with good sensitivity and drug tolerance; however we encountered problems with the confirmatory assay or the selectivity part of rhuMAb X immunogenicity evaluation. The confirmatory assay essentially had no selectivity as the addition of rhuMAb X to NC and LPC resulted in very similar percent signal inhibition (~83%). Various assay reagents and conditions were evaluated to improve selectivity in the re-developed MSD-based confirmatory assay.

3.1. Homogenous MSD-based clinical ATA assay

3.1.1. Evaluation of the rhuMAb X conjugates in the assay

The two conjugates, biotin- and Ru-labeled rhuMAb X, were evaluated individually or in combination to investigate the source of signal in the absence of a positive control. This experiment was conducted in assay buffer to eliminate any potential non-specific interference from serum. The MSD-based assay procedures in Section 2.3 were followed. When either biotin- or Ru-labeled rhuMAb X was added separately, minimal ECLU signals (similar to buffer alone) were observed (Fig. 2a and b); however when both conjugates were present together, signal was detected even in the absence of a positive control (Fig. 2c). Furthermore addition of unlabeled rhuMAb X to the mixture of biotin- and Ru-conjugated rhuMAb X eliminated this signal (Fig. 2d), suggesting unlabeled rhuMAb X competes with Ru-conjugated rhuMAb X for binding to the biotin–rhuMAb X.

To investigate whether the interaction between biotin and Ru conjugates of rhuMAb X, we tested each rhuMAb X conjugate with conjugates made from two unrelated monoclonal antibody therapeutics A and B (rhuMAb A and B). Biotin–rhuMAb X was paired with either Ru-rhuMAb A or B; Ru-rhuMAb X was also paired with either biotin–rhuMAb A or B in separate experiments. When either biotin- or Ru-conjugated rhuMAb X was paired with the complementary conjugate of rhuMAb A or B, minimal signals were observed. Elevated signals were only observed when the biotin and Ru conjugates of rhuMAb X were paired together (Fig. 2e). Therefore, the interaction between biotin- and Ru-conjugated rhuMAb X appears to be molecule specific. To overcome the non-specific interactions and increase the specificity of this assay various buffers were evaluated (see below).

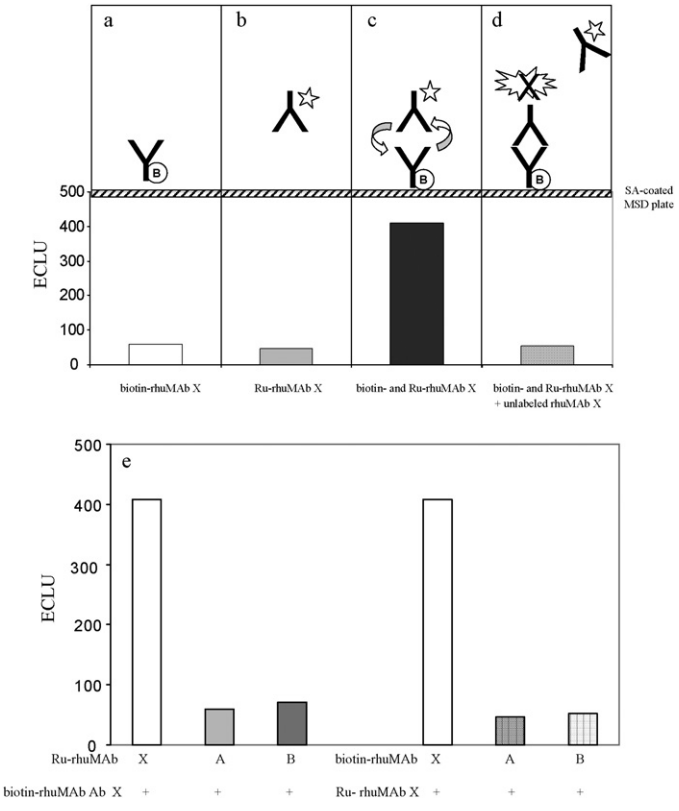

Fig. 2. Characterization of biotin and ruthenium labeled rhuMAb X conjugates. Two conjugates were evaluated separately (a) and (b), and in combination in the absence (c) or in the presence of 100 μg/mL soluble rhuMAb X (d). Each conjugate was also tested by pairing with complementary conjugates of monoclonal antibody therapeutics rhuMAb X, A and B (e). The ECLU signals were plotted against the conjugate(s) used in the experiments. The data are given as mean values of duplicate determinations.
1 μg/mL of biotin- and Ru-rhuMAb X were used for this experiment; rhuMAb A, B = two monoclonal antibody therapeutics other than rhuMAb X.

3.1.2. Optimization of assay conditions

Each assay reagent as well as various assay condition were examined to optimize the assay performance. These include conjugate challenge ratios and concentrations, incubation times and assay buffers. Biotin- and Ru-conjugated rhuMAb X at molar challenge ratios of 5:1 and 10:1 as well as at master mix concentrations of either 1 or 2 μg/mL were evaluated in the assay. Assay minimum dilutions at 1/10, 1/20 and 1/50 were compared. The final assay conditions of 1 μg/mL of biotin (5:1) and Ru (10:1) conjugated rhuMAb X at 1/10 minimum dilution were selected since these conditions gave the best signal to noise ratio (S/N), drug tolerance and separation between NC and low PC (LPC) in the confirmatory step.

We also investigated the impact of assay diluent composition on the binding interaction between biotin and Ru conjugates of rhuMAb X. Seven different assay buffers (buffers A to G listed in Table 1) were analyzed to select the condition with the highest percent competitive inhibition for the PC and more importantly the

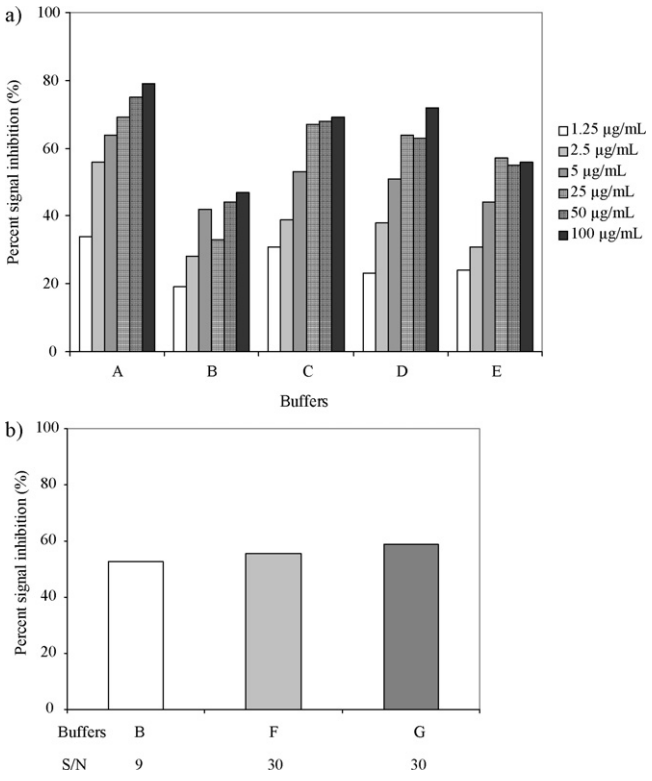

Fig. 3. Optimization of assay buffer conditions for MSD-based ECLA ATA assay. The percent signal inhibition for the NC in the presence of soluble rhuMAb X was evaluated, first in five assay buffers at various levels of rhuMAb X (a) and then in buffers B, F and G in the presence of 100 μg/mL of rhuMAb X (b). Buffers F worked best based on the consideration of good S/N as well as relatively lower percent signal inhibition for NC. The percent signal inhibition was plotted against the buffers used in the experiments. The data are given as mean values of duplicate determinations.

lowest percent competitive inhibition for the NC. Briefly, rhuMAb X was spiked at various levels into NC and minimally diluted into five different test buffers A to E (Fig. 3a). To this mixture, the master mix (1 μg/mL of biotin- and Ru-rhuMAb X) was added. This was done side by side with each buffer in the absence of rhuMAb X to calculate the percent competitive inhibition in each buffer at various rhuMAb X concentrations in the absence of a PC. The percent signal inhibitions of the PC were comparable in all buffer conditions (data not shown). The percent signal inhibition was observed for the NC with the addition of rhuMAb X at various levels for all buffers. Buffer B had the lowest percent competitive inhibition of NC at all rhuMAb X levels tested.

We then compared buffer B with two additional buffers: F and G (Fig. 3b). All three buffers had similar percent signal inhibition for the NC (between 50% and 60%) however, buffers F and G had much better S/N than buffer B (~30 for buffers F and G vs. ~9 for buffer B). Buffers F and G differ slightly from buffer B as both contained sarcosine (0.5% in buffer F and 1% in buffer G). Sarcosine is an anionic detergent and it aids in the solubility and separation of proteins and glycoproteins [17]. High pressure liquid chromatography (HPLC) analysis results revealed that 1.7% and 3.5% of aggregated protein were found in biotin- and Ru-rhuMAb X conjugates. Sarcosine was therefore added in the assay to improve rhuMAb X aggregation which was though to be a contributing factor to the immunodepletion of the NC. Ultimately buffer F was chosen to move forward for the homogenous bridging MSD ATA assay development, as increasing sarcosine to 1% (buffer G) did not improve assay performance beyond what was achieved with 0.5% sarcosine.

3.1.3. MSD-based ATA assay qualification parameters

The final MSD-based clinical ATA assay conditions are described at Section 2.3. The assay cutpoint multiplication factor was established based on signals from a panel of serum samples obtained from disease state patients ($n = 40$) and was calculated to be 1.2. A false-positive rate of approximately 5% was calculated for the population tested, based on this cutpoint multiplication factor. The relative sensitivity of the assay was estimated to be 32 ng/mL using 1C8, a CDR specific mouse monoclonal antibody raised against rhuMAb X. 500 ng/mL of 1C8 was detectable in the presence of 25–50 μg/mL rhuMAb X in neat serum.

The re-developed assay had better selectivity for rhuMAb X, although not as good a separation between NC and LPC as we have seen with other molecules in the past. Addition of soluble therapeutic to the samples resulted in signal inhibition of 84% for high PC (HPC), 76% for LPC and 58% for NC. A confirmatory cutpoint of 57% was calculated using a panel ($n = 24$) untreated patient serum samples. This was still much higher than the ~20% signal inhibition we typically observe with NC in our MSD-based clinical confirmatory ATA assays for other antibody therapeutics.

3.2. Homogenous ELISA-based clinical ATA assay

3.2.1. Optimization of assay conditions

As with the MSD platform, in order to optimize assay performance, each assay reagent as well as variety of assay conditions were examined, including conjugate challenge ratios, reagent concentrations, type of plates, incubation times, detecting reagent concentrations and assay buffers.

Different challenge ratios and concentrations of the biotin- and DIG-conjugated rhuMAb X were evaluated (2.5:1, 5:1 and 10:1 molar ratio of each conjugate at 1 and 2 μg/mL master mix concentrations, data not shown). Challenge ratio of 10:1 (tag:rhuMAb X) was selected for both DIG- and biotin-conjugated rhuMAb X at 1.25 μg/mL. Assay minimum dilutions at 1/10, 1/20 and 1/50 were evaluated, and 1/10 was selected since it generated the best S/N, drug tolerance and separation between NC and LPC in the confirmatory step. Assay performance using NA- versus SA-coated high binding Pierce plates were compared. NA-coated plates delivered comparable S/N but lower percent signal inhibition of NC therefore were chosen for final assay development.

Seven assay buffers (buffers A, B, F, H, I, J and K are listed in Table 1) were tested in the homogenous bridging ELISA format, to determine the best assay buffer condition with the goal of achieving minimum percent signal drop of NC and good assay S/N (Fig. 4a). Buffers B, F and K all had lower than 20% signal inhibition of NC in the presence of 100 μg/mL of rhuMAb X, however buffer B and K had better S/N. Buffer B was chosen as addition of fish gelatin (in buffer K) did not have significant impact on the assay performance.

Buffer B looked most promising among the seven assay buffers tested. However, we had a concern with the relatively high pH (8.9) of buffer B as it may compromise the detection of ATAs with low binding affinities in the assay. Assay performance using buffer B at pH levels of 7.4, 8.0 and 8.9 were evaluated (Fig. 4b). Using buffer B at pH 7.4 resulted in the highest percent signal inhibition of NC, which led to the worst separation between the NC and LPC2. This may be due to rhuMAb X aggregation since 7.4 is very close to the isoelectric point (p*I*) value (7.2) of rhuMAb X. Using buffer B at pH level of 8.0 the percent signal inhibition of NC increased slightly to 26% compared to less than 20% using buffer B at pH level of 8.9. Ultimately buffer B at pH of 8.0 was chosen as the final buffer for homogenous bridging ELISA assay development. In contrast to what

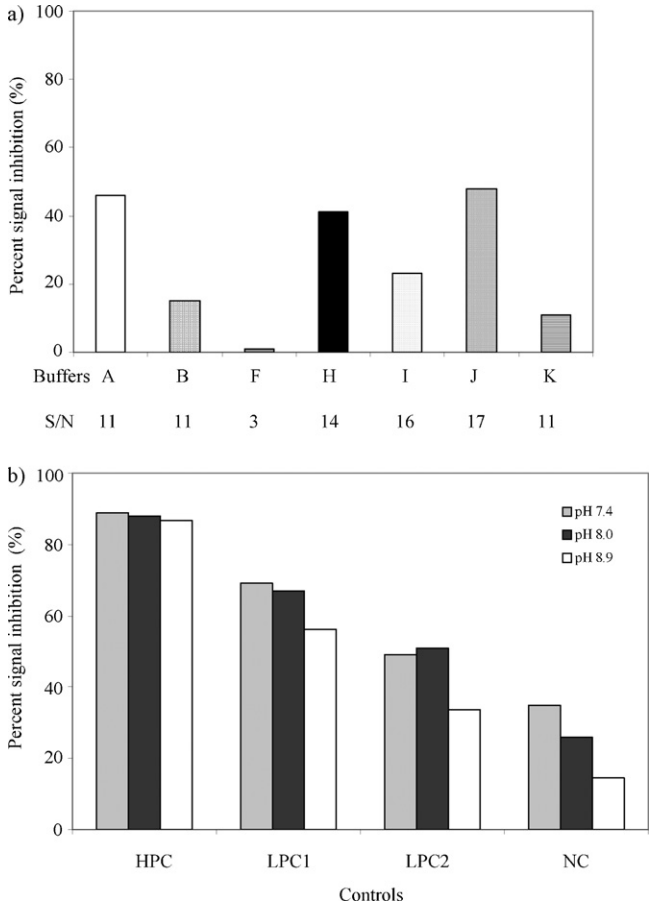

Fig. 4. Optimization of assay buffer conditions for homogenous bridging ELISA ATA assay. The percent signal inhibition for the NC in the presence of 100 μg/mL of soluble rhuMAb X was evaluated in seven assay buffers (a) and in buffer B at pH levels of 7.4, 8.0 and 8.9, respectively. Buffers B at pH level of 8.0 was selected based on the consideration of good S/N, relatively low percent signal inhibition for NC as well as preferred pH condition. The percent signal inhibition was plotted against the buffers used in the experiments. The data are given as mean values of duplicate determinations.

Table 2
Comparison of homogenous bridging ELISA versus ECLA MSD clinical assays.

	Homogenous bridging ELISA	MSD ECLA
Sensitivity	16 ng/mL	32 ng/mL
Screening cutpoint factor	1.2	1.2
Drug tolerance	25–50 μg/mL	25–50 μg/mL
Percent signal inhibition	NC: 26% drop LPC: 67% drop HPC: 89% drop	NC: 58% drop LPC: 76% drop HPC: 84% drop

was observed in the MSD-based assay (Fig. 2b), significant percent signal inhibitions were only observed in the positive controls (89% for HPC, 67% for LPC1 and 51% LPC2) but not in the NC (26%) in our homogenous bridging ELISA assay (Table 2).

3.2.2. Homogenous ELISA-based ATA assay qualification parameters

The final ELISA-based clinical ATA assay conditions are described at Section 2.4. The cutpoint multiplication factor of the assay was determined to be 1.2, based upon data from a panel of 40 serum samples from the target patient population; the relative sensitivity of the assay was estimated as 16 ng/mL using the 1C8 control; 500 ng/mL 1C8 is detectable in the presence of 25–50 μg/mL rhuMAb X in neat serum. A false-positive rate of approximate 5% (2 out of 40) was established for the panel of samples from untreated patients based on this cutpoint factor. The confirmatory cutpoint factor was established based on signals from a panel of untreated patient serum samples ($n = 24$) and was calculated to be 26%.

rhuMAb X clinical ATA assays were developed on MSD and ELISA assay platforms respectively. Comparison of major parameters of the two assays is summarized in Table 2. The optimized screening assays in both formats delivered very similar sensitivity and drug tolerance. However, the separations between the percent signal inhibition of the LPC and NC in the two confirmatory assays differed significantly. In the presence of 100 μg/mL of soluble rhuMAb X, the percent inhibitions of LPC and NC signals for the ELISA-based assay were 67% and 26% respectively versus 76% for LPC and 58% for NC in the MSD-based assay.

4. Discussion

A sensitive and drug tolerant clinical ATA screening assay for rhuMAb X was successfully developed using a homogeneous MSD platform. However during development of the confirmatory step of the assay, we encountered unexpected results. We observed a significant signal inhibition with the NC in the presence of soluble rhuMAb X. This led to a limited assay selectivity (i.e. separation between LPC and NC) making it less desirable for a confirmatory step. Confirmatory assay is an important part of clinical immunogenicity assessment as this step is designed to distinguish drug-specific ATA positive samples from false positive samples identified in the screening assay. The challenge with rhuMAb X clinical confirmatory assay development was to identify the best assay condition that would improve selectivity of the confirmatory step, yet maintain the assay sensitivity and individual variability which is essential in setting screening and confirmatory assay cut points.

To understand the cause of the high percent competitive inhibition of the NC observed with rhuMAb X, we evaluated various potential contributing factors including the assay platform, molecule characteristics, and assay conditions. We initially focused on the MSD platform as the potential cause. However our group has successfully developed multiple MSD-ECLA based clinical ATA confirmatory assays without problems. We then shifted our focus to investigating specific properties of our protein therapeutic rhuMAb X. We also concluded that the antibody subclass was not a contributing factor, since the effect was observed with antibody therapeutics of multiple immunoglobulin gamma (IgG) subclasses (data not included).

We then turned our attention to the key assay reagents. The effect of each reagent on the MSD signal was carefully evaluated. One observation was the high assay background in this assay compared to our other MSD-based ATA assays even with the best optimized condition. Experiments outlined in Fig. 2 suggested that there was "self-association" between the biotin- and Ru-rhuMAb X conjugates (Fig. 2c and 2d). This could explain the high assay background and the high percent signal inhibition of the NC signals. Although various molar ratios of the conjugates as well as buffer conditions were evaluated, we were not able to completely eliminate the "self-association" between the conjugates in the absence of PC. We concluded that the MSD-based assay worked well as a screening assay but may not be the best assay platform for a confirmatory assay in this case.

To ensure that the assay was delivered in time for sample analysis, we simultaneously developed the assay on a novel homogenous bridging ELISA platform while optimizing the MSD-based assay. This homogenous ELISA format combines the advantages of the BioVeris/MSD platforms, namely the homogeneous format, small tag combination, and overnight incubation. We chose DIG as a label to replace the ruthenium tag used in BioVeris/MSD formats. DIG is a small molecule with comparable molecular weight to biotin and has been widely used for protein labeling. Using the DIG/biotin combination, we were able to maintain the homogenous format of the assay and were hoping to achieve similar sensitivity and drug tolerance as with the BioVeris/MSD platforms. Indeed, the rhuMAb ATA screening assay developed using the homogenous bridging ELISA was found to be as sensitive and drug tolerant as the MSD-based assay. In addition, we were able to achieve a better separation between the NC and LPC, therefore improving the selectivity of the confirmatory assay compared to the MSD-based assay. We have now adopted this new homogenous ELISA format for immunogenicity evaluation of protein therapeutics at Genentech moving forward.

It is important to note that clinical assays are designed and are meant to be used through the clinical development of a therapeutic and beyond, which could be years. Therefore, a clinical assay not only needs to be robust and reproducible, but also should use reagents and technologies from sustainable sources. When the BioVeris platform was discontinued, our department as well as bioanalytical groups from other organizations were faced with the challenge of finding a replacement to this technology in a timely manner. This was due to the fact that BioVeris was the sole vendor for this technology and once it was discontinued there was no instrument or reagent support for this technology [18]. This experience, though painful for many companies, has emphasized the importance of choice of technology and reagents in clinical assay development. The homogenous bridging ELISA (DIG/biotin) platform proposed here has now been successfully used in a number of our projects. It is more economical than other formats and all the key materials for this assay platform such as labeling materials for making conjugates (biotin, DIG), detecting reagent (HRP-conjugated anti-DIG antibody), and other consumables (such as NA-coated microtiter plates) are widely available by multiple vendors.

5. Conclusion

Two clinical ATA assays for rhuMAb X were developed using a homogenous MSD as well as a homogenous bridging ELISA platforms, respectively. Both assays worked comparably as screening assays however, the homogenous bridging ELISA format worked more effectively in the confirmatory assay. The characterization

work summarized here suggests that our challenge with the confirmatory assay with rhuMAb X may be attributed to molecule specific properties, including neutral pI and potential aggregation tendencies of this molecule. Initially, we thought this was an isolated case, however we have now observed similar competitive inhibition issue on a number of other projects and therefore, our experience with rhuMAb X clinical confirmatory assay may be applicable to others for evaluating immunogenicity of therapeutic proteins. We believe that the homogenous bridging ELISA described here would be an excellent alternative to MSD/BioVeris platforms for immunogenicity assessment.

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