Research Article

When Assay Format Matters: a Case Study on the Evaluation of Three Assay Formats to Support a Clinical Pharmacokinetic Study

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Abstract. Data generated using various immunoassay methods are an integral part of the development of protein therapeutics. These assays are used in clinical and preclinical studies to establish the pharmacokinetic (PK) and pharmacodynamic (PD) characteristics as well as to assess the immunogenicity properties of a therapeutic. PK assays measure therapeutic levels post-administration which is essential for understanding the effective dose and dose regimen for a therapeutic. Anti-OX40L is a fully humanized monoclonal antibody designed for the potential treatment of an autoimmune disease. The anti-OX40L human PK assay is required to be sensitive, robust, and precise. To address challenges due to assay sensitivity and reproducibility, as well as assay technology limitations, during development of the anti-OX40L human PK assay, three different assays, including an MSD-based electrochemiluminescence assay (ECLA), a fluorometric enzyme-linked immunosorbent assay (ELISA), and a colorimetric ELISA, were evaluated. The MSD-based assay was the most sensitive but posed risk of inter-well signal crosstalk. The fluorescence ELISA fell short on reproducibility. The colorimetric ELISA was ultimately chosen for supporting sample analysis. This paper presents characterization data obtained from each of these assay formats, challenges that were encountered in the development of the assay, and the rationale for selecting the ultimate assay format.

KEY WORDS: assay crosstalk; clinical pharmacokinetic assay; electrochemiluminescence assay; enzyme-linked immunosorbent assay; monoclonal antibody therapeutic.

INTRODUCTION

In recent years, there has been an increase in biotechnology-derived therapeutics, including recombinant proteins, peptides, antibody therapeutics as well as nucleic acid based therapeutics. Monoclonal antibody (mAb) therapeutics show promising results in treating complex diseases due to their high specificity and selectivity for the therapeutic targets. More than 20 mAb therapeutics have been approved in the USA for treatment of a variety of disease indications (1). The success of mAb therapeutics has encouraged further research and development work, and many additional mAb therapeutics are currently being evaluated at various stages of clinical studies.

mAb therapeutics are designed to target specific antigens via noncovalent and reversible high-affinity binding to elicit pharmacological effects. Successful development of mAb therapeutics requires reliable bioanalytical methods to characterize pharmacokinetic (PK) properties of the therapeutic. PK assays quantitatively determine levels of a mAb in biological samples (fluids) post-administration and are essential for evaluation of PK/PD (pharmacodynamic) relationships, safety margin calculations, and eventual characterization of the exposure in the

clinic. It is therefore critical to establish analytical methods that are sensitive, precise, and robust as these methods may be used for years to support the lifecycle of such therapeutics (2, 3).

A target-binding format is commonly utilized for clinical PK assay development. In this format, the therapeutic target, which can be either a recombinant soluble full-length target protein or an extracellular domain portion of the target protein, is used as the capture reagent, and a monoclonal or polyclonal antibody (pAb) specific to the mAb therapeutic is often the preferred reagent for detection. Since a mAb therapeutic is typically divalent and has two independent antigen-binding sites, free (unbound), partially bound (one site bound), and fully bound (both sites bound) forms of mAb therapeutic may coexist in the circulation following treatment (4, 5). The free and partially bound forms of the mAb therapeutic are considered bioactive due to the availability of their target-binding site(s). In theory, only the free and partially bound forms of a mAb therapeutic can be captured in a target-binding assay. If the detection antibody is neutralizing or blocking the target binding site, only the free form of the mAb therapeutic can be detected; otherwise, both the free and partially bound forms can be detected. In addition, other assay conditions, such as sample dilution, incubation time, and binding affinity of a mAb therapeutic to its target, can also impact assay characteristics and the results generated, including what drug forms are indeed measured.

The essential parameters for PK assays include accuracy, precision, selectivity, sensitivity, reproducibility, limit of

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detection, and reagent stability (6). In addition, the continuing evolution of divergent analytical technologies provides opportunities to evaluate and incorporate newer technologies to achieve the most optimized assay performance, i.e., better sensitivity and more robust methods. To illustrate the challenges with developing sensitive, precise, and robust assays, a case study will be presented.

Anti-OX40 ligand (OX40L) is a fully humanized mAb designed for the potential treatment of an autoimmune disease, and it targets a soluble ligand OX40L. The phase I clinical study used a dose escalation approach to assess drug safety, starting with the lowest dose level at 2 $\mu\text{g/kg}$. Therefore, the desired PK assay sensitivity needed to be at a low nanogram per milliliter level in order to monitor the clearance profile and exposure of anti-OX40L. This sensitivity requirement was more stringent than a typical clinical PK assay requirement which is usually around 100–200 ng/mL for mAb therapeutics. In order to achieve this level of sensitivity, we evaluated three assay formats developed on two platforms, including an electrochemiluminescence assay (ECLA), a fluorometric enzyme-linked immunosorbent assay (FL ELISA), and a colorimetric (CL) ELISA.

Stepwise ELISA is one of the earliest platforms utilized for PK assay development (7). Typically, this is a heterogeneous assay with sequential reagent addition and wash steps. The assay readout can be absorbance, fluorescent, or chemiluminescent. An ECLA is 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 (Ru) metal ion. Analogous to chemiluminescence, electrochemiluminescence does not require the use of external light sources. Hence, background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light). The unique combination of chemiluminescence and electrochemistry in ECL provides many potential advantages, such as excellent assay sensitivity, limited matrix interference, and wide assay dynamic range (8, 9). The plate-based ECLA from Meso-Scale Discovery (MSD) located in Gaithersburg, MD, was used in this work. While both ECLA and ELISA platforms are broadly used for PK assay development, the MSD-ECLA platform was our first choice for developing the anti-OX40L clinical PK assay due to the low nanogram per milliliter assay sensitivity requirement. Although MSD-ECLA has been successfully used for PK assay development by various groups (10, 11), we encountered an unexpected inter-well crosstalk problem during the development of anti-OX40L clinical PK assay. A FL- and a CL-ELISA were then evaluated as possible assay formats.

This paper presents the challenges and strategies in developing the anti-OX40L clinical PK assay. We discuss our attempts to identify and understand the crosstalk issue in the MSD-based assay, our efforts to evaluate the assay performances using three approaches, and our considerations for selecting an assay format to support anti-OX40L clinical studies.

MATERIALS AND METHODS

Materials

Anti-OX40L is a fully human mAb generated at Genentech (South San Francisco, CA). Individual serum samples from the target patient population and pooled normal human sera (NHS)

were purchased from Bioreclamation (Hicksville, NY) and BioChemed (Winchester, VA). The recombinant human OX40L (rhuOX40L) was generated at Genentech. MAb 1G6, a complementarity determining region (CDR) specific mouse mAb raised against anti-OX40L, was produced 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 [(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate) was from Research Organics (Cleveland, OH); and ProClin 300 was from Supelco (Bellfonte, PA). Streptavidin (SA)-coated MSD plates and 4X Read Buffer T with surfactant were purchased from Meso-Scale Discovery (Gaithersburg, MD).

Preparation of Conjugates of rhuOX40L and Detection Antibody 1G6

rhuOX40L and mAb 1G6 were buffer exchanged into phosphate buffered saline (PBS) prior to conjugation using an illustra™ Nap-10 column following the manufacturers' instructions (GE Healthcare).

Buffer-exchanged rhuOX40L and mAb 1G6 were conjugated with biotin at a challenge ratio of 10:1 (biotin:rhuOX40L or 1G6) using EZ-Link SulfoNHS-LC-Biotin (Pierce, IL). MAb 1G6 was also conjugated with Ru at a challenge ratio of 10:1 (Ru:1G6) using sulfo-TAG (MSD, MD). All conjugations were prepared according to the manufacturer's instructions followed by a buffer exchange step into storage buffer (20 mM Sodium Phosphate, 0.35 M Sodium Chloride, 6% Sucrose, 0.25% Polysorbate 20, 0.25% CHAPS, 0.05% Proclin 300, 0.5% BSA, pH 5.2 $\pm$ 0.2).

Characterization of Assay Detection Antibody 1G6

A Nunc Maxisorp 96-well ELISA plate was coated with 1 $\mu\text{g/mL}$ of human immunoglobulin G (IgG), rhuOX40L, or anti-OX40L in PBS, pH 7.4 at 2–8°C overnight. The next day, the plate was washed three times with wash buffer (PBS, 0.05% polysorbate 20, pH 7.2 $\pm$ 0.2), blocked with 200 μL per well of assay buffer (PBS, 0.5% BSA, 0.05% polysorbate 20, 15 ppm Proclin 300, pH 7.2 $\pm$ 0.2), incubated at room temperature (RT) for an hour with agitation, and then washed another three times with wash buffer. Anti-OX40L at 250 ng/mL in assay buffer was then applied to the rhuOX40L-coated wells; assay buffer was added to the rest of the wells respectively at 100 μL per well. The plate was incubated at RT for an hour with agitation and then washed three times with wash buffer. MAb 1G6 prepared in assay buffer at 1 $\mu\text{g/mL}$ were then applied to the entire plate at 100 μL per well. The plate was again incubated at RT for an hour with agitation and then washed three times with wash buffer. Detection antibody, 50 ng/mL of horseradish peroxidase (HRP)-conjugated anti-mouse IgG in assay buffer, was added at 100 μL per well and incubated at RT for an hour with agitation. The plates were washed three times with wash buffer; 100 μL of tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added per well and incubated at RT for 15–20 min. The reaction was stopped by adding 100 μL of 1 M phosphoric acid (Sigma) to each well, and the absorbance was measured at 450 nm using 650 nm as reference on a plate reader (Molecular Devices, Sunnyvale, CA).

MSD-ECLA Based Anti-OX40L Clinical PK Assay

In the MSD ECLA-based assay (Fig. 1a), 200 μ L per well of assay diluent (PBS, 3% BSA, 0.05% polysorbate 20, 15 ppm Proclin 300, pH 7.2 $\pm$ 0.2) was added to SA-coated MSD plates and incubated at RT for 1–2 h with agitation to block wells. The plates were then washed three times with wash buffer. Biotin-conjugated rhuOX40L at 2 μ g/mL was applied to SA-coated MSD plates and incubated at RT for 1 h with agitation. Human serum samples and controls were prepared at 1:4 dilutions in the assay diluent. An anti-OX40L standard curve, ranging from 0.488 to 125 ng/mL (in-well concentrations) using 1:2 serial dilutions, was prepared in the standard diluent (assay diluent plus 25% pooled NHS). The plates were then washed three times, and 50 μ L per well of the prepared standards, controls, and samples were added. The plates were incubated at RT for 2 h with agitation, followed with six washes. Ru-conjugated mAb 1G6 at 2 μ g/mL in assay diluent was then applied at 50 μ L per well for detection. The plate was incubated at RT for an hour with agitation in the dark. Plates were washed six times prior to addition of 150 μ L of 1x Read Buffer T with surfactant (MSD) per well and were immediately analyzed using a MSD Imager (Sector Imager 6000 reader) (Fig. 1a).

FL-ELISA Based Anti-OX40L Clinical PK Assay

In the FL-ELISA (Fig. 1b), 0.5 μ g/mL of rhuOX40L in PBS, pH 7.4 was coated on Nunc MaxiSorp® flat-bottom 96-well plates and incubated at 2–8°C overnight. The next day, the plates were washed three times with wash buffer, blocked with 200 μ L per well of assay buffer, and incubated at RT for 1–2 h with agitation. Human serum samples and controls were prepared at 1:5 dilutions in the assay buffer. An anti-OX40L standard curve, ranging from 2.95 to 1,800 ng/mL (in-well concentrations) using 1:2.5 serial dilutions, was prepared in the standard diluent (assay buffer plus 20% pooled NHS). The plates were then washed three times with wash buffer, and 100 μ L per well of the prepared standards, controls, and samples were added. The plates were incubated at RT for 2 h with agitation, followed with six washes. Biotin-conjugated mAb 1G6 at 200 ng/mL in assay buffer was applied at 100 μ L per well for detection and incubated at RT for 1 h with

agitation. The plates were washed six times followed by addition of 500 ng/mL of SA- β -galactosidase (Gal) in assay buffer at 100 μ L per well and incubated at RT for 30 min with agitation. 4-Methylumbelliferyl- β -D-glucuronide (4-MUG) substrate (Sigma) at 340 μ g/mL in 0.1 M sodium phosphate, 1 mM MgCl₂, pH 7.4 was then applied at 100 μ L per well for detection. After 1-h incubation at RT in the dark, the reaction was stopped by the addition of 100 μ L/well of 0.3 M glycine, pH 10.5. The fluorescent signals were immediately analyzed using a Molecular Devices SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA) with a 360-nm excitation filter and a 460-nm emission filter (Fig. 1b).

CL-ELISA-Based Anti-OX40L Clinical PK Assay

Similar to the FL-ELISA, the CL-ELISA used the same reagents and conditions for the coat and detection steps. The sample/control dilution was increased from 1:5 to 1:20 for the CL-ELISA. An anti-OX40L standard curve, ranging from 0.20 to 400 ng/mL (in-well concentrations) using 1:2 serial dilutions, was prepared in the standard diluent (assay buffer plus 5% NHS). The CL signals were visualized after addition of 20 ng/mL of SA-HRP at 100 μ L per well and incubated at RT for an hour with agitation. Plates were washed six times with wash buffer, 100 μ L of TMB substrate was applied to each well, and then incubated at RT for 15–20 min. The reaction was stopped by adding 100 μ L of 1 M phosphoric acid to each well. The absorbance was measured at 450 nm using 650 nm as reference with a Molecular Devices SpectraMax 384 plate reader (Fig. 1b).

RESULTS

Characterization of the Assay Detection Reagent

The detection antibody mAb 1G6 was characterized by testing its specificity and binding ability to anti-OX40L in the presence and absence of OX40L. The results indicate that mAb 1G6 is specific to anti-OX40L and does not cross-react with endogenous human IgG in human serum (Fig. 2). To determine binding ability in the presence of OX40L, excessive rhuOX40L was applied to ensure anti-OX40L molecules were all bound to rhuOX40L. The molar ratio of rhuOX40L

Fig. 1. Anti-OX40L clinical PK assay developed on **a** MSD-ECLA: schema of the MSD-ECLA; **b** FL- or CL-ELISA: schema of the ELISA. *MSD* Meso-Scale Discovery; *Ru* ruthenium; *SA* streptavidin; *SA- β -gal* streptavidin-beta galactosidase; *SA-HRP* streptavidin-horseradish peroxidase

Fig. 2. Characterization of assay detection antibody 1G6 based on the assay signal-to-noise (S/N) ratios. MA b 1G6 bound specifically to anti-OX40L and the complex of OX40L-anti-OX40L

to anti-OX40L was at 17 to 1. The data indicated that mAb 1G6 detects anti-OX40L similarly in the presence and absence of excessive target rhuOX40L (Fig. 2).

MSD-ECLA PK Assay

MSD-ECLA PK Assay Performance

This assay can detect anti-OX40L over a broad range of concentrations in the serum (1.95–500 ng/mL), and a representative standard curve is presented in Fig. 3. The minimum quantifiable concentration (MQC) of the assay was determined to be 4 ng/mL. Accuracy of the assay was assessed during assay qualification using anti-OX40L controls prepared in the human serum and found to be acceptable with the average percent recovery ranging from 98 to 115% of the nominal levels. Overall, the mean inter- and intra-assay precisions for assay controls were determined to be in the range of 8–13 and 5–8% (Table I), respectively. Specificity of the assay was confirmed by measuring the reactivity of an unrelated recombinant humanized mAb (full length) and two generic purified human IgG samples (full length). None of these IgGs cross-reacted in the assay (data not shown), demonstrating the high specificity. OX40L interference was observed in the presence of 50 ng/mL or greater level of rhuOX40L. Since the soluble OX40L in circulation was determined to be at low picogram per milliliter levels (internal unpublished data), the presence of OX40L is unlikely to have any impact on the drug level measurement in clinical samples. While most of the assay parameters met the standard acceptance criteria, a problem with dilutional linearity was observed during assay development. As described in the next section, the problem was proven to be platform-related. Therefore, this assay format was not considered adequate for further evaluation.

Crosstalk Observed in the MSD Format

As part of the assay qualification, dilutional linearity was evaluated to determine if serum concentrations of anti-OX40L could be accurately determined in samples need to be prepared by more than the minimum dilution in order to fall into the assay quantitative range. The acceptance criteria

were that samples that are within the assay quantitative range should recover between 80 and 120% of the nominal value. A problem with dilutional linearity was observed during assay development, which prompted an investigation to identify the source of the problem. In the experiment, a serum sample containing 440 µg/mL of anti-OX40L was prepared. A set of dilutional linearity samples was prepared by initially diluting the serum sample at the minimum dilution (1:4), followed by three 1:10 serial dilutions to reach the top end of the assay dynamic range, and then further diluted using eight serial 1:2 dilutions to target the entire assay dynamic range. Blank samples consisting of standard diluent (assay diluent plus 25% pooled NHS) without anti-OX40L were also prepared. The blank and dilutional linearity samples were then tested side-by-side in two plates utilizing different plate layouts. Each sample was tested in duplicate. The wells containing standard curves and controls were positioned as indicated in Table II. For the purpose of demonstration, only the results of the blank and dilutional linearity samples are presented.

When reading the ECLA signal of a 96-well MSD-plate, the MSD Sector Imager 6000 reader takes an image of a four row×four column section at a time (a total of 16 wells per image). Thus, it takes a total of six separate images to completely read a 96-well plate (Table III). These six image sections can be broken down as follows: section 1 (wells A1–D4), section 2 (wells A5–D8), section 3 (wells A9–D12), section 4 (wells E1–H4), section 5 (wells E5–H8), and section 6 (wells E9–H12). To verify whether extremely high ECLA signal from samples containing high level of anti-OX40L would have inter-well crosstalk signal into adjacent wells within an image section or even adjacent plate image sections, the dilutional linearity samples were applied to two plates using different plate layouts (Table II).

In plate 1, dilutional linearity samples were plated in image sections 2 and 3, in wells A8 to H9 as well as A11 to D12 of the plate (Table II). None of the plate image sections 1, 4, 5, and 6 contained samples with readouts greater than one million electrochemiluminescence unit (ECLUs) or blank samples with greater than 70 ECLUs. However, in image sections 2 and 3, the blank ECLA signals increased nearly 3-fold (~200 ECLU) in the presence of anti-OX40L samples with more than one million ECLUs in the same image section. In addition, dilutional linearity samples with lower anti-OX40L levels (plated in A11 to D12) were also affected by this crosstalk within an image section, leading to significant over recovery of the anti-OX40L (143–201% of nominal, [Table IV]).

In plate 2, the same set of dilutional linearity samples were applied to different well locations than plate 1. Here, the samples were added across image sections 4, 5, and 6 in wells E1 to F12 (Table II). Similar to that observed in plate 1, the blank sample signals in image section 6 were elevated due to crosstalk with the high-level anti-OX40L samples (> one million ECLUs) present within the same image section. In this case, the dilutional linearity samples with lower levels of anti-OX40L plated in image section 4 were not affected by crosstalk, exhibiting anti-OX40L percent recovery within our acceptance criteria (80–120% of nominal, [Table IV]).

Fig. 3. Representative assay standard curve for the three assay formats

This plate layout comparison experiment indicated that the assay had good dilutional linearity; however, the plate readouts could be impacted in the presence of samples with more than one million ECLU. Additionally, multiple factors were investigated to verify if the crosstalk issue was assay specific or instrument- or platform-related. Different lots of MSD plates and MSD Imagers were tested and compared. There was a joint effort with the vendor for troubleshooting, including an attempt of utilizing algorithm incorporated in the MSD software to minimize the crosstalk. However, none of the efforts resolved the inter-well crosstalk issue successfully in the presence of samples with signals over one million ECLUs (Fig. 4).

FL-ELISA PK Assay

This assay detects anti-OX40L over a broad range of concentrations in the serum (14.75–3,600 ng/mL), and a representative standard curve is presented in Fig. 3. The MQC of the assay was determined to be 50 ng/mL. Accuracy of the assay was assessed during assay qualification using anti-OX40L controls prepared in the human serum and found to be acceptable with the average recovery ranging from 98 to 117% of the nominal levels. Overall, the mean inter- and intra-assay precisions for the assay controls were determined to be in the range of 7–19 and 5–11% (Table 1), respectively. Specificity of the assay was confirmed by measuring the reactivity of another unrelated recombinant humanized mAb (full length) and two generic purified human IgG samples (full length). None of these IgGs cross-reacted in the assay (data not shown), demonstrating the high specificity of this assay for anti-OX40L. Interference was observed in the presence of 4 ng/mL or more of rhuOX40L. This should not impact clinical sample analysis results due to reasons described earlier under “MSD-ECLA PK Assay Performance.” The dilutional linearity was considered acceptable (80–120% of nominal). Unlike the MSD-ECLA format, the FL-ELISA assay did not exhibit a crosstalk issue. Although the assay had slightly higher number of failed runs during validation, it still met all standard acceptance criteria and passed the validation. However, the assay reproducibility issue worsened during production (data not shown) and not suitable for long-term support.

CL-ELISA PK Assay

This assay detects anti-OX40L over a broad range of concentrations in the serum (15.6–2,000 ng/mL), and a representative standard curve is presented in Fig. 3. The MQC of the assay was determined to be 36 ng/mL. Accuracy of the assay was assessed during assay qualification using anti-OX40L controls prepared in the human serum and found to be acceptable with the average recovery ranging from 85 to 100% of the nominal levels.

Table 1. Comparison of Three Assay Formats

	MSD assay	FL-ELISA	CL-ELISA
Minimum dilution	1:4	1:5	1:20
Dynamic range (in-well), ng/mL	0.49–125	2.95–720	0.78–100
Quantitative range (neat serum), ng/mL	1.95–500	14.8–3,600	15.6–2,000
Sensitivity (MQC in serum), ng/mL	4	50	36
Accuracy ^a (based on nominal conc.), percent recovery ^b	98–115%	98–117%	85–100%
Precision, intra-assay percent CV ^a	5–8%	5–11%	6–11%
Precision, inter-assay percent CV ^a	8–13%	7–19%	7–12%

MSD Meso-Scale Discovery, FL-ELISA fluorometric enzyme-linked immunosorbent assay, CL-ELISA colorimetric enzyme-linked immunosorbent assay, MQC minimum quantifiable concentration, CV coefficient of variation, GTR great than range, LTR less than range
^a Accuracy, intra-assay percent CV, and inter-assay percent CV were calculated based on ten independent runs of serum control samples
^b Percent recovery=(interpolated dilution corrected concentration)/(nominal concentration)×100

Table II. Plate Layouts and Results of the Two Dilutional Linearity Experiments

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	54	45	262	211	255	1690000	1690000	260	768	814
B	STD2	STD2	54	66	191	272	222	1600000	1600000	252	500	459
C	STD3	STD3	51	52	168	295	225	781000	748000	222	377	304
D	STD4	STD4	46	Ctrl 1	Ctrl 1	176	196	34600	36700	203	188	208
E	STD5	STD5	54	Ctrl 2	Ctrl 2	52	148	12700	13300	176	53	55
F	STD6	STD6	62	Ctrl 3	Ctrl 3	58	51	6340	6530	57	64	51
G	STD7	STD7	53	Ctrl 4	Ctrl 4	57	58	2710	2780	57	58	57
H	STD8	STD8	58	Ctrl 5	Ctrl 5	59	54	1270	1260	57	58	56

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	70	67
B	STD2	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	65	71
C	Ctrl 1	Ctrl 2	Ctrl 3	Ctrl 4	Ctrl 5	Ctrl 1	Ctrl 2	Ctrl 3	Ctrl 4	Ctrl 5	59	57
D	53	53	57	59	55	50	56	56	54	50	58	50
E	108	182	311	621	1280	2760	6400	12200	32900	761000	1560000	1650000
F	113	181	315	608	1240	2540	6100	13700	34200	724000	1580000	1640000
G	48	56	51	54	57	58	58	58	266	466	374	392
H	49	47	54	54	54	48	51	59	260	287	306	269

Light-shaded wells are results of duplicate samples prepared for dilutional linearity experiment; dark-shaded wells are blank samples within an image section affected by crosstalk; non-shaded wells are blank samples within an image section not affected by cross-talk

Table III. MSD Plate Reader Reads a 96-well Plate in Six Image Sections

	1	2	3	4	5	6	7	8	9	10	11	12
A B C D	Section 1 (wells A1 to D4)				Section 2 (wells A5 to D8)				Section 3 (wells A9 to D12)			
E F G H	Section 4 (wells E1 to H4)				Section 5 (wells E5 to H8)				Section 6 (wells E9 to H12)			

Overall, the mean inter- and intra-assay precisions for the assay controls were determined to be in the range of 7–12 and 6–11% (Table I), respectively. Interference was observed in the presence of 4 ng/mL or more of rhuOX40L. Again this should not impact clinical sample analysis results due to reasons described earlier under “MSD-ECLA PK Assay Performance.” The dilutional linearity was considered acceptable (within 80–120% of nominal). Unlike the MSD-ECLA format, the CL-ELISA assay did not exhibit a crosstalk issue. The assay was further tested during validation with more extensive tests and met all the standard acceptance criteria.

Table IV. Percent Recovery of Dilution Linearity Samples Using Different Plate Layouts

Dilution corrected [anti-OX40L] (ng/mL)	Dilution, fold	Plate 1			Plate 2		
		ECLA Signals (ECLU)	Measured [anti-OX40L], ng/mL	% Recovery ^a	ECLA Signals (ECLU)	Measured [anti-OX40L], ng/mL	% Recovery ^a
440,000	4	1690000	GTR	GTR	16500000	GTR	GTR
440,000	40	1600000	GTR	GTR	1570000	GTR	GTR
440,000	400	765000	GTR	GTR	743000	GTR	GTR
440,000	4,000	35600	506,000	115	33500	528,000	120
440,000	8,000	13000	447,000	102	13000	458,000	104
440,000	16,000	6440	502,000	114	6250	480,000	109
440,000	32,000	2750	492,000	112	2650	444,000	101
440,000	64,000	1260	494,000	112	1260	444,000	101
440,000	128,000	791	627,000	143	615	434,000	99
440,000	256,000	480	716,000	163	313	395,000	90
440,000	512,000	341	883,000	201	182	326,000	74
440,000	1,020,000	198	LTR	LTR	111	LTR	LTR

Light-shaded results are for dilutional linearity samples using plate layout 1; dark-shaded results are for dilutional linearity samples using plate layout 2

ECLA electrochemiluminescence assay

^a% Recovery=(interpolated dilution corrected concentration)/(nominal concentration)×100

DISCUSSION

The clinical anti-OX40L PK assay was developed using three formats in two platforms: ECLA and ELISA. MSD-ECLA was robust and was identified as the most sensitive with the best signal-to-noise (S/N) among the three assays (Fig. 3). However, significant inter-well crosstalk was observed in the presence of samples with signals greater than one million ECLUs. The MSD detector reads plates in six steps with each step measuring an image section area of four rows×four columns (16 wells). Inter-well crosstalk was observed when the image section contained sample(s) with more than one million ECLUs (Table II). In clinical sample analysis, samples are often blinded and samples producing signals of more than one million ECLUs could be positioned randomly on the plate. Therefore, the quantitation of other samples located within the same image section can be impacted due to crosstalk, therefore compromising all 16 data points within the image section. As mentioned in the Results section, multiple efforts were used, including using algorithms to compensate for the crosstalk, without success. Therefore, MSD-ECLA assay was not considered suitable for

analyzing (blinded) clinical samples. This inter-well crosstalk issue was first identified in the anti-OX40L clinical PK assay; it was later also observed in several other MSD-based assays. For example, in one experiment of running a MSD-based nonclinical anti-therapeutic antibody (ATA) assay, eight samples were plated in one section of four rows×four columns with each sample analyzed in duplicates. In the presence of one sample with readouts of more than one million ECLU, other seven samples were all tested as ATA positive. In the absence of this sample, significant signal reductions (76–90%) were observed in all seven samples (unpublished data), and six of the seven samples were confirmed to be ATA negative instead. In this case, inter-well crosstalk issue compromised assay readouts and conclusions. Observations in this ATA assay were consistent with our experience in the MSD-based anti-OX40L PK assay. In addition, inter-well crosstalk of the MSD-based assay was reported in an ECLA review article (12); however, there was no reference citations and detailed description included. Our experience seems not to be an isolated case, and others encountered similar problems with MSD-based assays.

Fig. 4. Dilutional linearity results obtained with and without crosstalk for the anti-OX40L clinical PK assay developed on MSD-ECLA

Inter-well crosstalk was not observed in the FL-ELISA and CL-ELISA. The FL-ELISA has the largest dynamic range among the three assays (Table I), which is an advantage for production due to fewer sample dilution steps. The assay sensitivity is not as good as the MSD-based assay but adequate to confirm therapeutic exposure even for the group with the lowest dose evaluated. However, this assay was less reproducible than the other two assays. The total error was higher across the entire standard curve, especially at the lower portion of the curve. This assay reproducibility issue worsened at the contracting lab and resulted in a higher failure rate than the other two assay formats. Therefore, the assay was determined to be unsuitable for long-term support.

The CL-ELISA has the same format as FL-ELISA except using a colorimetric substrate for signal development and higher sample minimum dilution. The CL-ELISA has better sensitivity with smaller assay dynamic range compared to the FL-ELISA, and the sensitivity was adequate for supporting the study needs. The assay also proved to be both robust and reproducible during validation and production. Therefore, the CL-ELISA was ultimately chosen for supporting the study.

CONCLUSION

Anti-OX40L human PK assay was developed using three different approaches, including an MSD-based ECLA, a FL-ELISA, and a CL-ELISA. The ECLA was the most sensitive assay but exhibited a significant inter-well crosstalk issue. The FL-ELISA had the largest dynamic range but fell short in reproducibility during production. Both ECLA and FL-ELISA are widely used

formats for bioanalytical assay development. ECLA-based ATA assays and biomarker assays often have good drug tolerance and sensitivity. FL-ELISA assay is known for its large dynamic range and good cost efficiency and can be supported by basic laboratory instrument technology. However, neither ECLA nor FL-ELISA was determined to be the best format for our application in this case. Our work demonstrated that each technology has its benefits and constraints, and a fit for purpose approach should be utilized in selecting a platform during assay development. In this instance, the CL-ELISA assay proved to be the superior format with adequate sensitivity and reproducibility required for long-term support. Therefore, the CL-ELISA was ultimately chosen as the final PK assay format to support our clinical sample analysis.

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