APPLICATION NOTE

Immunogenicity assessment using the AlphaLISA technology.

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Introduction

Biological drug products often elicit an immune response in patients. Clinical consequences of the presence of anti-drug antibodies (ADA) can vary from mild to serious adverse events. Therefore, the presence of ADA is a major safety and efficacy concern and should be evaluated and correlated with any pharmacological or toxicological observations. The development of rapid and sensitive assay platforms for ADA detection is an essential step of the drug development process. Current methods exhibit limitations related to assay sensitivity, robustness and drug tolerance. We report here the development of an AlphaLISA™ bridging assay for the detection of ADA in serum samples. The use of this technology in immunogenicity testing was successfully demonstrated with an antibody model system. We demonstrate here, by a full qualification study, that the AlphaLISA mix-and-read homogeneous assay permits the sensitive detection of ADA in the low ng/mL range, maintaining excellent performance in the presence of human serum and exhibiting high drug tolerance at µg/mL concentrations of free drug.

Materials and methods

Materials

Mouse monoclonal IgG2b antibody (the drug) was purchased from AbD Serotec (Cat # MCA2472). The ADA positive control antibody (PC) was a polyclonal goat anti-mouse IgG (mouse IgG) antibody obtained from Jackson ImmunoResearch (Cat # 115-005-062). The matrix consisted of a pooled normal human serum (PNHS) and individual lots of normal human serum purchased from Bioreclamation (Cat # HMSRM).

AlphaLISA Acceptor beads and streptavidin-coated Donor beads (SA-Donor) were from Revvity (Cat # 6772002 and Cat # 6760002, respectively). The ChromaLink™ biotinylating reagent was obtained from SoluLink™ (Cat # B1001-105). Zeba Spin desalting columns were from Thermo Scientific (Cat # 89883). OptiPlate™-96 white opaque 96-well microplates were from Revvity (Cat # 6005290).

The assay buffer was composed of 50 mM Tris-HCl pH 7.75, 150 mM NaCl, 0.1% Tween-20, 0.5% BSA, and 0.05% bovine γ-globulin. The neutralization buffer was prepared by mixing the assay buffer with 1 M Tris-HCl pH 9.5 at a ratio of 30:70 (v/v).

Preparation of drug-conjugated AlphaLISA Acceptor beads

Drug immobilization on the AlphaLISA Acceptor beads was performed using a simple standard aldehyde group coupling procedure. Briefly, 0.025 mg of drug antibody, 0.0625% of Tween-20, 1.25 mg of AlphaLISA beads and 1.25 mg/mL of $NaBH_3CN$ were mixed together. The reaction volume was completed to 0.05 mL with 130 mM Na Phosphate pH 8.0 and the reaction was incubated for 18 hours at 37 ˚C. The reaction was stopped by the addition of 2.5 µL of a 65 mg/mL CMO solution and incubation for one hour at 37 ˚C. Beads were then washed twice by centrifugation for 15 minutes (14,000 rpm/4 ˚C) and the bead pellet was resuspended in 0.5 mL of 100 mM Tris pH 8.0. After a third centrifugation step, the beads were resuspended at 5 mg/mL in PBS pH 7.2 containing 0.05% Proclin-300.

Drug biotinylation

Drug biotinylation was performed with the ChromaLink biotinylating reagent using standard biotinylation and purification procedures. Briefly, 0.025 mg of antibody and 1.9 µL of biotinylating reagent (2 mg/mL) were mixed together at a 30:1 molar biotin/antibody ratio. The reaction volume was completed to 0.05 mL with PBS pH 7.4 and the reaction was incubated for 2 hours at 23 ˚C. Purification of the biotinylated antibody was performed using a Zeba 0.5 mL desalting column. The ratio of biotinylation of the final product and the protein recovery were determined from absorbance readings at 354 nM and 280 nM, respectively.

AlphaLISA ADA assay

The AlphaLISA ADA assay was performed as follows

In an OptiPlate-96 microplate, all samples (PC, blank and test samples) were diluted to 10% with acetic acid (600 mM) and then incubated for 60 minutes on a plate shaker set at 200 rpm in an incubator set at 23 ˚C. Then 50 µL of neutralization buffer containing 4 nM of biotinylated drug and 80 µg/mL of AlphaLISA drug-Acceptor beads was added to the plate. The plate was then incubated for 60 minutes on a plate shaker set at 200 rpm, in an incubator set at 23 ˚C. Finally 100 µL of SA-Donor beads (40 µg/mL) diluted in assay buffer was added to the plate which was then incubated for 60 minutes on a plate shaker set at 200 rpm, in an incubator set at 23 ˚C. After this incubation, the plate was read using the EnVision™ 2103 Multilabel Plate Reader and the AlphaLISA relative counts were measured.

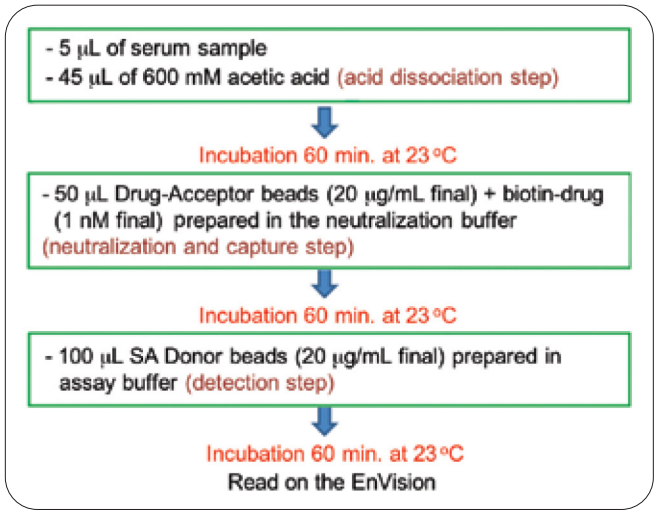

Figure 1: AlphaLISA assay protocol.

Assay configuration

The AlphaLISA ADA detection assay was developed using a bridging assay configuration. The assay is dependent on bivalent binding of anti-drug antibodies to biotinylated drug molecules captured by the Streptavidin (SA)-Donor beads and drug antibodies immobilized on the Acceptor beads. This bridging assay format eliminates the need for species-specific secondary antibodies. The presence of anti-drug antibodies will bridge the two drug molecules and, consequently, bring the two beads in close proximity.

Laser excitation of the Donor beads will provoke the release of singlet oxygen molecules that trigger a cascade of energy transfer in surrounding Acceptor beads, thus resulting in a time-delayed sharp peak of light emission at 615 nM.

Acid induced dissociation of immune complexes was implemented during sample preparation in order to improve drug tolerance.

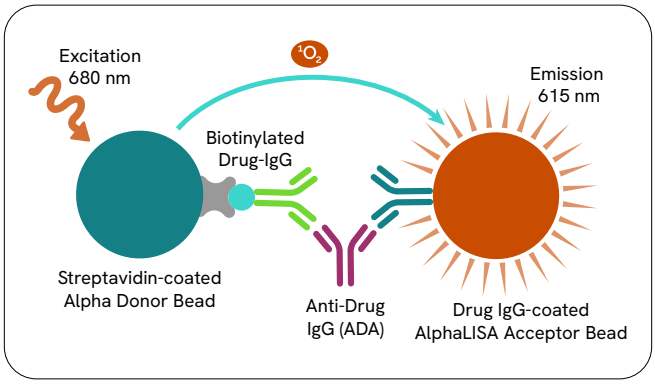

Figure 2: AlphaLISA bridging assay configuration.

Results

The AlphaLISA qualification study was performed at Charles River Laboratories Preclinical Services Montreal Inc. (Quebec, Canada) using a standardized approach for qualification of immunogenicity assays. The analytical method was subjected to the following experimental assessments to ensure it was suitable for its intended use: determination of the screening cut-point (CP), determination of the confirmatory assay cut-point (CCP), intra- and inter-assay precision, sensitivity, specificity, selectivity, drug tolerance and prozone effect.

For this study, a model system was used in which the drug is mimicked by a monoclonal mouse IgG. The Positive Control (PC) used in this procedure is an affinity purified Goat anti-mouse IgG antibody prepared at different concentration levels in neat pooled normal human serum (PNHS).

Screening cut-point

The screening cut-point (CP) was used to determine the threshold for identifying samples as negative (below the CP) or potential positive (equal or above the CP) for the presence of anti-drug antibodies. Fifty individual lots of normal human serum (25 males and 25 females) were analyzed each in duplicate on a total of 4 occasions performed by two analysts using a balanced design to collect mean relative counts values. On each occasion, the average values of the two corresponding relative counts measurements were used in the data analysis.

The normal distribution was assumed for the CP determination. The CP value for each occasion was determined to be the mean relative counts value of the individual serum lots plus 1.645 x SD, which represents the 95th percentile of a normal distribution. All serum lots with values higher than the 95th percentile on more than 50% of the CP determination occasions were removed from the calculation. They were defined as true or false positives based on the confirmatory assay results (see next section). The final CP value was calculated in the second iteration, using the remaining individual serum lots.

Twelve replicates of Blank prepared with the pooled normal human serum (PNHS) were included on each plate. The mean relative counts value of the 12 replicates, a correction factor (CF) and the overall mean relative counts value of PNHS were determined for each occasion. The mean CF value was used as normalization factor for all assays in Qualification. The plate specific cut-point (PSCP) was calculated by multiplying the mean CF value with the mean relative counts value of PNHS (Blank wells, n = 12).

Table 1: Screening cut-point determination. The CP was determined using 50 individual lots of PNHS, analyzed each in duplicate on a total of four occasions. The CP was calculated as the mean counts plus 1.645 x SD. Two samples were removed for the second iteration.

	1st iteration screening cut-point	2nd iteration screening cut-point
Mean (counts)	532	509
n	50	48
SD	127	55
Cut-Point (counts)	741	600
Mean Blank (counts)	470	470
Correction Factor	1.57	1.28

Two human serum lots were identified as outliers, since their mean relative count values were superior to the CP on all four occasions and were excluded from the final CP calculation. These lots were analyzed in the confirmatory assay and one lot was confirmed positive (true positive), whereas the other one was confirmed negative (false positive). The mean CF, defined at 1.280, was used in the qualification. The PSCP for each assay plate was determined in all subsequent experiments using the following formula:

PSCP = Mean Blank Relative Counts (RC) (after removal of maximum 2 outliers) x 1.280

Confirmatory cut-point

The confirmatory assay was validated to determine the CCP (% signal inhibition) which was used to verify whether samples identified as potentially reactive in the anti-drug antibody screening assay are confirmed positive or negative. CCP determination was performed in 4 occasions, by two analysts, using a balanced assay design. Fifty individual lots of normal human serum (i.e. same lots tested for CP in the previous section) were spiked with the drug and then analyzed in duplicates. The CCP blank and PCs were loaded only in one plate among the 4 plates. Two PC levels (LPC and HPC), Blank (PNHS) and the individual lots of human serum (same lots as used for the CP determination), were spiked with the drug at a final concentration of 25 µg/mL (spiked samples). The overall mean of PC and blank samples loaded on the plate and the individual lots of human serum analyzed for the CP determination on the same plate served as control unspiked samples. The spiked and unspiked samples were incubated for 1 hour at 23 °C and then tested. The % signal inhibition was determined for each assay control and serum lot, using the following formula:

% Signal Inhibition = [1-(spiked sample/control unspiked sample)] x 100

For calculation of the drug competition test cut-point, normal distribution of the data was assumed. On each occasion, the % signal inhibition was calculated as outlined above for each individual lot tested. Then the mean and SD were computed using the % inhibition of all the individual lots tested on a particular occasion. The mean +2.33 x SD, which represents the 99th percentile of a normal distribution, was calculated for each occasion. All serum lots with values higher than the 99th percentile on at least 50% of the occasions were identified as outliers and removed from the drug competition test cut-point calculation. The mean +2.33 x SD value for each occasion was calculated again in the second iteration using the remaining individual serum lots. Finally, the fixed drug competition cut-point obtained on each occasion was averaged to obtain the final drug competition test cut-point.

Acceptance criteria

The final drug concentration (25 µg/mL) should generate a reduction (% signal inhibition) higher than or equal to the CCP when comparing the mean relative counts of the drug-spiked PC samples (LPC and HPC) to the corresponding mean relative counts of the unspiked PC samples.

The final drug concentration (25 µg/mL) should generate a reduction (% signal inhibition) lower than the CCP when comparing the mean relative counts of the drug-spiked Blank to the corresponding mean relative counts of the unspiked Blank.

Table 2: Confirmatory cut-point. The CCP was determined using 50 individual lots of PNHS spiked with 25 µg/mL of the drug, analyzed each in duplicate on a total of four occasions. It was calculated as the mean of percentage inhibition plus +2.33 x SD. One sample was removed for the second iteration.

	1st iteration confirmatory cut-point	2nd iteration confirmatory cut-point
Mean	7.0	6.1
n	50	49
SD	10.5	8.4
Cut-Point (counts)	31.5	25.7

One human serum lot was identified as an outlier during the screening cut-point determination and produced % signal inhibition values higher than the 99th percentile on the four occasions; therefore it was excluded from the CCP calculation. The mean CCP calculated as the average of all the CCP obtained from all occasions was defined at 25.7%. The LPC and HPC spiked with 25 µg/mL drug final concentration generated a % inhibition superior to the CCP. The blank spiked with 25 µg/mL drug final concentration generated a reduction lower than or equal to the CCP.

Table 3: Controls for the CCP.

Control results for the CCP				
	Mean counts			Status with regards to CCP
Samples	Spiked	Unspiked	% inhibition	
LPC	590	1361	56.4	≥ CCP
HPC	9128	59928	84.4	≥ CCP
PHNS	469	451	-4.0	≥ CCP

Both positive control samples showed percentage of inhibition higher than the CCP confirming that these samples contain specific ADA.

Assay sensitivity and prozone effect

The assay sensitivity is relative to the characteristics of the positive controls used in the assay. In the present assay, the affinity-purified goat anti-mouse IgG antibody (PC) was used to evaluate the sensitivity of the assay.

The assay sensitivity was tested on six occasions, performed by two analysts using a balanced design, at the LPC concentration (15 ng/mL) prepared in PNHS. The assay sensitivity was evaluated based on the inter- and intra-assay precision results. Another indicator of method sensitivity is the assay's ability to detect approximately 5% false positives among the lots of human serum that were tested for CP determination and specificity assessment.

Acceptance criteria

The sensitivity was defined as the lowest PC concentration meeting the acceptance criteria described later for the inter- and intra-assay precision. The lowest PC sample that met the acceptance criteria was selected for the samples analysis and subsequent qualification assays. It should be noted that drug tolerance and drug competition results may justify selecting an alternative PC level even if the LPC meets the above mentioned criteria.

To investigate the possibility of assay deficiency at very high concentrations of ADA, the prozone effect was assessed. For the evaluation of the prozone effect, a sample was prepared by diluting a stock of affinity purified anti-mIgG antibody (PC) in PNHS at a concentration 10 times greater than the HPC. The sample was serially diluted (2-fold dilution) in PNHS to reach the PSCP and analyzed in one experiment in duplicate for each dilution, on one occasion.

Acceptance criteria

The mean counts value obtained for each prozone dilution higher than or equal to the PSCP should be greater than or equal to the overall mean relative counts value of the subsequent dilution.

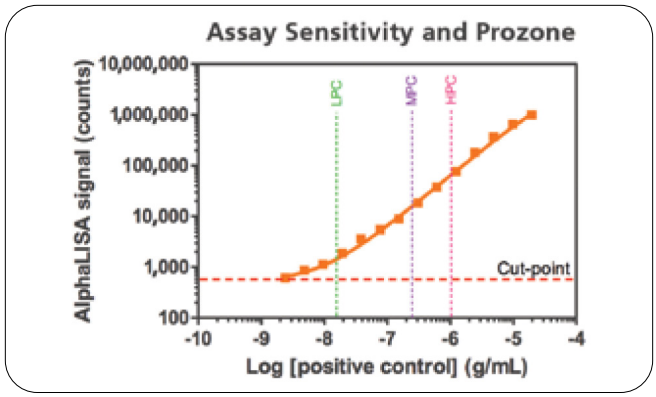

Figure 3: Prozone and sensitivity. The PNHS was spiked with increasing concentrations of the positive control antibody to generate ADA standard curves. Three PC concentrations were selected for the study (LPC, low level PC: 15 ng/mL, MPC, medium level PC: 250 ng/mL and HPC, high level PC: 1000 ng/mL). For this experiment, the plate specific cut-point (PSCP) was calculated at 576 counts.

The sensitivity tested at the LPC (15 ng/mL) met the inter-and intra-assay acceptance criteria (see intra- and inter-assay precision section) and consistently produced a positive signal (i.e. > PSCP) on all tested occasions. Based on the affinity purified goat anti-mouse IgG positive control, the assay sensitivity was set at the LPC (15 ng/mL in neat serum). The percentage of false positive (1 out of 50 lots tested; 2.0%) obtained in this qualification from the individual lots tested to define the screening CP and the specificity is in line with the recommended rate of 5% described by Mire-Sluis et al., 2004. No prozone effect was detected when the affinity purified rabbit anti-drug antibody (PC) was spiked in neat pooled normal human serum at a concentration of 20 µg/mL (20 times higher than HPC) and titrated in 2-fold serial dilutions. The mean RC value obtained for each prozone dilution was greater than or equal to the overall mean RC value of the subsequent dilution up to 20 µg/mL.

Drug tolerance

Samples containing circulating drug could exhibit assay interference due to competition between the circulating drug and the antigen in the assay system for anti-drug antibodies. In order to mimic the study, 2x-concentrated LPC and HPC samples were prepared in PNHS. Equal volumes of each PC level were then spiked with equal volumes of different drug concentrations (prepared 2 times more concentrated in PNHS: 400, 200, 100, 40, 20, 10 and 2 µg/mL). After mixing with the PC samples, the final tested drug concentrations in neat serum were 200, 100, 50, 20, 10, 5 and 1 µg/mL.

Corresponding unspiked samples were prepared by adding an equivalent volume of PNHS. All unspiked and spiked samples were incubated for 1 hour at 23 ˚C. All drug tolerance samples were tested at least once in duplicate on one occasion.

The % difference between the PC samples with and without the addition of drug was calculated using the following formula:

$$\% \text{ Difference} = \frac{(\text{Mean relative counts of spiked} - \text{Mean relative counts of unspiked}) \times 100}{\text{Mean relative counts of unspiked}}$$

Acceptance criteria

The drug tolerance was defined as the lowest concentration of drug that inhibits detection of the PC sample and that brings the mean relative counts value below the PSCP.

Table 4: Drug tolerance. The positive control antibody was diluted in PNHS and spiked with different amounts of drug (1, 5, 10, 20, 50, 100 and 200 µg/mL). The samples were incubated 1 hour before analysis to allow binding.

Samples	Final drug conc. in neat sample (µg/mL)	Mean counts	Status with regards to the PSCP
HPC (1000 ng/mL)	0	61217	-
	1	48465	≥ PSCP
	5	24918	≥ PSCP
	10	16774	≥ PSCP
	20	9747	≥ PSCP
	50	4118	≥ PSCP
	100	2318	≥ PSCP
	200	1109	≥ PSCP
LPC (15 ng/mL)	0	1202	-
	1	1057	≥ PSCP
	5	830	≥ PSCP
	10	686	≥ PSCP
	20	611	≥ PSCP
	50	526	< PSCP
	100	518	< PSCP
	200	498	< PSCP

The tested drug concentrations of 20 µg/mL at LPC level and 200 µg/mL at HPC level did not affect the detection of anti-mIgG in human serum. Since the LPC (15 ng/mL) was selected as the low PC level for this qualification assays, the drug tolerance was set at > 20 µg/mL drug in PNHS.

It should be noted that the drug tolerance evaluated using the PC is highly dependent on the affinity of the positive control and may not be representative for all study samples.

Specificity and selectivity

Ten individual lots of normal human serum (5 males and 5 females) were used for the assessment of specificity and selectivity. These individual lots were analyzed unspiked on one occasion for the specificity assessment.

For the evaluation of selectivity, each of the individual lots of human serum was spiked with the PC (affinity purified goat anti-drug antibody) at the following concentrations in neat human serum: 15 ng/mL (LPC), 250 ng/mL (MPC) and 1,000 ng/mL (HPC). In addition, PNHS was spiked at the LPC and HPC levels similarly to the individual lots of serum to serve as reference samples to calculate the recovery. The mean Relative Counts were compared between individual serum lots and the reference samples. The selectivity was evaluated on one occasion.

$$\% \text{ Difference (recovery)} = \frac{(\text{Mean RC of PC individual lot} - \text{Mean RC of PC PNHS}) \times 100}{\text{Mean RC of PC PNHS}}$$

Acceptance criteria

For the specificity, at least 80% of the unspiked human serum lots should be below the PSCP. True positive samples should be excluded from the specificity acceptance criteria.

For the selectivity, at least 80% of the individual lots of human serum spiked with the PC should be within ±25% difference of the corresponding PC prepared in PNHS. Individual normal human serum lots confirmed true positive during specificity assessment should be excluded from the selectivity acceptance criteria.

Ten individual normal human serum lots were analyzed unspiked for the assessment of specificity and spiked with the PC at LPC and HPC levels for the evaluation of selectivity. All 10 individual lots analyzed unspiked were below the PSCP and the specificity acceptance criteria were met.

The selectivity acceptance criteria were met at LPC and HPC levels, as 10 serum lots (100%) for LPC, and 8 serum lots (80%) for HPC were within ±25% difference from the corresponding PC samples prepared in PNHS.

Table 5: Assay specificity. Ten individual serum lots were analyzed unspiked and compared to the PSCP level.

	Specificity	
Serum Lot	Mean counts	Status with regards to PSCP
PNHS	466	-
Serum 1	482	< PSCP
Serum 2	464	< PSCP
Serum 3	406	< PSCP
Serum 4	474	< PSCP
Serum 5	472	< PSCP
Serum 6	558	< PSCP
Serum 7	555	< PSCP
Serum 8	582	< PSCP
Serum 9	518	< PSCP
Serum 10	543	< PSCP

Table 6: Assay selectivity. Ten individual serum lots were analyzed spiked with purified anti-drug antibody at LPC and HPC level and compared to control samples spiked in PNHS.

Selectivity (recovery)				
	LPC		HPC	
Serum lot number	Mean counts	% Difference	Mean counts	% Difference
PNHS	1243	-	49304	-
Serum 1	1179	-5.1	48355	-1.9
Serum 2	1346	8.3	56036	13.7
Serum 3	1171	-5.8	47750	-3.2
Serum 4	1154	-7.1	63536	28.9
Serum 5	1291	3.9	48484	-1.7
Serum 6	1281	3.1	63168	28.1
Serum 7	1265	1.8	47031	-4.6
Serum 8	1383	11.3	59847	21.4
Serum 9	1222	-1.7	43074	-12.6
Serum 10	1221	-1.8	55941	13.5

Intra- and inter-assay precision

The PC samples were assayed in replicates of 3 (n = 3), each one in duplicate, performed on six occasions by two analysts using a balanced design. The group mean, SD and coefficient of variation (%CV) of the mean relative counts obtained for each assay were calculated and used to determine the intra-assay precision. The group mean, SD and %CV of all the intra-assay mean relative counts values obtained over all occasions were calculated and used to determine the inter-assay precision. The data from this evaluation was used for the assessment of assay sensitivity. Precision, expressed as the %CV, was calculated using the formula:

$$\%CV = \frac{\text{Standard Deviation (SD)} \times 100}{\text{Mean Relative Counts}}$$

Inter- and intra-assay precision was evaluated by testing the following control samples:

i. Negative control sample of PNHS (Blank)

ii. PC samples at three concentrations levels (LPC: 15 ng/mL, MPC: 250 ng/mL, HPC: 1000 ng/mL).

Acceptance criteria

For the intra-assay precision to be acceptable, the mean relative counts of positive controls of 2 or more replicates at each level, on each occasion, should be greater than or equal to the blank mean relative counts and equal to or above the PSCP value (calculated retrospectively). The coefficient of variation (%CV) should be lower than or equal to 25% for all PCs and the global mean relative counts of LPC should be less than the global mean relative counts of MPC and less than the global mean relative counts of HPC.

For the inter-assay to be acceptable, the %CV of the mean relative counts of intra-assay means (at all assay levels) should be greater than or equal to 25%.

Table 7: Assay precision. Twelve independent experiments using triplicate data points were performed by two analysts using a balanced design to determine the intra- and interassay variability.

Samples	Mean counts	Mean intra-assay precision %CV	Inter-assay precision (n=12) %CV
PNHS	466	6.3	8.7
LPC	1339	4.3	5.8
MPC	14973	3.7	9.1
HPC	62538	6.7	9.0

The intra-assay precision results for all LPC, MPC and HPC samples were within the acceptance criteria (i.e. %CV was within 25% on all occasions at all levels). The global mean RC values of the PC samples (LPC, MPC and HPC) were greater than PSCP on all occasions, and the global mean RC value of LPC < the global mean RC value of MPC < the global mean RC value of HPC. The inter-assay precision results for the LPC (5.8%), MPC (9.1%) and HPC (9.0%) samples were within the acceptance criteria (i.e. %CV within 25%).

The assay low PC level was set at 15 ng/mL based on the drug interference data. The overall inter-assay precision for the blank samples, calculated for information purposes only, was ≤ 8.7%.

Conclusions

The results of this qualification study were the followings:

- The correction factor was established at 1.280.
- The CCP was established at 25.7% inhibition. The spiked LPC and HPC samples produced signal inhibition greater than 25.7% while spiked blank samples produced signal inhibition lower than or equal to 25.7%.
- The intra-assay and inter-assay precision met the acceptance criteria at all PC levels.
- The assay sensitivity was deemed to be 15 ng/mL in neat serum, based on the affinity purified Goat antimouse IgG. The calculated false positives rate was 2.0%, which is in line with the recommended range (i.e. 5%).
- The specificity and selectivity assessments met the acceptance criteria.
- The tested drug concentrations of 20 µg/mL at LPC, and 200 µg/mL at HPC level did not affect the detection of ADA in human serum.
- Since the LPC (15 ng/mL) was selected as the low PC level for this qualification assay, the drug tolerance was set at ≤ 20 µg/mL of drug in neat serum.
- No prozone effect was observed at up to 20 µg/mL of ADA in neat serum.
- During qualification, the assay failure rate was 0%.

In light of these results, the AlphaLISA method for the detection of ADA in human serum has been successfully qualified using a model system and standard qualification procedures in place at Charles River Laboratories.
The results of this study prove the excellent performance of the assay, which offers significant advantages over current platforms for immunogenicity screening assays: 1) Fully homogeneous, simple protocol, 2) Superior sensitivity, 3) High drug tolerance and 4) Excellent reproducibility.

Very importantly, the fully homogeneous AlphaLISA format eliminates the requirement for wash steps, shortening hands-on time to execute the assay and improving the likelihood for detection of low affinity anti-drug antibodies. This assay platform therefore represents an important analytical tool offering distinct advantages for immunogenicity testing applications.

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