Automation of a Homogeneous Proximity Assay for Immunogenicity Testing of Biological Drug Products

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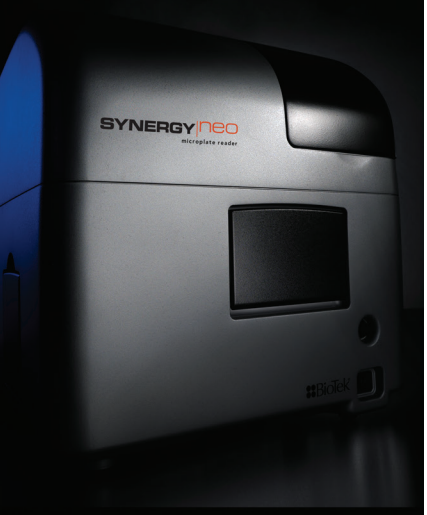

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Bridging Assay
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ADA

Introduction

Several challenges have surfaced during clinical evaluation of biological drug products due to a commonly associated immune response in patients. Anti-drug antibodies (ADA) are known to be frequently generated during administration of humanized monoclonal antibody therapeutics. These ADAs are nearly indistinguishable from antibody drug therapeutics thus requiring robust selective methods to determine the extent to which they impact safety and efficacy during treatment[1]. A commonly used technology platform for assessment of immunogenicity relies on the bridging immunogenicity assay format typical of the Enzyme-Linked Immunosorbent Assay (ELISA). Other methods have been used to provide simpler work flows and higher sensitivity, such as Electrochemiluminescence (ECL) assays using streptavidin ECL plates to create the classic bridging assay[2].

Here we present a homogeneous assay based on using a bridging assay format where all reagents and sample are in solution. This facilitates automation of reagent addition and simplifies the work flow without sacrificing sensitivity. Additionally, we compare the automation of the AlphaLISA® ADA assay with a solution ELISA ADA assay using liquid handling and dispensing instrumentation and a high performance multi-mode microplate reader which can be used to detect the presence of ADA activity in a model system[3]. The study will describe the assays, reagent preparation and automated methods used and will demonstrate several validation experiments comparing the performance of the two methods described above. Adaptation of the AlphaLISA ADA assay to be performed in a high-density 384-well format will also be described including serum screening data as well as additional analysis required for a full validation study using the AlphaLISA ADA assay technology with automated methods.

The AlphaLISA ADA assay utilizes bivalent binding of anti-drug antibodies to biotinylated drug which is then captured on streptavidin (SA)-coated Donor beads and drug antibody immobilized on Acceptor beads (Figure 1). The resulting complex is formed in the presence of ADAs resulting in the two beads coming into close proximity. Laser excitation of Donor beads at 680 nm results in singlet oxygen generation and as the beads are in close proximity, energy transfer to Acceptor beads is facile resulting in light emission at 615 nm. The formation of the complex in solution eliminates washing steps and secondary detection antibodies typically required with standard sandwich ELISA methods.

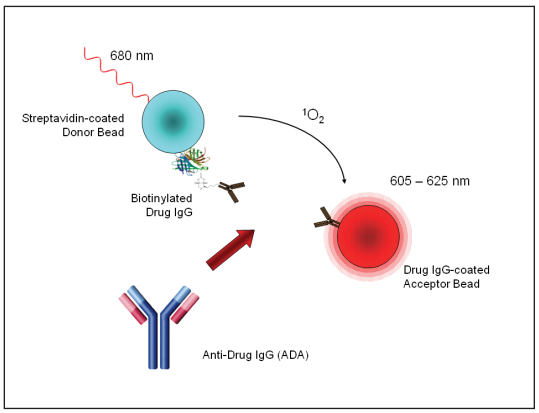

Figure 1. **Assay schematic for AlphaLISA used in the detection of host antibodies against biotechnology products.** Upon excitation, the AlphaLISA donor bead generates singlet oxygen molecules that trigger a cascade of energy transfer to an Acceptor bead, provided the Acceptor beads are in close proximity to the Donor beads.

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The solution ELISA also relies on bivalent binding of anti-drug antibodies to biotinylated- and digoxigenin-labeled drug. Upon complex formation, the complex is captured on a streptavidin-coated microplate (Figure 2). Assay quantification is accomplished by complex identification by an anti-digoxigenin monoclonal antibody HRP conjugate and subsequent measurement of chemiluminescent signal intensity.

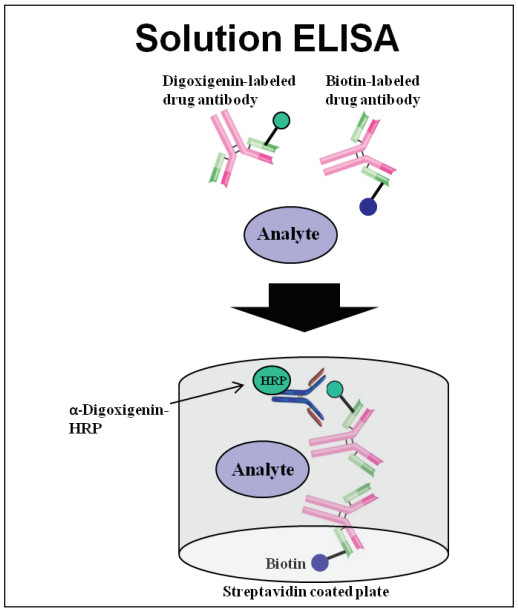


Figure 2. **Assay schematic for solution ELISA used in the detection of host antibodies against biotechnology products.** Similar to a standard ELISA, a solution ELISA assay relies on a bridging assay format. Dissimilarly, formation of labeled drug-ADA complexes occurs in solution. The use of biotin- and digoxigenin-labeled drug during complex formation allow capture and detection of the complex by use of a streptavidin coated plate and an anti-digoxigenin-HRP conjugate, respectively. Luminescent signal is generated during assay development for quantification.

Materials and Methods

Instrumentation

Precision™ Microplate Pipetting System

Precision is an affordable solution for automated 96- or 384-well microplate liquid handling. The instrument was used to transfer serum samples and controls from a master plate to assay plates, bulk addition of acid dissociation reagent, and replicate sample transfer from 96- to 384-well assay plate formats (AlphaLISA assay).

MultiFlo™ Microplate Dispenser

The MultiFlo Microplate Dispenser offers up to four reagents dispensed in parallel with one compact instrument. The instrument was used to dispense assay specific reagents to the 96- and 384-well assay plates.

ELx50™ Microplate Strip Washer

The ELx50 Washer is a flexible platform that provides a variety of washing capabilities. The washer was used for all wash steps associated with the solution ELISA assay workflow.

Synergy™ Neo HTS Multi-Mode Microplate Reader

The Synergy Neo is a high performance multi-mode microplate reader designed specifically for providing rapid analysis without compromising sensitivity. Synergy Neo possesses dual PMT optics for rapid ratiometric assays such as FRET, TR-FRET and fluorescence polarization and a dedicated laser for excitation of AlphaLISA assays. The Synergy Neo also incorporates BioTek's unique patented Hybrid Technology™ utilizing monochromator-based detection for ultimate wavelength flexibility.

Reagents

Pooled neat human serum (PNHS) and individual lots of human serum were purchased from Bioreclamation, LLC (Catalog No. HMSRM, Westbury, NY, USA). Anti-DIG-HRP (Catalog No. 200-032-156) and the positive control antibody, polyclonal goat anti-mouse IgG (Catalog No. 115-005-062) was purchased from Jackson Immunoresearch Labs., Inc. (West Grove, PA, USA). Goat anti-mouse HRP (Catalog No. 12349MI), Pierce ECL substrate (Catalog No. PI-32109), and Zeba Spin desalting columns (Catalog No. 89883) were purchased from Thermo Scientific (Waltham, MA, USA). Carboxy-methoxylamine (Catalog No. C13408), sodium cyanoborohydride (Catalog No. 152159), bovine γ-globulin (Catalog No. G5009) and Proclin-300 (Catalog No. 48912-U) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NHS-ChromaLink-biotinylating reagent (Catalog No. B1001-105) and ChromaLink Digoxigenin One-Shot Antibody Labeling Kit (Catalog No. B-9014-009K) was purchased from Solulink (San Diego, CA, USA). Mouse monoclonal IGg2b was purchased from AbD Serotec (Raleigh, NC, USA). AlphaLISA Streptavidin (SA) Donor (Catalog No. 6760002S) and Acceptor beads (Catalog No. 6772003) were purchased from PerkinElmer (Waltham, MA, USA).

Preparation of Drug-Conjugates

A portion of drug was either biotinylated, digoxigenin-labeled or conjugated to AlphaLISA Acceptor beads as per manufacturer's protocol as previously described[1]. Briefly, for biotinylation of drug antibody, NHS-ChromaLink-biotinylating reagent was used in PBS at a 30:1 molar ratio of biotin reagent to antibody to label 25 µg of mouse monoclonal IGg2b. Purification was performed using standard procedures and analyzed for labeling efficiency by absorbance spectroscopy using an Epoch™ Microplate Reader and Take3™ Micro-Volume Plate accessory (BioTek Instruments, Inc., Winooski, VT, USA) as previously described[4]. Additionally the purified product was assessed for purity by SDS-PAGE with silver staining (data not shown).

For digoxigenin labeling of drug antibody, ChromaLink Digoxigenin One-Shot Antibody Labeling Kit was used and analyzed as above by absorbance spectroscopy.

For conjugation of antibody to AlphaLISA Acceptor beads a coupling ratio of 50:1 (w/w) beads to antibody, 1 mg beads to 0.02 mg antibody (drug), was used at an antibody concentration of 0.5 mg/mL.

Assay Plates

AlphaLISA®

OptiPlate™ -384 white opaque 384-well microplates were from PerkinElmer (Catalog No. 6007299, Waltham, MA, USA).

Solution ELISA

Forty-eight Pierce Streptavidin Plates were from Thermo Scientific (Catalog No. 15502, Rockford, IL, USA).

Instrument Setting

The Synergy Neo HTS Multi-Mode Microplate Reader was used for all determination with the settings shown in Tables 1 and 2.

Synergy Neo Read Parameters (AlphaLISA)	
Mode	Alpha
Gain	200
Delay after plate movement	0 msec
Excitation time	80 msec
Delay after excitation	120 msec
Integration time	160 µsec
Read Height	8.00 mm

Table 1. AlphaLISA signal was read on the Synergy Neo. AlphaLISA reading parameters used in Gen5™ Data Analysis software.

Synergy Neo Read Parameters (Solution ELISA)	
Mode	LUM
Optics position	Top
Gain	225
Integration time	1 sec
Delay after plate movement	100 msec
Read Height	1.00 mm

Table 2. Luminescence signal was read on the Synergy Neo. Luminescence reading parameters used in Gen5 Data Analysis software.

AlphaLISA Assay Setup

The AlphaLISA assay was performed as previously described with the following modification[3]. Briefly, serum and control samples were subject to acid dissociation by addition of 600 mM acetic acid for 60 minutes at RT with shaking in a 96-well polypropylene microplate. Samples were then transferred to 384-well assay plate for the neutralization and capture step. The addition of 2x drug-acceptor bead (final concentration, 20 µg/mL) and biotin-drug (final concentration, 1 nM) mix was added followed by incubation at RT for 60 minutes w/shaking. SA Donor beads were added during the detection step to a final concentration of 20 µg/mL. For spiked sample during confirmatory cut point (CCP) determination experiments an additional 2 µL of drug/PNHS or PNHS was added to serum and control samples prior to the acidification step using the MultiFlo.

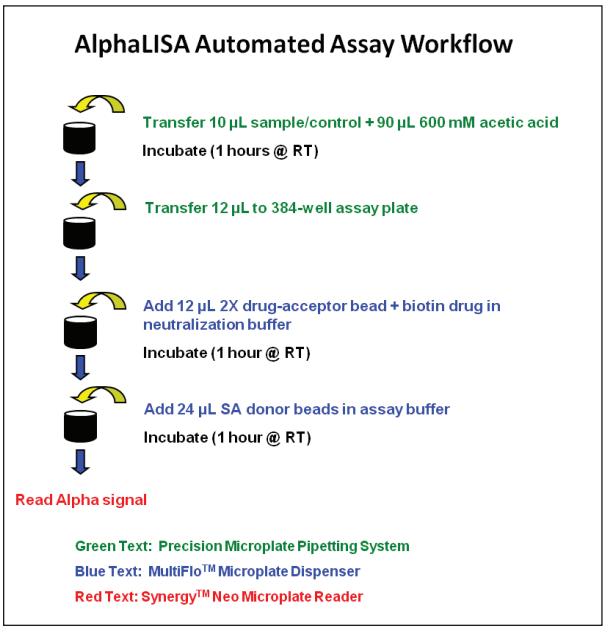


Figure 3. AlphaLISA Automated Assay Procedure. Transfer of sample/controls accomplished by Precision. Assay specific reagents dispensed using MultiFlo. Detection of AlphaLISA signal accomplished using the Synergy Neo.

Solution ELISA Assay Setup

The solution ELISA assay was performed as previously described[2]. Briefly, serum and control samples were subject to acid dissociation by addition of 80 mM acetic acid for 30 minutes at RT with shaking in a 96-well polypropylene microplate. Samples were then transferred to 96-well assay plate for the neutralization and labeling step. The addition of 3X labeling mix (1 M Tris-HCl, pH=8.0, 3 µg/mL biotin-drug and 3 µg/mL DIG-drug) was followed by incubation overnight at 4°C. The samples were then transferred for capture to streptavidin coated assay plates prewashed 3 x 200 µL PBS/0.1% Tween20 followed by incubation at RT for 60 min. The plate was washed 4 x 300 µL PBS/0.1% Tween20 followed by addition of anti-DIG HRP conjugate diluted 1:40,000 in PBS-casein buffer and incubated at RT for 2 hours.

The final wash was 4 x 300 µL PBS/0.1% Tween20 followed by addition of 100 µL ECL luminescent reagent. For spiked sample during CCP determination experiments an additional 2 µL of drug/PNHS or PNHS was added to serum and control samples prior to acidification step using the MultiFlo.

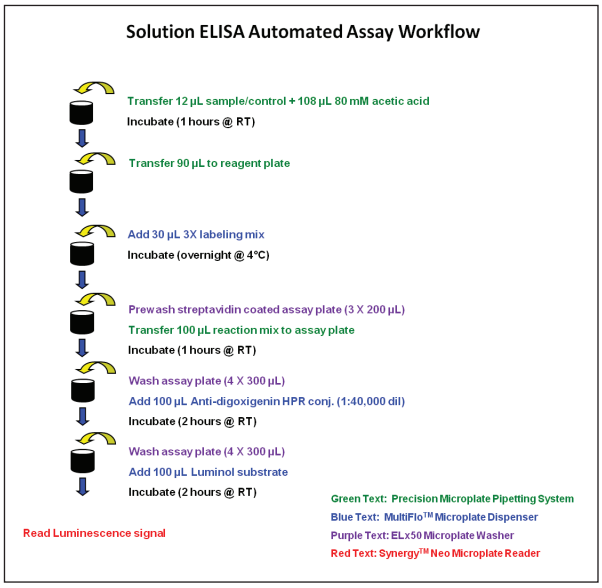


Figure 4. Solution ELISA Automated Assay Procedure. Transfer of sample/controls accomplished by Precision. Assay specific reagents dispensed using MultiFlo. Plate washing was accomplished by ELx50. Detection of luminescence signal accomplished using the Synergy Neo.

Results and Discussion

Z'-Factor Determination

Twenty-four replicates of either HPC or PNHS were assayed to determine the Z'-factor for both assay formats. The calculated Z'-factors were 0.74 and 0.57 for the AlphaLISA and solution ELISA assay formats, respectively where a Z'-factor >0.5 is indicative of a robust assay. The AlphaLISA assay format appears to provide significantly less variability when compared to the solution ELISA format with the added benefit of a simpler workflow.

Screening Cut-Point

The screening cut-point (CP) is used for determination of a threshold for identification of a sample as either negative (<CP) or potentially positive (≥CP) for presence of ADAs[1]. Analysis of 50 lots of normal human serum (NHS, 25 male, 25 female) was performed. Blanks were prepared with pooled normal human serum (PNHS) and the average of the replicates was used; a normal distribution was assumed.

CP was calculated as follows:

CP = mean + 1.645 x SD (95th percentile)

Correction factor was calculated as follows:

CF = CP/ Mean Blank (counts)

Serum lots with values higher than CP on greater than 50% of CP determination occasions were removed from the second iteration calculation and analyzed in the confirmatory assay as true or false positives. The final CP was calculated using the remaining lots (Table 3).

A

Screening Cut-Point (AlphaLISA)	1st interation Screening Cut-Point	2nd interation Screening Cut-Point
Mean counts	11891	11723
n	50	46
SD	921	749
Cut-Point (counts)	13406	12954
Mean Blank (counts)	11149	11149
Correction Factor	1.20	1.16

B

Screening Cut-Point (Solution ELISA)	1st interation Screening Cut-Point	2nd interation Screening Cut-Point
Mean RLUs	9306	5207
n	50	46
SD	15624	1438
Cut-Point (RLUs)	35009	7572
Mean Blank (RLUs)	5797	5797
Correction Factor	6.0	1.3

Table 3. Screening Cut-Point Determination. A. AlphaLISA determination of CP using 50 individual lots of NHS, analyzed in quadruplicate on a total of two occasions. Four samples were removed for the second iteration. B. Solution ELISA determination of CP using the same 50 individual lots of NHS, analyzed on a total of two occasions. Four samples were removed for the second iteration.

Determination of the second iteration CP resulted in a correction factor (CF) determinant of 1.16 and 1.3 for AlphaLISA and solution ELISA, respectively. The plate specific cut-point (PSCP) is determined in all subsequent experiment as follows:

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

Confirmatory Cut-Point

Determination of the confirmatory cut-point (CCP) (% signal inhibition) is used to confirm positives or negatives of samples identified as potentially reactive in CP screening[3]. The same 50 individual lots used in CP screening were spiked with the drug and analyzed. CCP blanks and positive controls (PCs), spiked and unspiked, were present on each plate. Two PCs, low PC (LPC) and high PC (HPC), Blank (PNHS), and individual lots were spiked with 25 µg/mL drug. All samples were run concurrently with unspiked samples used in the PC determination. The overall mean of sample, PC and Blanks were calculated for both spiked and unspiked samples.

The percent signal inhibition was calculated as follows:

% Signal Inhibition= [1-(spiked sample or control/ unspiked sample or control)] x100

The mean and standard deviation were calculated using the percent inhibition of all the lots in each experiment.

The CCP was then calculated as follows:

CCP=mean +2.33 x SD (99th percentile)

Lots with a percent inhibition greater than the CCP were removed for the second iteration calculation. The final CCP used for final drug competition test cut-point was the result of the first iteration calculation as the percent inhibition for all lots fell below that of CCP (Table 4).

Acceptance criteria requires percent inhibition of HPC>LPC>CCP>Blanks (PNHS) (Table 5).

A

Confirmatory Cut-Point (AlphaLISA)		
	1st interation Confirmatory Cut-Point	2nd interation Confirmatory Cut-Point
Mean	-0.9	N/A
n	50	N/A
SD	6.7	N/A
Cut-Point	14.7	N/A

B

Confirmatory Cut-Point (Solution ELISA)		
	1st interation Confirmatory Cut-Point	2nd interation Confirmatory Cut-Point
Mean	14	10
n	50	47
SD	21	12
Cut-Point	62	39

Table 4. Confirmatory Cut-Point. A. AlphaLISA determination of CCP using 50 individual lots of NHS, both unspiked and spiked with drug, analyzed in quadruplicate on a total of two occasions. No samples were removed for the second iteration. B. Solution ELISA determination of CCP using the same 50 individual lots of NHS, both unspiked and spiked with drug, analyzed on total of two occasions. Three samples were removed for the second iteration.

A

Control results for the CCP (AlphaLISA)				
	Mean Counts			Status with
Samples	Spiked	Unspiked	% Inhibition	regards to
LPC	17094	33817	49.5	≥ CCP
HPC	38727	128609	69.9	≥ CCP
PNHS	11052	11149	0.9	< CCP

B

Control Results for the CCP (Solution ELISA)				
	Mean RLUs			Status with
Samples	Spiked	Unspiked	% Inhibition	regards to
LPC	7965	18883	57.8	≥ CCP
HPC	30162	140296	78.5	≥ CCP
PNHS	4703	5797	18.9	< CCP

Table 5. Results of Controls for CCP. A. Controls for CCP determination for. B. Solution ELISA.

Four human serum lots were identified as outliers during CP analysis for each assay method. The four lots identified in the AlphaLISA assay produced percent inhibition signal values lower than the 99th percentile and were therefore included in the CCP calculation. Three of the four lots identified in the solution ELISA assay produced percent inhibition signal values higher than the 99th percentile and were therefore excluded in the CCP calculation. False positives due to non-specific binding (NSB) were identified at a rate of 20% and 2%, for AlphaLISA and solution ELISA, respectively. The CCPs calculated were 14.7% and 39%, for AlphaLISA and solution ELISA, respectively. The LPC, HPC and PNHS spiked with 25 µg/mL drug meet the acceptance criteria described above for both assays.

Drug Tolerance

Drug tolerance determines the extent to which circulating drug interferes with assay performance due to competition between circulating drug and the assay antigen for ADAs. Each PC was prepared at a 2X concentration and spiked with a 2:1 serial dil. of drug at 2X the final assay concentrations of 0-200 µg/mL in PNHS. Unspiked samples were also prepared and all were incubated for 1 hr at RT. For the AlphaLISA assay format each PC was tested in quadruplicate, while for the solution ELISA assay format, each PC was tested in duplicate (Table 6). The percent difference was calculated for PC both with and without drug as follows:

% Difference= ((Mean relative counts of spiked –Mean relative counts unspiked sample) x100)/Mean relative counts of unspiked

A

Samples	Final drug conc. in neat sample (µg/mL)	Mean counts	Status with regards to the PSCP
HPC (1000 ng/mL)	0.0	129202	-
	0.4	111454	≥PSCP
	0.8	100623	≥PSCP
	1.6	88553	≥PSCP
	3.1	64844	≥PSCP
	6.3	47014	≥PSCP
	12.5	33701	≥PSCP
	25.0	22179	≥PSCP
	50.0	14013	≥PSCP
	100.0	12041	≥PSCP
	200.0	10372	< PSCP
LPC (100 ng/mL)	0.0	20073	-
	0.4	18119	≥PSCP
	0.8	18449	≥PSCP
	1.6	17405	≥PSCP
	3.1	15011	≥PSCP
	6.3	14478	≥PSCP
	12.5	12457	≥PSCP
	25.0	11658	≥PSCP
	50.0	10093	< PSCP
	100.0	10258	< PSCP
	200.0	9090	< PSCP

B

Samples	final drug conc. in neat sample (µg/mL)	Mean RLUs	Status with regards to the PSCP
HPC (1000 ng/mL)	0.0	1111870	-
	0.4	942404	≥PSCP
	0.8	814129	≥PSCP
	1.6	641684	≥PSCP
	3.1	487839	≥PSCP
	6.3	339779	≥PSCP
	12.5	165670	≥PSCP
	25.0	76457	≥PSCP
	50.0	46153	≥PSCP
	100.0	23789	< PSCP
	200.0	13975	< PSCP
LPC (100 ng/mL)	0.0	149602	-
	0.4	143289	≥PSCP
	0.8	124244	≥PSCP
	1.6	109111	≥PSCP
	3.1	78063	≥PSCP
	6.3	55926	≥PSCP
	12.5	34973	≥PSCP
	25.0	23786	< PSCP
	50.0	16496	< PSCP
	100.0	11287	< PSCP
	200.0	9016	< PSCP

Table 6. Drug Tolerance. **A.** Drug tolerance was determined using quadruplicate determinants on one occasion using the AlphaLISA assay format and compared to the PSCP. **B.** Solution ELISA drug tolerance was examined using duplicate data points on one occasion and compared to the PSCP.

Drug tolerance can be defined by the lowest concentration of drug inhibiting detection of the PC and has mean relative counts below the PSCP. For the AlphaLISA assay, the drug concentration of 25 µg/mL at the LPC and 100 µg/mL at the HPC did not show interference. For the solution ELISA assay, the drug concentration of 12.5 µg/mL at the LPC and 50 µg/mL at the HPC did not show interference. Thus, for both assay formats the LPC was set at 100 ng/mL and drug tolerance was set at >25 µg/mL and >12.5 µg/mL for AlphaLISA and solution ELISA, respectively.

Assay Sensitivity

The assay sensitivity evaluates the characteristics of the PC to determine the lowest concentration meeting the acceptance criteria for inter- and intra-assay precision, described below, as well as the ability to detect ~5% false positives among samples. To evaluate assay sensitivity an 11-point 1:2 serial titration of the PC at 10x the HPC (10 µg/mL) and a zero point in PNHS were prepared to generate ADA standard curves (Figure 5).

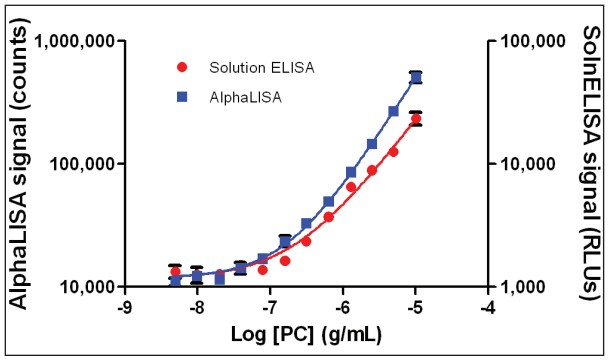
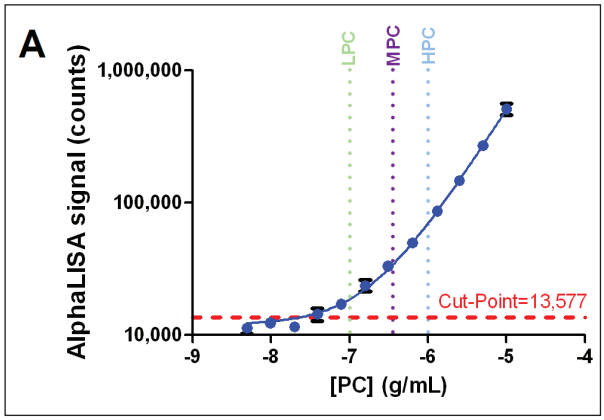


Figure 5. Assay Sensitivity. Assay sensitivity was assessed by performing an 11-point 1:2 serial dilution of the positive control starting at a concentration of 10x the HPC plus a zero concentration point.

As can be seen in Figure 5, a shift in the PC dilution series curve to the left at higher PC concentrations when performing the ADA assay in AlphaLISA format, as compared to solution ELISA, is indicative of slightly enhanced assay sensitivity. However, the enhancement diminishes at lower concentration and the limit of detection is nearly identical when comparing the two methods as can be determined from the nearly identical inflection points of the two curves at ~$1x10^{-7}$ g/mL PC.

Prozone Effect

The prozone effect evaluates assay performance at very high ADA concentrations. To evaluate the prozone effect an 11-point 1:2 serial titration of the PC, described above, beginning at 10x the HPC (10 µg/mL) including a zero point in PNHS, was used (Figure 6).



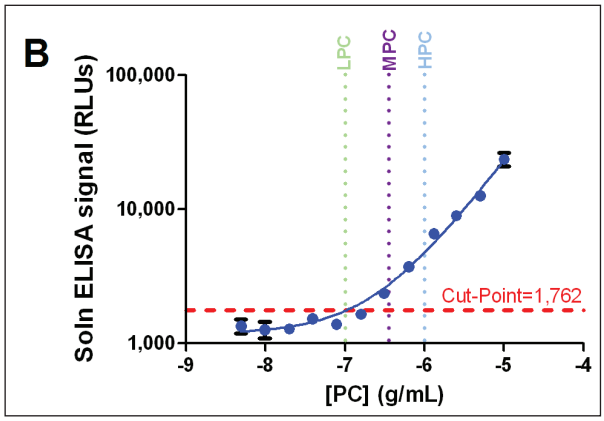


Figure 6. Assessment of Prozone Effect. The prozone effect was assessed by performing an 11-point 1:2 serial dilution of the positive control starting a concentration of 10x the HPC plus a zero concentration point. **A)** AlphaLISA and **B)** Solution ELISA.

No prozone effect was detected when the PC was spiked in PNHS at 10x the HPC and serial diluted as can be determined by the nearly linear response at very high PC concentrations (Figure 6). From this standard curve three PC concentrations can be selected for determination of inter- and intra-assay precision, drug tolerance, and assay specificity and selectivity. Identical PC concentrations were selected for comparison of the two assay methods.

Inter- and Intra-Assay Precision

The data from inter- and intra-assay precision testing is used in conjunction with the above Assay Sensitivity data for determination of assay sensitivity. Three PC concentrations were selected at 100, 350, and 1000 ng/mL (LPC, MPC and HPC) for sensitivity testing to determine the concentration meeting the inter- and intra-assay acceptance criteria and then used to set the LPC.

For the AlphaLISA assay format each PC was tested in quadruplicate on six (6) occasions whereas for the solution ELISA assay format, each PC was tested in quadruplicate on two (2) occasions. The group mean, SD and %CV were calculated for each experiment and used to determine intra-assay precision (Table 7).The group mean, SD and %CV of all experiments were calculated and represent inter-assay precision (Table 7). Acceptance criteria requires that the RC of PCs of two (2) or more replicates ≥ blank mean RCs and PSCP (calculated retrospectively), %CV ≤25% for all PCs, and global mean RCs of LPC ≤ MPC and HPC.

A **Intra- and Inter-assay Precision (AlphaLISA)**

Samples	Mean counts	Mean intra-assay precision %CV	Inter-assay precision (n=6) %CV
PNHS	10502	6.1	6.8
LPC	20507	6.2	7.9
MPC	47073	3.8	5.0
HPC	269873	6.3	7.8

B **Inter- and Intra-assay Precision (Solution ELISA)**

Samples	Mean counts	Mean intra-assay precision %CV	Inter-assay precision (n=6) %CV
PNHS	7579	3.2	9.9
LPC	14473	10.3	15.3
MPC	45114	9.0	17.5
HPC	197304	10.4	12.7

Table 7. Assay Precision. A. The inter- and intra-assay variability was analyzed using quadruplicate determinants on six (6) occasions using the AlphaLISA assay format. B. Solution ELISA inter- and intra-assay variability was examined using quadruplicate data points on two (2) occasions.

As can be seen in Table 7, the assay precision results for all PCs were within the acceptable limits as defined above. The PSCPs were calculated for each method at 12,202 counts and 11,258 RLUs for AlphaLISA and Solution ELISA, respectively.

Assay Specificity and Selectivity

The Assay specificity and sensitivity data was only determined for the AlphaLISA assay format. Assay specificity and sensitivity was determined by analyzing 10 individual normal human serum lots (5 male, 5 female). For specificity testing each individual lot was tested unspiked (Table 8). For sensitivity testing each individual lot was spiked with PC in PNHS at LPC (100 ng/mL), and HPC (1000 ng/mL) concentrations (Table 9). Each lot was tested in quadruplicate on one occasion for the AlphaLISA assay format. Controls were prepared by spiking LPC and HPC in PNHS.

For selectivity, the individual lots were compared to reference samples by calculating percent difference as follows:

% Difference (recovery) = ((Mean RC of PC–Mean RC of PC PNHS) x100)/Mean RC of PC PNHS

Specificity

Serum Lot #	Mean counts	Status with regards to the PSCP
PNHS	8013	-
Serum 1	8672	< PSCP
Serum 2	12206	≥PSCP
Serum 3	9086	< PSCP
Serum 4	9121	< PSCP
Serum 5	8509	< PSCP
Serum 6	9131	< PSCP
Serum 7	8912	< PSCP
Serum 8	8627	< PSCP
Serum 9	9892	≥PSCP
Serum 10	8601	< PSCP

Table 8. Assay Specificity. Assay specificity was determined by analyzing10 individual serum lots (5 male, 5 female) on one occasion using quadruplicate determinants.

The acceptance criterion for specificity requires at least 80% of unspiked human serum lots be below the PSCP. This requirement was met with 80% of the serum lots being below the PSCP.

Selectivity (recovery)

Serum Lot #	LPC		HPC	
	Mean counts	% Difference	Mean Counts	% Difference
PNHS	13707	-	80574	-
Serum 1	18040	31.6	67234	-16.6
Serum 2	20833	52.0	97805	21.4
Serum 3	15339	11.9	71596	-11.1
Serum 4	15253	11.3	86548	7.4
Serum 5	14751	7.6	82986	3.0
Serum 6	16669	21.6	84531	4.9
Serum 7	11674	-14.8	80810	0.3
Serum 8	15794	15.2	82003	1.8
Serum 9	15573	13.6	85979	6.7
Serum 10	14493	5.7	85035	5.5

Table 9. Assay Sensitivity. Assay selectivity was determined by comparing 10 individual serum lots (5 male, 5 female) spiked with LPC or HPC on one occasion to the reference sample using quadruplicate determinants.

The acceptance criteria for selectivity requires at least 80% of individual human serum lots spiked with PC lie within ±25% difference of the corresponding PC in PNHS. The selectivity requirements were met at the LPC (80%) and HPC (100%).

Conclusion

The study demonstrated assay robustness by determination of the Z'-factor. A determination of ≥0.5 is indicative of robust assay performance. While the resulting Z'-factors of 0.74 and 0.57 for the AlphaLISA assay and solution ELISA formats, respectively, indicate robust assay performance for both formats, significantly less variability is seen for the AlphaLISA when compared to the solution ELISA assay format.

When tested in the AlphaLISA assay format, drug concentrations of 25 µg/mL at LPC and 100 µg/mL at HPC showed no adverse affect on detection of ADA in pooled neat human serum (Table 3). For the solution ELISA assay, drug concentrations of 12.5 µg/mL at the LPC and 50 µg/mL at the HPC showed no interference (Table 3). Thus, for both assays the LPC was set at 100 ng/mL and drug tolerance set at >25 µg/mL and 12.5 µg/mL for AlphaLISA and solution ELISA, respectively. The prozone effect was investigated to determine if the assay may be affected by very high concentrations of ADA; typically evaluated at 10 times the HPC concentration. No prozone effect was detected as noted by a nearly linear response of the ADA standard curves up to the highest concentration evaluated for both assay formats (Figure 5).

The assay sensitivity was investigated based on the characteristics of the positive control; in this model system the affinity-purified goat anti-mouse IgG antibody was used for this purpose. Assay sensitivity is determined as described above based on the lowest concentration meeting the acceptance criteria for inter- and intra-assay precision, drug tolerance data, as well as, the ability to detect ~5% false positives among samples; with detection of false positives calculated retrospectively. Both the AlphaLISA and solution ELISA assay formats met the acceptance criteria at all PC levels for intra- and inter-assay precision with %CV of mean RCs within 25% (Table 7).

Together these data indicate that both the AlphaLISA and solution ELISA assay format workflows can be simplified by integrating automated methods with good results. The AlphaLISA assay provides for increased throughput and decreased overall time requirements when compared to the Solution ELISA. Furthermore, considerable reagent savings can be achieved when scaling the AlphaLISA ADA assay to be performed in a 384-well format. This can be extremely important when working with precious small quantities of labeled drug samples during drug development and screening applications.

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