State-of-the-Art Technologies for the Detection of Anti-Drug Antibodies in Serum Samples

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1 Abstract

Immunogenicity testing of therapeutic proteins is an essential step of the drug development process. There is a definite need for rapid and sensitive assay platforms for the detection of anti-drug antibodies (ADA), as current methods exhibit limitations related to assay sensitivity, robustness and drug tolerance. We report here the development of AlphaLISA® and DELFIA® bridging assays for the detection of ADA in serum samples. The use of these technologies in immunogenicity testing was successfully demonstrated with model antibody systems. We demonstrate here with a full qualification study that the AlphaLISA mix-and-read homogeneous assay permits the sensitive detection of drug antibody 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. The sensitivity of the DELFIA bridging assay also makes it a valuable technique for immunogenicity testing.

2 Materials & Methods

AlphaLISA qualification study

The AlphaLISA qualification study was performed at Charles River Laboratories Preclinical Services Montreal Inc. (Quebec, Canada) using a standardized approach for qualifications of immunogenicity assays.

Model description

- **Drug**: mouse monoclonal IgG2b antibody (AbD Serotec #MCA2472).
- **ADA**: the positive control antibody used in these studies was a polyclonal goat anti-mIgG antibody (Jackson ImmunoResearch #115-005-062).

- Matrix: pooled normal human serum and individual lots of normal human serum (Bioreclamation #HMSRM)
- AlphaLISA Acceptor beads and streptavidin-coated Donor beads were from PerkinElmer (#6772002 and #6760002, respectively).
- 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.
- OptiPlate™-96 white opaque 96-well microplates were from PerkinElmer #6005290

- Drug immobilization on the AlphaLISA Acceptor beads was performed using a simple standard aldehyde groups coupling.
- Drug biotinylation was performed with a NHS linker (SoluLink #B1001-105) using standard biotinylation and purification procedures.

DELFIA feasibility study

The DELFIA feasibility study was performed at PerkinElmer.

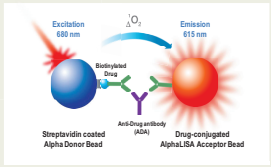
Model description

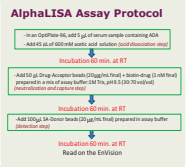
- **Drug**: mouse polyclonal IgG antibody (Jackson ImmunoResearch #015-000-003).
- **ADA**: the positive control antibody used in these studies was a polyclonal goat anti-mIgG antibody (Jackson ImmunoResearch #115-005-062).
- Human serum was purchased from Lonza #14-402E.

- DELFIA assay buffer was from PerkinElmer #4002-0010
- DELFIA Wash Solution was from PerkinElmer #1244-114
- DELFIA Enhancement Solution was from PerkinElmer #1244-105

- Streptavidin-coated 384-well plates were from PerkinElmer #CC11-H10
- The drug molecules were conjugated with Europium chelate using the Eu-N1-ITC product (PerkinElmer #1244-301) according to standard labeling procedures.
- Drug biotinylation was performed as described previously for the AlphaLISA study.

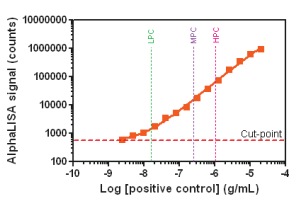
3 AlphaLISA ADA Detection Assay





The drug was covalently attached to the AlphaLISA Acceptor beads, and was also biotinylated and captured by Streptavidin coated Donor beads. Divalent anti-drug antibodies (ADA) simultaneously bind to the labeled drug molecules and consequently create a bridge between the two beads. A specific AlphaLISA signal is then produced as a result of the close proximity of the two beads: laser excitation of the Donor beads provokes 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.

4 Assay Sensitivity and Prozone



The pooled normal human serum (PNHS) was spiked with increasing concentrations of the positive control antibody to generate ADA standard curves. Highly intense signals and excellent sensitivity (4.9 ng/mL neat serum) were obtained. Three positive control (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). The sensitivity and prozone plate specific cut-point (PSCP) was calculated at 576 counts. The signal was still proportional to positive control antibody at a level 10-fold higher than the HPC, indicative of the high dynamic range of the assay.

5 Screening Cut-Point & Confirmatory Assay Cut-Point Determination

Parameters	1st iteration		2nd iteration	
	Screening Cut-Point	Confirmatory Cut-Point	Screening Cut-Point	Confirmatory Cut-Point
	counts	% inhibition	counts	% inhibition
Mean	532	7.0	509	6.1
n	50	50	48	49
SD	127	10.5	55	8.4
Cut-Point	741	31.5	600	25.7
Mean Blank	470	-	470	-
Correction Factor	1.57	-	1.28	-

Samples	Control results for the CCP			
	Mean Counts		% Inhibition	Status with regards to CCP
	Spiked	Unspiked		
LPC	590	1361	56.4	≥ CCP
HPC	9128	89928	84.4	≥ CCP
PNHS	469	451	-4.0	< CCP

The screening cut-point (CP) was determined using 50 individuals lots of normal human serum, analyzed each in duplicate on a total of four occasions. The CP was calculated as the mean counts plus 1.645xSD. On each occasion, replicates of the PNHS were included and a correction factor was calculated, corresponding to the ratio between the CP and the mean counts of PNHS. Outliers (defined as lots with mean counts or % inhibition above the CP or CCP on more than 50% of the occasions) were removed for the 2nd iteration calculations. The 2nd iteration correction factor was used as a normalization factor for all assays in the qualification.
The confirmatory cut-point (CCP) was determined using the same 50 lots as for the CP, except that each lot was spiked with 25 µg/mL of the drug. The CCP was calculated as the mean of the percentage inhibition plus 2.33xSD. The LPC and HPC positive control samples were included to confirm the CCP value. Outliers were also removed for the 2nd iteration calculations.

6 Drug Tolerance

Samples	Final drug concentration 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	685	≥ PSCP
	20	611	≥ PSCP
	50	525	< PSCP
	100	518	< PSCP
	200	498	< PSCP

Samples containing circulating drug could exhibit assay interference, as a result of the competition between the circulating drug and the labeled drug of the assay system for binding to anti-drug antibodies. In order to mimic biological samples, the positive control antibody was diluted in PNHS and spiked with different amounts of drug. The samples were incubated 1 hour before analysis to allow for binding. Our results indicated that the assay could tolerate up to 20 µg/mL of free drug at the LPC level, whereas the HPC sample still produced a signal above the PSCP in the presence of 200 µg/mL of free drug. This result demonstrates that the AlphaLISA ADA detection assay exhibits an excellent drug tolerance.

7 Specificity & Selectivity

Serum Lot	Specificity	
	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

Serum Lot Number	Selectivity (recovery)			
	LPC		HPC	
	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	-3.1
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	13.4

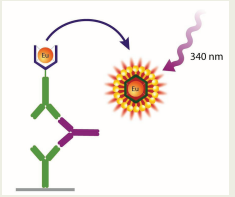
The specificity was determined using ten lots of normal human serum. These individual lots were analyzed unspiked and compared to the PSCP level. All unspiked samples were below the PSCP level, indicating a good assay specificity.
The selectivity was determined using the same lots of serum, except that each lot was spiked with the LPC or HPC concentrations. In addition, PNHS was spiked with the LPC and HPC levels to serve as reference for recovery determination. The mean counts were compared between individual serum lots and the reference samples. We observed 18 samples of 20 with count levels within a 25% difference of the corresponding PC prepared in PNHS, reflecting outstanding assay selectivity.

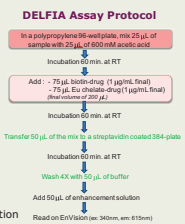
8 Assay Reproducibility

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

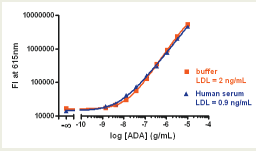
Twelve independent experiments using triplicate data points were performed by two analysts using a balanced design to determine the reproducibility of the assay. The group mean, SD and coefficient of variation (%CV) of the mean counts obtained for each assay were calculated and used to calculate the intra-assay precision. The group mean, SD and %CV of all the intra-assay mean counts obtained over all occasions were calculated and used to determine the inter-assay precision. As shown in the table above, %CV values lower than 10% were obtained for all conditions, indicating a robust and reproducible assay.

9 DELFIA ADA Detection Assay

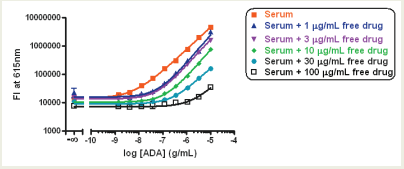




DELFIA is a proven, robust immunodetection platform based on time-resolved fluorescence (TRF) detection in a standard sandwich format.



As shown in the above standard curves, the DELFIA assay delivers very good sensitivity (2 ng/mL) and is not affected by the presence of human serum.



Serum samples were spiked with increasing concentrations of ADA and a fixed concentration of free drug, and were incubated for 2 hours before the assay. A high drug tolerance was obtained where a specific signal was detected in the presence of 30 µg/mL of free drug.

10 Summary

An AlphaLISA assay has been successfully qualified for the detection of anti-drug antibodies in human serum samples, using a model system and standard qualification procedures in place at Charles River Laboratories. The results of this study proves 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 homogenous 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.

In addition, we demonstrated the feasibility of using the DELFIA platform for ADA detection. DELFIA is a simple and cost-effective alternative to standard ELISA. Our results showed excellent sensitivity, full serum compatibility and very good drug tolerance. The DELFIA technology offers promising characteristics that could meet or exceed the requirements for immunogenicity testing.

Those two assay platforms are therefore important analytical tools offering distinct advantages for immunogenicity testing applications.