APPLICATION NOTE

Development of AlphaLISA technology assays for the detection of neutralizing antibodies in human serum.

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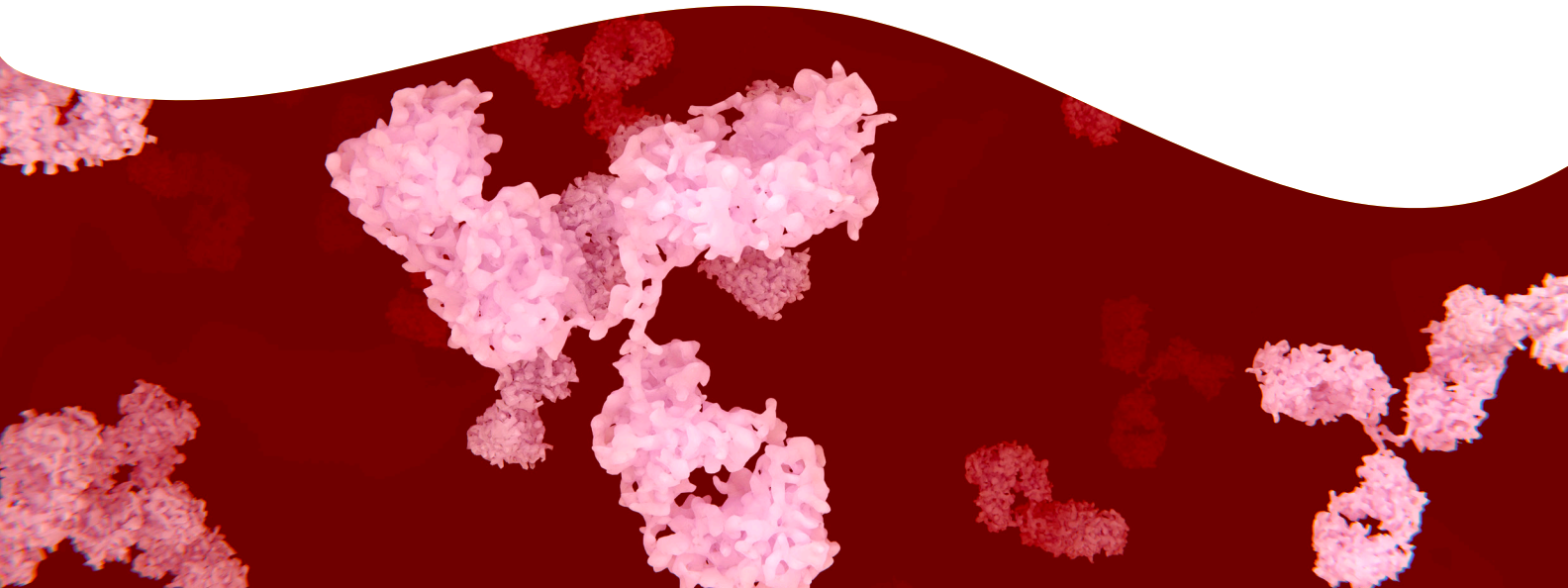
Introduction

Administration of biological therapeutics may result in the development of an immune response in patients. The presence of the anti-drug antibodies (ADA) does not necessarily cause serious events. However, it is important that the presence of ADA is evaluated and correlated with any potential pharmacological or toxicological observations.

We have previously demonstrated the application of the AlphaLISA™ technology for ADA screening and confirmatory assays.[1] The usual step after the screening process is to characterize the ADA response by determining if the ADA could neutralize the activity of the drug. Generally, the ADA binds to the drug without affecting its efficacy. However, the presence of neutralizing ADA (NAb) is of great concern since the efficacy and the pharmacokinetic profile of the biotherapeutic drug could be greatly affected. Another concern will arise if the drug has an endogenous counterpart, since the presence of NAb could lead to an autoimmune-like deficiency.

Neutralizing antibodies are usually detected using a functional cell-based assay in which the drug induces a specific intracellular signal. The presence of the NAb in the serum sample will inhibit the drug from triggering this activation. However, as there is a need to decrease the assay complexity and improve the assay reproducibility and robustness, biochemical (cell-free) NAb assays are of increasing interest.



We report here the development of two AlphaLISA functional assays, a cell-based and a biochemical assay, for the detection of NAb in human serum samples.

We selected the EGF receptor (EGF-R) activation as the model system for this proof-of-principle study and the performance of the AlphaLISA assays was assessed by performing a pre-validation study.

Materials and methods

Materials

Recombinant EGF (to mimic the drug) was purchased from R&D Systems (Part No. 236-EG). The NAb positive control antibody (PC) was a monoclonal mouse anti-EGF antibody obtained from R&D Systems (Part No. MAB236). The recombinant EGF receptor (for the biochemical assay) was obtained from Fitzgerald (Part No. 30R-AE012). The biotinylated EGF (for the biochemical assay) was obtained from Invitrogen (Part No. E3477). The antibodies for the capture of intracellular Y845 phosphorylated EGF receptor (for the cell-based assay) were obtained from R&D Systems and Cell Signaling Technology (Part Nos. AF231 and 6963, respectively). The CHO cell line stably expressing the EGF receptor was engineered in house. Pooled normal human serum (PNHS) and individual lots of normal human serum were purchased from Bioreclamation (Part No. HMSRM). AlphaLISA Acceptor beads and streptavidin-coated Donor beads (SA-Donor) were from Revvity (Part No. 6772002 and Part No. 6760002, respectively). The ChromaLink biotinylating reagent was obtained from SoluLink (Part No. B1001-105). Zeba Spin desalting columns were from Thermo Scientific (Part No. 89891). ½ AreaPlate™-96 white opaque 96-well microplates were from Revvity (Part No. 6005560).

The assay buffer for the biochemical assay was composed of 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween-20, 0.5% BSA and 0.05% bovine γ-globulin. The assay buffer for the cell-based assay was HBSS (Gibco Part No. 1072021) supplemented with 0.1% BSA. The AlphaLISA cell lysis buffer 5X was from Revvity (Part No. AL003C).

Preparation of conjugated AlphaLISA acceptor beads

Antibody or EGF-R immobilization on the AlphaLISA Acceptor beads was performed using a simple aldehyde group coupling procedure. Briefly, 0.025 mg of antibody or EGF-R, 0.0625% final concentration of Tween-20, 1.25 mg of AlphaLISA beads and 1.25 mg/mL final of $NaBH_3CN$ were mixed together. The volume was completed to 0.05 mL with 100 mM Hepes pH 7.4 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 carboxymethylamine hemihydrochloride solution and incubation for one hour at 37 ˚C. Beads were then washed twice by centrifugation for 15 min (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.

Antibody biotinylation

Antibody 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 molar ratio of 30:1 (biotin:antibody). The reaction volume was completed to 0.05 mL with PBS pH 7.4 and the reaction was incubated for 2 hr at 23 ˚C. Purification of the biotinylated antibody was performed using a Zeba™ 0.5 mL desalting column. The biotinylation efficiency and the protein recovery were determined from absorbance readings at 354 nM and 280 nM, respectively.

AlphaLISA NAb assays

The AlphaLISA NAb cell-based assay was performed as follows:

The serum samples (10 µL) were added to the ½ AreaPlate-96 white microplate. Then, 10 µL of EGF at 1 nM final (diluted in HBSS) and 20 µL of CHO cells expressing the EGF receptor (6,000 cells / well, in HBSS) were added to the plate. The plate was incubated for 15 min at RT to allow stimulation of the receptor. Then, 10 µL of AlphaLISA cell lysis buffer (5X) was added followed by 10 min incubation. The detection was performed by adding 50 µL of a mix of anti-phospho EGF-R conjugated Acceptor beads at 10 µg/mL final and biotinylated anti-EGF-R at 1 nM final (diluted in HBSS). The plate was then incubated for 60 minutes in an incubator set at 23 ˚C. Finally, 50 µL of SA-Donor beads at 20 µg/mL (diluted in assay buffer) was added to the plate, which was then incubated for 60 min in an incubator set at 23 ˚C. After this incubation, the plate was read using the EnVision™ Multimode Plate Reader (v. 2103) and the AlphaLISA relative counts were measured.

The AlphaLISA NAb biochemical assay was performed as follows:

The serum samples (5 µL) were added to the ½ AreaPlate-96 white microplate. Then, 20 µL of the biotinylated EGF at 1 nM final (diluted in assay buffer) and 25 µL of the EGF-R conjugated Acceptor beads at 20 µg/mL final (diluted in assay buffer) were added. The plate was then incubated for 60 min in an incubator set at 23 °C. Finally, 50 µL of SA-Donor beads at 20 µg/mL (diluted in assay buffer) were added to the plate, which was then incubated for 60 min in an incubator set at 23 °C. After this incubation, the plate was read using the EnVision Multimode Plate Reader (v. 2103).

Assay configuration

The two AlphaLISA NAb detection assays were developed as follows:

The cell-based assay uses CHO cells stably expressing the EGF receptor. Cell stimulation with EGF triggers internal phosphorylation of the receptor. The presence of a neutralizing anti-EGF antibody (positive control NAb) in the samples hampers receptor activation and, as a consequence, the resulting intracellular signaling is abolished. The detection step is performed after cell lysis by capturing the phosphorylated EGF-R protein using specific antibodies (total EGF-R and pEGF-R antibodies). The bridge formed by the protein captured by the two antibodies will 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.

The biochemical assay uses a recombinant EGF receptor that is directly conjugated to the Acceptor beads. The signal is generated by the presence of biotinylated EGF that binds both the immobilized receptor and the Streptavidin-Donor beads. In the presence of NAb, the signal is reduced proportionally to the amount of NAb present in the samples to measure.

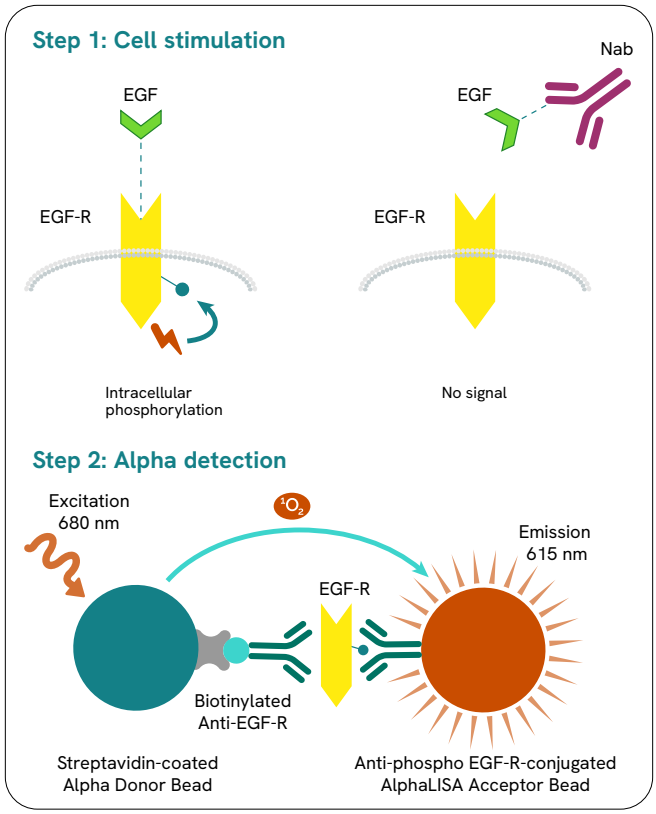


Figure 1: AlphaLISA cell-based NAb assay configuration.

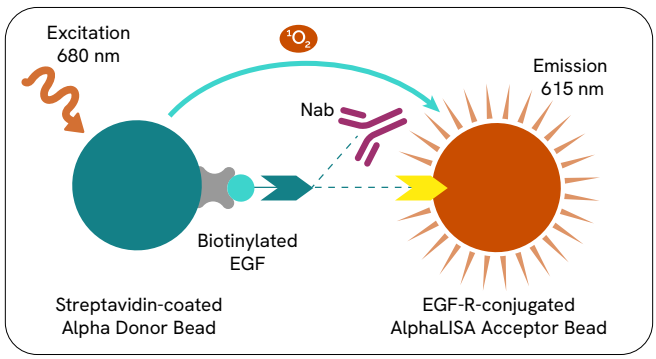


Figure 2: AlphaLISA biochemical NAb assay configuration.

Table 1: Cut-point determination. A) Cell-based assay, B) Biochemical assay. The CP was determined using 50 individual lots of human serum, analyzed each in duplicate on a total of three occasions. The CP was calculated as the mean counts minus 1.645 x SD. Two samples were removed for the second iteration for each assay. 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 counts of the 12 replicates were determined for each occasion. A correction factor corresponding to the ratio between the CP and the overall mean counts of PNHS was determined for each occasion. The mean CF value was used as normalization factor for all subsequent assays. The plate specific cut-point (PSCP) was calculated by multiplying the mean CF value with the mean counts value of PNHS (Blank wells, n = 12).

A. Cell-based assay			B. Biochemical assay		
	1st iteration	2nd iteration		1st iteration	2nd iteration
mean	374448	379846	mean	80910	81308
n	50	48	n	50	48
sd	67521	51026	SD	7880	7326
cut-point	306927	328820	cut-point	73029	73981
PNHS	367858	367858	PNHS	78858	78858
correction factor	0.83	0.90	correction factor	0.93	0.94

Results

In order to determine if the cell-based and biochemical NAb AlphaLISA assays could meet the minimal requirements for NAb detection, we have performed a pre-validation study using general guidelines found in white papers.[2,3,4,5]

The following experiments were performed: determination of the cut-point (CP), assay sensitivity, assay selectivity and intra- and inter-assay precision.

In our model system, the NAb (positive control) is mimicked by a monoclonal mouse anti-EGF antibody prepared at different concentrations in neat pooled normal human serum (PNHS).

Screening cut-point

The cut-point (CP) was used to determine the threshold for identifying samples as negative (above the CP) or potentially positive (equal or below the CP) for the presence of NAb. Fifty individual lots of normal human serum were analyzed each in duplicate on a total of 3 occasions. On each occasion, the average values of the two corresponding 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 counts of the individual serum lots minus 1.645 x SD, which represents the 95th percentile of a normal distribution.

Two human serum lots were identified as outliers, since their mean counts were inferior to the CP on all three occasions and were excluded from the final CP calculation. Interestingly, the two outliers were different in the two assays, indicating that the format used for the NAb detection can impact the final results. The mean Correction Factor (CF), defined at 0.90 and 0.94 for the cell-based and biochemical assays, respectively, was used for subsequent experiments. The plate specific cut-point (PSCP) for each assay plate was determined in all subsequent experiments using the following formula: *PSCP = Mean Blank (pooled serum) Relative Counts x 0.90 (or 0.94).*

Assay sensitivity

To determine this parameter, the monoclonal anti-EGF antibody (PC) was spiked at different concentrations in the PNHS and tested in each assay.

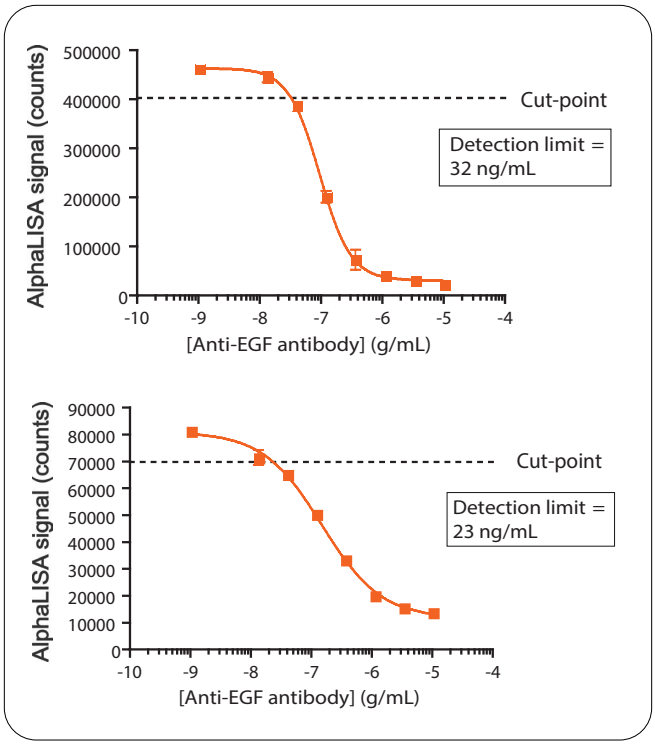


Figure 3: Assay Sensitivity. The PNHS was spiked with increasing concentrations of the positive control antibody to generate NAb standard curves. The assay sensitivity was determined as the concentration of PC, which generated a signal at or below the cut-point.

Based on this preliminary determination, the sensitivity was determined to be 32 and 23 ng/mL for the cell-based and biochemical assays, respectively. This level of sensitivity is sufficient for determination of NAb in serum samples, as the minimal requirement for assay sensitivity has been set at 250 ng/mL by regulatory agencies.[6]

Assay selectivity

The selectivity is defined as the ability of an assay to measure the molecule of interest independently of any possible matrix effect. For evaluation of the selectivity of the NAb assays, each of the individual lots of human serum was spiked with PC at two concentrations: 100 ng/mL and 2,500 ng/mL. In addition, PNHS was spiked at the same levels to serve as reference samples to calculate the recovery.

Overall, the calculated percentage recoveries were within acceptable difference from the corresponding PC samples prepared in PNHS. We observed similar performance for both the cell-based and biochemical assays.

Table 2: Assay selectivity: A) Cell-based assay, B) Biochemical assay. Ten individual serum lots were analyzed spiked with the PC at two concentrations and compared to control samples spiked in PNHS. The percentage recovery was calculated with the following formula: % Difference (recovery) = ((counts for PC individual lot counts of PC PNHS) x 100)/counts of PC PNHS.

A. Cell-based assay					B. Biochemical assay				
sample	PC at 100 ng/mL	% difference	PC at 2500 ng/mL	% difference	sample	PC at 100 ng/mL	% difference	PC at 2500 ng/mL	% difference
PNHS	235627	N/A	39190	N/A	PNHS	48931	N/A	14993	N/A
1	241621	2.5	43261	10.4	1	63420	29.6	18688	24.6
2	221491	-6.0	27079	-30.9	2	55770	14.0	15979	6.6
3	254803	8.1	36107	-7.9	3	57671	17.9	17350	15.7
4	244301	3.7	30627	-21.8	4	60715	24.1	18567	23.8
5	192840	-18.2	42803	9.2	5	61789	36.2	21257	41.8
6	255302	8.4	48010	22.5	6	48946	0.0	11782	-21.4
7	221370	-6.1	42657	8.8	7	52149	6.6	16181	7.9
8	250666	6.4	60734	55.0	8	53466	9.3	13525	-9.8
9	245609	4.2	41220	5.2	9	47536	-2.9	13854	-7.6
10	233801	-0.8	39055	-0.3	10	56991	16.5	17215	14.8

Intra- and inter-assay precision

Precision is a quantitative measure of the random variation between a series of measurements. To determine the precision of the assay, six independent experiments using triplicate data points were performed. 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. Inter- and intra-assay precision was evaluated by testing the three PC concentrations in the PNHS.

In summary, these results indicate a significantly lower variability with the biochemical assay. This was expected, due to the lower assay complexity and the absence of cellular background. The variability was generally lower with samples generating counts near both ends of the curve. The lowest PC concentration tested (100 ng/mL) produced counts below the cut-point in all six experiments, indicating that both assay formats could be used reproducibly for NAb testing. Overall, these values are within acceptable range and would typically meet the requirements for NAb detection assays.

Conclusions

Simple, robust and sensitive technologies are increasingly required for the immunogenicity assessment of biotherapeutic drugs. We have already demonstrated that the AlphaLISA platform could be advantageously used for ADA screening and confirmatory assays. We now demonstrate in this application note a proof-of-principle study of two assay formats for NAb testing.

Although the cell-based approach is still widely used, the biochemical assay could become a more popular choice as the cell-free format offers several advantages in terms of simplicity and reproducibility. We have performed several pre-validation experiments that showed very good performance of both assay formats.

Table 3: Assay precision. Six independent experiments using triplicate data points were performed to determine the intra- and inter-assay variability.

	A. Cell-based assay			B. Biochemical assay		
	Mean counts	Mean intra %CV	Inter inter %CV	Mean counts	Mean intra %CV	Mean inter %CV
PNHS	367858	6	9	78858	10	10
PC at 100 ng/mL	200963	12	24	52978	7	9
PC at 500 ng/mL	48009	28	18	29175	10	9
PC at 2500 ng/mL	30031	17	10	16771	8	8

The use of the AlphaLISA technology offers significant advantages over current platforms for immunogenicity screening assays: 1) Fully homogeneous, simple protocol, 2) Excellent sensitivity, 3) Very good reproducibility. Very importantly, the fully homogenous AlphaLISA format eliminates the requirement for wash steps, shortening hands-on time to execute the assay. This assay platform therefore represents an important analytical tool offering distinct advantages for immunogenicity testing applications.

References

1. Immunogenicity Assessment using the AlphaLISA Technology. Application Note (2011), Revvity.

2. Gupta, S., Devanarayan, V., Finco, D., Gunn III, G. R., Kirshner, S., Richards, S., Rup, B., Song, A., Subramanyam, M. Recommendation for the Validation of Cell-based Assays Used for the Detection of Neutralizing Antibody Immune Responses Elicited Against Biological Therapeutics. Journal of Pharmaceutical and Biomedical Analysis (2011) 55: 878-888.

3. Mire-Sluis A. R., Barrett Y. C., Devanarayan V., Koren E., Liu H., Maia M., Parish T., Scott G., Shankar G., Shores E., Swanson S.J., Taniguchi G., Weirda D. and Zuckerman L.A. Recommendation for the Design and Optimization of Immunoassays Used in the Detection of Host Antibodies Against Biotechnology Products. Journal of Immunological Methods (2004) 289: 1-16.

4. Shankar G., Devanarayan V., Amaravadi L., Barrett Y., Bowsher R., Finco-Kent D., Fiscella M., Gorovits B., Kirschner S., Moxness M., Parish Th., Quarmby V., Smith H., Smith W., Zuckerman L. A., Korren E. Recommendations for the Validation of Immunoassays Used for Detection of Host Antibodies Against Biotechnology Products. Journal of Pharmaceutical and Biomedical Analysis. (15 Dec 2008) Volume 48, issue 5: 1267-1281.

5. Koren E., Smith H.W., Shores E., Shankar G., Finco-Kent D., Rup B., Barrett Y-C., Devanarayan V., Gorovits B., Gupta S., Parish T., Quarmby V., Moxness M., Swanson S.J., Taniguchi G., Zuckerman L.A., Stebbins C.C., Mire-Sluis A.. Recommendations on Risk-based Strategies for Detection and Characterization of Antibodies Against Biotechnology Products. Journal of Immunological Methods (2008) 333: 1-9.

6. FDA – Draft Guidance for Industry – Assay Development for Immunogenicity Testing of Therapeutic Proteins (Dec. 2009).

7. AlphaLISA Immunogenicity Assay Development Guide (2012), Revvity.

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