TECHNICAL NOTE

Detection of human PD-L2 in serum - Comparison of AlphaLISA and ELISA immunoassays.

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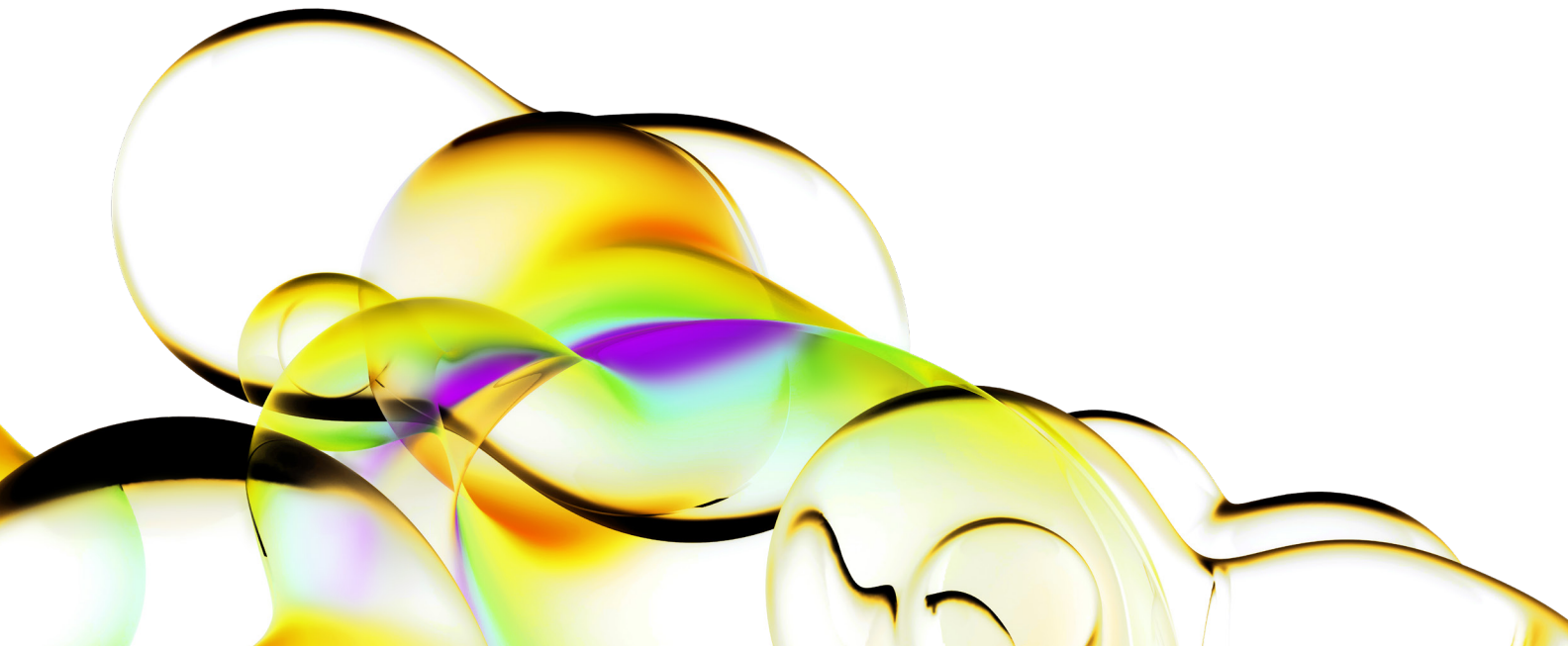
Introduction

While ELISA (enzyme-linked immunosorbent assay) technology is considered the gold standard of immunoassays, it requires numerous wash steps which make it laborious and time-consuming. In the field of drug discovery, technologies that give robust, sensitive, automatable, and reproducible immunoassays at a lower cost are highly desirable. AlphaLISA™ (amplified luminescent proximity-based homogeneous) is a bead-based homogeneous immunoassay technology that offers an alternative to standard ELISA. We compared performance of an AlphaLISA PD-L2 assay to a colorimetric ELISA PD-L2 assay using human serum samples (Figure 1). AlphaLISA offers the benefits of a wider dynamic range and a significantly faster and easier protocol, while yielding results that correlate to results obtained from ELISA experiments.



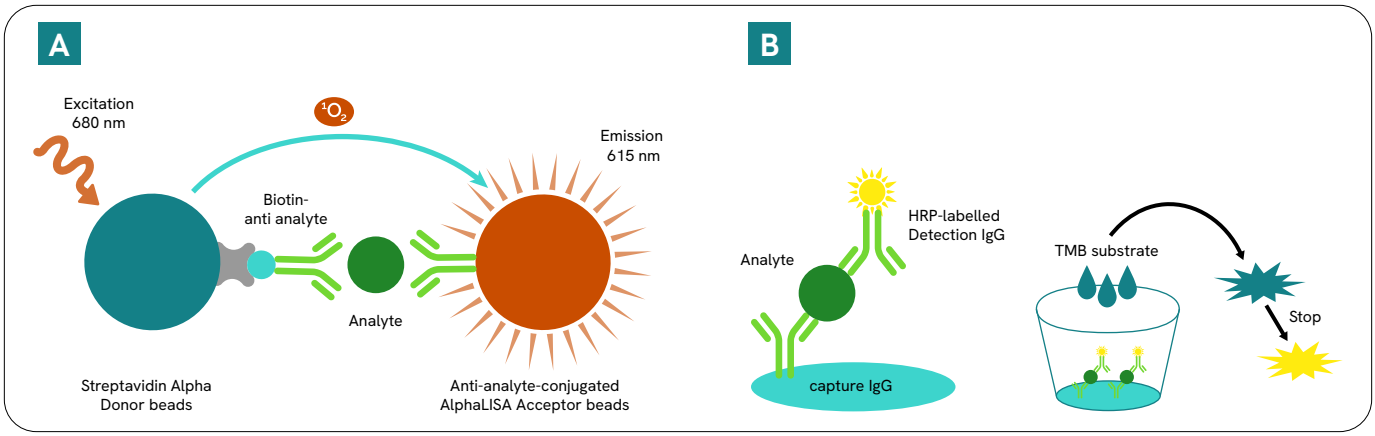


Figure 1: Comparison of AlphaLISA and ELISA immunoassay configurations. A) In the AlphaLISA assay, one anti-analyte antibody is biotinylated and a second anti-analyte antibody is directly conjugated to AlphaLISA Acceptor beads. In the presence of the analyte of interest, Streptavidin Donor and antibody-conjugated Acceptor beads are brought into proximity, allowing the AlphaLISA signal to be generated following laser excitation at 680 nM. B) In the ELISA assay, a capture IgG specific to the antigen of interest is coupled to the microplate whereas another IgG is labeled with horseradish peroxidase (HRP, detection IgG). After a series of blocking and wash steps to remove excess detection IgG non-specifically bound, TMB (a blue colored substrate for HRP) is added. Upon reaction of TMB and HRP, a yellow colored product is generated which is proportional to the amount of analyte in the sample.

Materials

The AlphaLISA PD-L2 kit (#AL3093C), EnVision™ multimode reader, EnSpire™ multimode reader, and white 384-well OptiPlates™ (#6007290) were supplied by Revvity. The colorimetric ELISA PD-L2 kit and microplates were obtained from an established ELISA kit provider. Kits were run according to each manufacturer's protocol. Normal human serum was from Innovative Research (#IPLA-SER-22834). Serum from hepatocellular carcinoma (HCC) was from BioIVT (#HMSRM-HCC). Fetal bovine serum was from Gibco (#26140079).

Results

Validation of AlphaLISA PD-L2 assay for serum samples

In order to accurately quantitate levels of PD-L2 in a complex matrix such as human serum, it is necessary to create a standard curve of the analyte in a matrix (diluent) that closely matches the sample type. Linearity experiments and spike-and-recovery experiments were performed to test fetal bovine serum (FBS) as a potential diluent for the standard curve for the AlphaLISA PD-L2 assay using human serum samples. PD-L2-spiked (3000 ng/mL) normal human serum samples were diluted with 100% FBS, and the assay was performed along with a standard curve using the recombinant PD-L2 analyte prepared in 100% FBS. Concentrations of PD-L2 in diluted human serum were determined by interpolating from the standard curve.

Excellent dilution linearity (R^2 > 0.997) was achieved in the PD-L2-spiked human serum samples that were diluted ≥ 2 fold. Results are shown in Figure 2.

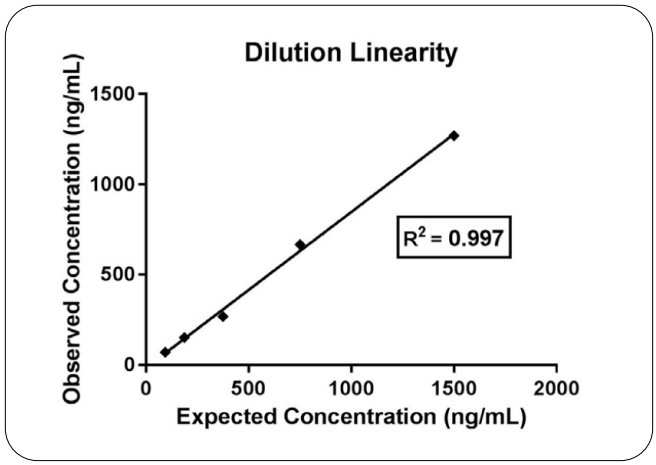


Figure 2: Results from linearity experiments for the AlphaLISA PD-L2 serum assay. Linearity was assessed using 100% FBS as a diluent. Excellent dilution linearity (R^2 > 0.997) was achieved in the PD-L2-spiked human serum samples that were diluted ≥ 2 fold.

For spike-and-recovery, three known amounts of PD-L2 (300, 100, and 30 ng/mL) were spiked into normal human serum. Samples were then diluted 2-fold into 100% FBS. The recovery of PD-L2 was determined and the results are shown Table 1. Excellent recovery (>85%) was observed for each concentration of spiked PD-L2. This validates the use of 100% FBS as a diluent for the standard curve in the AlphaLISA PD-L2 assay when testing human serum samples.

Correlation of AlphaLISA and ELISA results in human serum

Next, AlphaLISA and ELISA PD-L2 assays were used to quantitate the amount of PD-L2 in human serum samples. Two sources of human serum [serum from hepatocellular carcinoma (HCC) and normal human serum (NHS)] were tested with each assay. For the AlphaLISA assay, 100% FBS was used as a diluent for the standard curve. For the ELISA assay, sample diluent provided with the ELISA kit was used as a diluent for the standard curve and sample dilution. Results are show in Table 3. Data indicate good correlation between the AlphaLISA and ELISA assays in determining the amount of PD-L2 in human serum samples.

Table 1: Recovery data for AlphaLISA PD-L2 human serum assay performed using FBS as a diluent. The PD-L2 standard was prepared in 100% FBS. All other reagents were prepared in 1X AlphaLISA immunoassay buffer.

	Diluent: 100% FBS	
	Spiked sample (Normal human serum)	
Spike (ng/mL)	Concentration (ng/mL)*	Recovery (%)
No spike	5.5	N/A
300	258	86
100	102	102
30	29	98

*Recoveries were calculated after the no spike PD-L2 level was subtracted (in this case, 5.5 ng/mL in Normal Human Serum).

Comparison of assay protocols

AlphaLISA is a homogeneous assay technology that doesn't require wash or separation steps. A comparison of the AlphaLISA and ELISA PD-L2 assay protocols is shown in Table 3. As seen in the table, the AlphaLISA PD-L2 assay can be run in less than 3 hours with no washes. The ELISA PD-L2 assay has a total assay time of 5-6 hours, and requires eight washes. AlphaLISA is faster and simpler to perform compared to ELISA, with fewer pipetting steps.

Table 2: Quantitation of PD-L2 in HCC and NHS serum samples using AlphaLISA and ELISA assays.

	AlphaLISA	ELISA
[PD-L2] (ng/mL) in HCC serum (n=6)	8.8 (standard deviation=1.7)	11.6 (standard deviation=3.7)
[PD-L2] (ng/mL) in NHS serum (n=2)	6.4 (standard deviation=1.4)	10.1 (standard deviation=1.8)

Table 3: Comparison of AlphaLISA and ELISA protocols

Step	AlphaLISA assay protocol	ELISA assay protocol
1	Add 5 µL standard or sample	Add 90 µL sample diluent
2	Add 10 µL of 5X AlphaLISA Anti-PD-L2 Acceptor beads	Add 10 µL of standard or sample
3	Incubate 30 minutes at room temperature	Add 50 µL biotin conjugate.
4	Add 10 µL of Biotinylated Anti-PD-L2 Antibody	Incubate 120 minutes on orbital microplate shaker (500 ± 50 rpm).
5	Incubate 60 minutes at room temperature	Wash plate 4X.
6	Add 25 µL of 2X Streptavidin Donor beads	Add 100 µL streptavidin-HRP conjugate.
7	Incubate 30 minutes in dark	Incubate 60 minutes on orbital microplate shaker (500 ± 50 rpm).
8	Measure on EnVision plate reader	Wash plate 4X.
9		Add 100 µL TMB substrate solution.
10		Incubate 30 minutes at room temperature in the dark.
11		Add 100 µL stop solution.
12		Read on EnSpire™ plate reader at 450 nM within 30 minutes.
Overall	**2.5 hours overall, no wash steps**	**5-6 hours overall, 8 washes**

Conclusions

The comparison between the AlphaLISA and ELISA PD-L2 assays revealed that the kits yield comparable results when quantitating PD-L2 in human serum samples. However, because the AlphaLISA kit does not require any wash steps, both the elapsed time and hands-on time required to perform the assay was much less than for the ELISA kit. Overall, the AlphaLISA kit offers the benefits of a significantly faster and easier protocol, with equivalent or better performance. These workflow benefits, combined with the generally lower cost of AlphaLISA reagents, make the AlphaLISA assay platform particularly advantageous over traditional immunoassays such as wash-based ELISAs.

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