AlphaLISA assays for immune checkpoint detection and quantitation.

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Introduction

Immune checkpoints are molecular markers that can regulate an immune system attack on cancer cells, and they have become highly regarded as promising candidates for immunotherapies. These molecules can be either functional stimulatory (e.g. CD28) or inhibitory checkpoint molecules (e.g. CD276, CD272, CTLA-4, LAG3, PD-1, TIM-3 and VISTA). AlphaLISA biomarker detection kits are designed to detect and quantify the levels of these molecules in cell culture media, serum and cell lysates.

AlphaLISA® technology allows for the detection and quantitation of these immune checkpoints in buffer, cell culture media, serum, and cell lysates in a highly sensitive, reproducible and user-friendly way. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in a sharp peak of light emission at 615 nM (Figure 1). This light emission can then be detected on an Alpha-enabled reader, such as the EnVision® multilabel plate reader.
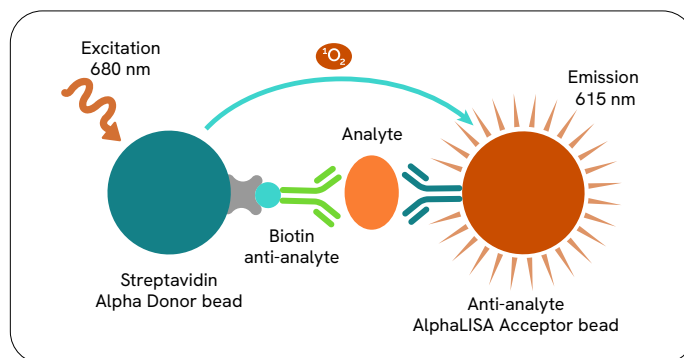

Figure 1: Assay principle for AlphaLISA detection assay.

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Experimental protocol

Add 5 μL of each analyte standard dilution or 5 μL of samples

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Add 20 μL of 5x MIX of AlphaLISA Anti-Analyte Acceptor beads (10 μg/mL final) + biotinylated Anti-Analyte antibody (1 nM final)

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Incubate 60 minutes at 23 ˚C

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Add 25 μL of 2x SA-Donor beads (40 μg/mL final)

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Incubate 30 minutes at 23 ˚C in the dark

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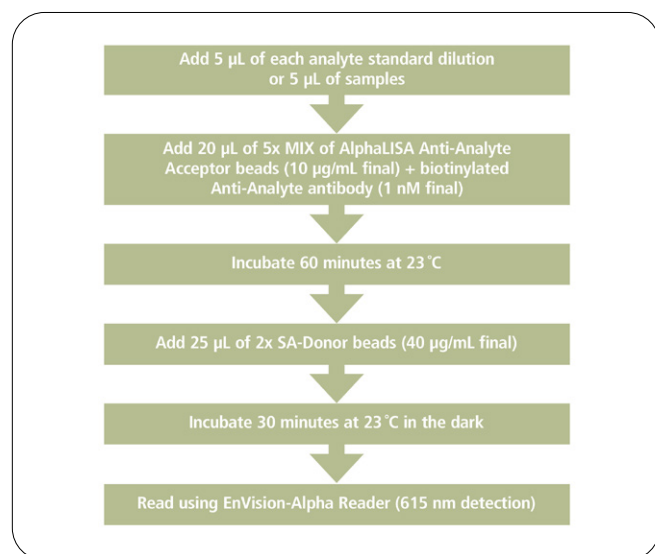Read using EnVision-Alpha Reader (615 nm detection)

Figure 2: General workflow for an AlphaLISA detection assay. Procedures may vary slightly for each individual kit.
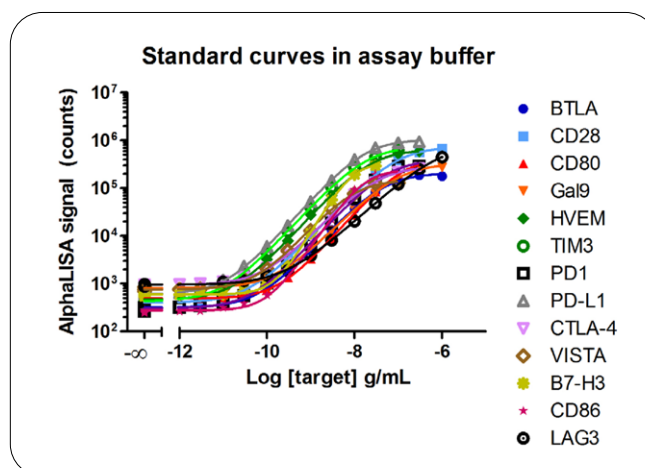
Standard curves

Figure 3: Standard curves for AlphaLISA immune checkpoint detection assays. AlphaLISA assays provide high signal-to-background and high sensitivity with broad dynamic range.

Assay performance

Table 1: Assay performance for AlphaLISA kits.

Target	Part number	LDL[1]	LLOQ[2]	Dynamic range	%CV[3] (Intra)	%CV[3] (Inter)
B7-H3 (CD276)	AL3060	42 pg/mL	104 pg/mL	42 - 100,000 pg/mL	5	7
BTLA (CD272)	AL3062	11 pg/mL	38 pg/mL	11 - 300,000 pg/mL	6	10
CTLA-4	AL3050	34 pg/mL	121 pg/mL	34 - 100,000 pg/mL	4	7
CD28	AL3044	9 pg/mL	26 pg/mL	9 - 1,000,000 pg/mL	3	4
CD86	AL3045	25 pg/mL	75 pg/mL	25 - 100,000 pg/mL	4	5
CD80	AL3055	36 pg/mL	126 pg/mL	36 - 300,000 pg/mL	6	8
GAL9 (Galectin)	AL3051	9 pg/mL	42 pg/mL	9 - 1,000,000 pg/mL	5	10
HLA-C	AL3061	56 pg/mL	193 pg/mL	56 - 300,000 pg/mL	4	5
HLA-E	AL3057	76 pg/mL	326 pg/mL	76 - 3,000,000 pg/mL	4	11
HVEM	AL3054	3 pg/mL	11 pg/mL	3 - 300,000 pg/mL	3	8
LAG-3	AL3058	74.1 pg/mL	331.1 pg/mL	74 - 1,000,000 pg/mL	7	13
PD-1	AL343	9 pg/mL	37 pg/mL	9 - 100,000 pg/mL	5	15
PD-L1	AL355	2 pg/mL	10 pg/mL	2 - 300,000 pg/mL	4	6
TIM-3	AL3052	2.5 pg/mL	8 pg/mL	2.5 - 100,000 pg/mL	6	6
VISTA	AL3059	14.2 pg/mL	56.6 pg/mL	14.2 - 100,000 pg/mL	5	9

[1] The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.

[2] The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve.

[3] % CV data were calculated using two different kit lots. For each lot, the standard curves were prepared in kit assay buffer. Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well format. The intra-assay precision was determined using a total of seven independent determinations in triplicate, shown as CV%. The inter-assay precision was determined using a total of seven independent determinations with 21 measurements, shown as CV%.

Validated sample matrices

| Table 2: Validated sample matrices for AlphaLISA immune checkpoint detection assays.

Target	Part number	Buffer	DMEM	RPMI	Serum
B7-H3 (CD276)	AL3060	✓	✓	✓	✓
BTLA (CD272)	AL3062	✓	✓	Not recommended	✓
CTLA-4	AL3050	✓	✓	✓	✓
CD28	AL3044	✓	✓	✓	Not recommended
CD86	AL3045	✓	✓	Not recommended	✓
CD80	AL3055	✓	✓	Not recommended	✓
GAL9 (Galectin)	AL3051	✓	✓	Not recommended	✓
HLA-C	AL3061	✓	✓	Not recommended	Not recommended
HLA-E	AL3057	✓	✓	Not recommended	Not recommended
HVEM	AL3054	✓	✓	Not recommended	✓
LAG-3	AL3058	✓	✓	✓	✓
PD-1	AL343	✓	✓	✓	✓
PD-L1	AL355	✓	✓	✓	✓
TIM-3	AL3052	✓	✓	✓	✓
VISTA	AL3059	✓	✓	Not recommended	✓

Instrumentation

All immune checkpoint AlphaLISA kits were developed using the EnVision multilabel plate reader. In addition to fast, sensitive Alpha technology detection, the EnVision plate reader provides fluorescence intensity, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence detection technologies. The system is based on hybrid technology, combining filters and a monochromator for enhanced flexibility. It incorporates proprietary Direct Double Optics™ technology for high speed and sensitivity in simultaneous filter-based readouts, such as FRET assays, and for TRF, lamp-based excitation is standard, with the option of a high energy laser for higher speed and sensitivity.

| Figure 4: EnVision multilabel plate reader.

Products and part numbers

| Table 3: Products for AlphaLISA immune checkpoint detection assays.

Revvity product	Format	Catalog number
B7-H3 (CD276) (human) AlphaLISA detection kit	500 assay points	AL3060C
	5000 assay points	AL3060F
BTLA (CD272) (human) AlphaLISA detection kit	500 assay points	AL3062C
	5000 assay points	AL3062F
CD28 (human) AlphaLISA detection kit	500 assay points	AL3044C
	5000 assay points	AL3044F

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Table 3: Products for AlphaLISA immune checkpoint detection assays. (Cont.)

Revvity product	Format	Catalog number
CD80 (human) AlphaLISA detection kit	500 assay points	AL3055C
	5000 assay points	AL3055F
CD86 (human) AlphaLISA detection kit	500 assay points	AL3045C
	5000 assay points	AL3045F
CTLA-4 (human) AlphaLISA detection kit	500 assay points	AL3050C
	5000 assay points	AL3050F
GAL9 (Galectin) (human) AlphaLISA detection kit	500 assay points	AL3051C
	5000 assay points	AL3051F
HLA-C (human) AlphaLISA detection kit	500 assay points	AL3061C
	5000 assay points	AL3061F
HLA-E (human) AlphaLISA detection kit	500 assay points	AL3057C
	5000 assay points	AL3057F
HVEM (human) AlphaLISA detection kit	500 assay points	AL3054C
	5000 assay points	AL3054F
LAG-3 (human) AlphaLISA detection kit	500 assay points	AL3058C
	5000 assay points	AL3058F
PD-1 (human) AlphaLISA detection kit	500 assay points	AL343C
	5000 assay points	AL343F
PD-L1 (human) AlphaLISA detection kit	500 assay points	AL355C
	5000 assay points	AL355F
TIM-3 (human) AlphaLISA detection kit	500 assay points	AL3052C
	5000 assay points	AL3052F
VISTA (human) AlphaLISA Detection Kit	500 assay points	AL3059C
	5000 assay points	AL3059F
½ AreaPlates (96-well, white)	50 plates	6005560
	200 plates	6005569
OptiPlate® (96-well, white)	50 plates	6005290
	200 plates	6005299
OptiPlate (384-well, white)	50 plates	6007290
	200 plates	6007299
AlphaPlate® (384-well, gray)	50 plates	6005350
	200 plates	6005359
AlphaPlate (1536-well, gray)	50 plates	6004350
TopSeal®-A PLUS	Box of 100	6050185

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