TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

PD-1 and PD-L1 (Human) Binding AlphaLISA Kit

Product number: AL356 HV/C/F

Caution: For Laboratory Use. A research product for research purposes only.

Contents

Product Information

Application: This kit is designed for the detection of binding activity between Human PD-1 and PD-L1, using a homogeneous AlphaLISA assay (no wash steps). This assay can facilitate the design and development of antibody therapetics by using competitive binding to PD-1/P-L1.

Sensitivity: $K_{d(app)}$: 2.5 nM (average) using 5 nM PD-1

Signal to background ratio: 1600 (average) using 5 nM PD-1

Kit contents: The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human PD-1, His tagged human PD-L1 and AlphaLISA Immunoassay buffer.

Storage: The kit components must be stored at +4 ˚C and in the dark for the beads.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

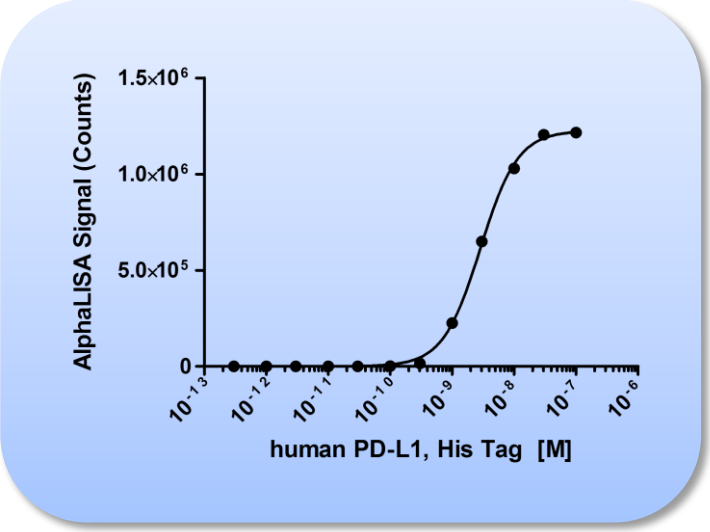


Figure 1. Typical Binding curve (5nM of PD-1) in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals and the apparent binding dissociation constant $K_{d(app)}$ were measured on the EnVision Multilabel Plate Reader with Alpha option using the protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum counts may vary between bead lots and the instrument used, with no impact on $K_{d(app)}$ measurement.

Analyte of Interest

Programmed cell death protein 1 (PD-1), also known as cluster of differentiation 279 (CD279), belongs to immunoglobulin superfamily and is a transmembrane receptor protein. Programmed death ligand 1 (PD-L1), also known as cluster of differentiation 274 (CD274) or B7 homolog1 (B7-H1) belongs to the growing B7 family of immune proteins. PD-L1, together with PD-L2, are two ligands for PD-1. By binding to PD-1 on activated T-cells and B-cells, PD-L1 may inhibit ongoing T-cell responses by inducing apoptosis and arresting cell-cycle progression. Accordingly, it leads to growth of immunogenic tumor growth by increasing apoptosis of antigen specific T cells and may contribute to immune evasion by cancers. Therefore blocking PD-1 and PD-L1 binding has been considered as promising therapeutic target for human autoimmune disease and malignant cancers.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a biotinylated PD-1 binds to the Streptavidin-coated Alpha Donor beads, while His tagged PD-L1 is captured by Anti-His AlphaLISA Acceptor beads. When PD-L1 binding to PD-1 happens, Donor beads and Acceptor 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 in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2).

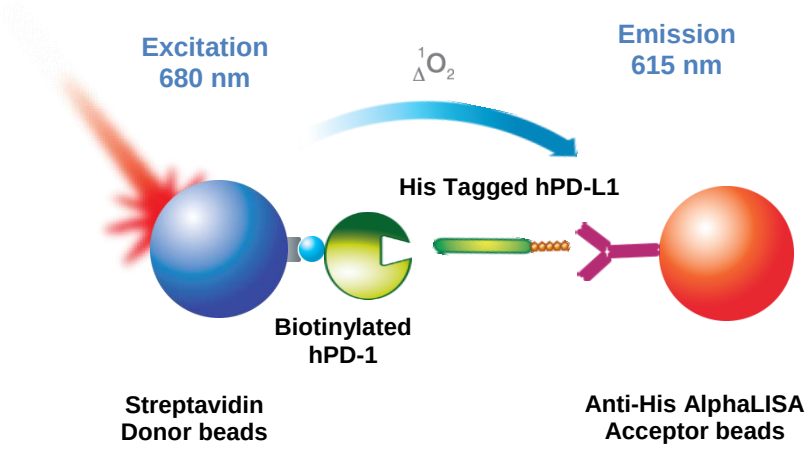


Figure 2. AlphaLISA Assay Principle.

Precautions

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous. The analyte included in this kit is from a human source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated sample contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL356HV*** (100 assay points)	AL356C*** (500 assay points)	AL356F**** (5000 assay points)
Anti-6xHis AlphaLISA Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	40 µL @ 5 mg/mL (1 brown tube, white cap)	200 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	80 µL @ 5 mg/mL (1 brown tube, black cap)	400 µL @ 5 mg/mL (1 brown tube, black cap)
Lyophilized Human PD-1(Biotinylated)*	2.13 µg, lyophilized (1 tube, clear cap)	2.13 µg, lyophilized (2 tubes, clear caps)	2.13 µg, lyophilized (10 tubes, clear caps)
Lyophilized Human PD-L1(His tagged)*	1.3 µg, lyophilized (1 tube, clear cap)	1.3 µg, lyophilized (2 tubes, clear caps)	1.3 µg, lyophilized (10 tubes, clear caps)
AlphaLISA Immunoassay Buffer (10X)**	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute hPD-1 and PD-L1 in 100 µL Milli-Q® grade H_2O respectively. The reconstituted proteins should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles.

** Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL356HV), 40 µL in 96- or 384-well assay plates

**** The number of assay points is based on 20 µL in 384 well ProxiPlate using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	PerkinElmer Inc.	6050185
EnVision®-Alpha Reader	PerkinElmer Inc.	-

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
Anti-human PD-1 antibody	BioLegend	329912
Anti-human PD-L1 neutralizing antibody	ACRO Biosystems	PDL-NA002
Anti-human PD-L1 antibody (no blocking)	Sino Biological Inc.	10084-R015

Recommendations

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec). Re-suspend the beads by vortexing before use. Do not vortex the proteins.
- Use Milli-Q® grade H_2O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized proteins.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.

Competition Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The protocol described below is an ***example*** for generating three inhibition curves in a 40 µL final assay volume (150 wells, triplicate determinations). If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

			Volume				
Format	**# of data points**	**Final**	**Inhibitor Or Antibody**	**Biotinylated PD-1**	**His Tag PD-L1**	**Mix of SA-Donor beads Acceptor beads**	**Plate recommendation**
AL356HV	100	100 µL	25 µL	25 µL	25 µL	25 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL356C	200	100 µL	25 µL	25 µL	25 µL	25 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	40 µL	10 µL	10 µL	10 µL	10 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 000	20 µL	5 µL	5 µL	5 µL	5 µL	ProxiPlate™-384 Plus (cat # 6008280)
	2 000	10 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL356F	5 000	20 µL	5 µL	5 µL	5 µL	5 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	10 000	10 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	ProxiPlate-384 Plus (cat # 6008280)

One Incubation Step Protocol described as below:

1) Preparation of 1X AlphaLISA Immunoassay Buffer (for 10 mL):
   Add 1 mL of 10X AlphaLISA Immunoassay Buffer to 9 mL H_2O.

2) Preparation of serial dilutions of 4X anti-human PD-L1 or PD-1 antibody in 1x Immunoassay buffer as follows:

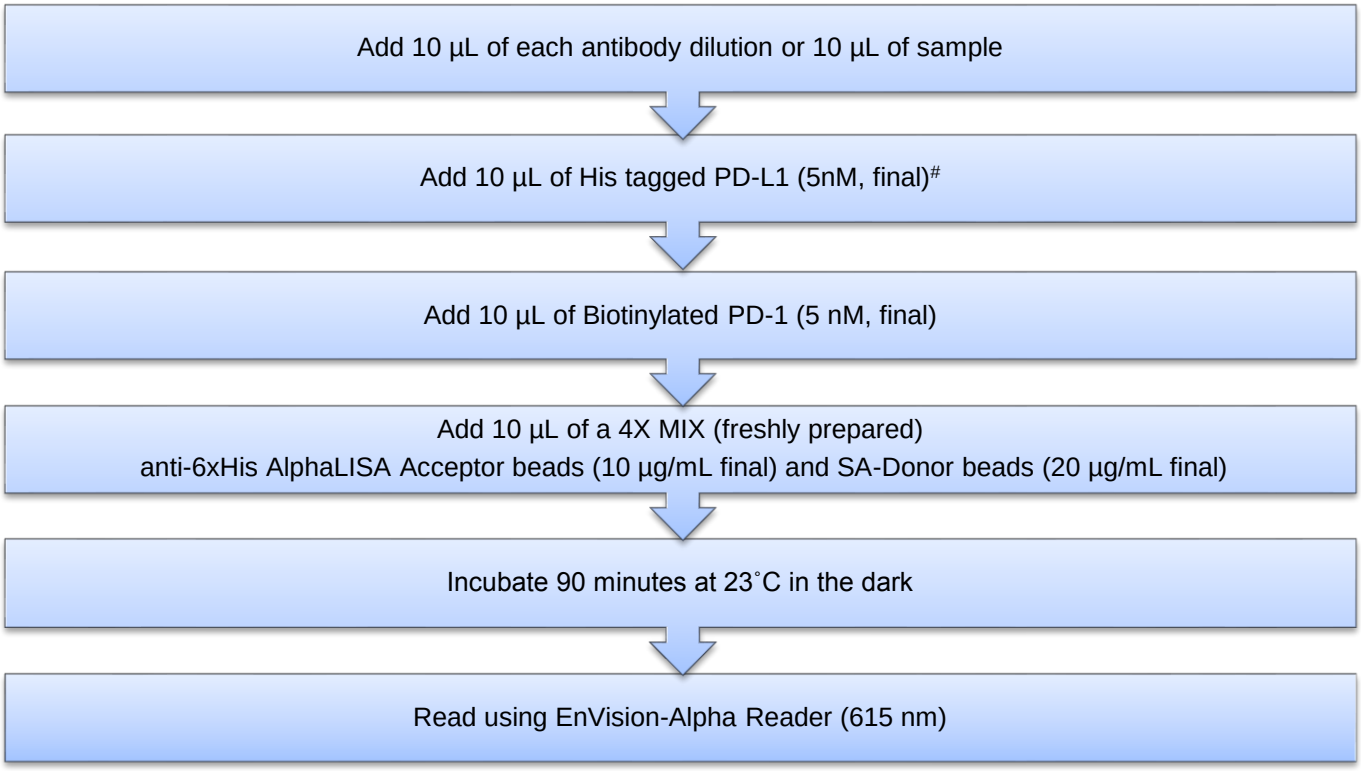
Tube	Volume of Antibody	Volume of 1X buffer	[Ab] (g/mL) (4X)	[Ab] (g/mL) (1X)
A	40 μg/mL stock	0	4.00E-05	1.00E-05
B	30 µL of tube A	70 µL	1.20E-05	3.00E-06
C	30 µL of tube B	60 µL	4.00E-06	1.00E-06
D	30 µL of tube C	70 µL	1.20E-06	3.00E-07
E	30 µL of tube D	60 µL	4.00E-07	1.00E-07
F	30 µL of tube E	70 µL	1.20E-07	3.00E-08
G	30 µL of tube F	60 µL	4.00E-08	1.00E-08
H	30 µL of tube G	70 µL	1.20E-08	3.00E-09
I	30 µL of tube H	60 µL	4.00E-09	1.00E-09
J	30 µL of tube I	70 µL	1.20E-09	3.00E-10
K	30 µL of tube J	60 µL	4.00E-10	1.00E-10
L	30 µL of tube K	70 µL	1.20E-10	3.00E-11
M	30 µL of tube L	60 µL	4.00E-11	1.00E-11
N	30 µL of tube M	70 µL	1.20E-11	3.00E-12
O	30 µL of tube N	60 µL	4.00E-12	1.00E-12
P	30 µL of tube O	70 µL	1.20E-12	3.00E-13

3) Preparation of 4X His tagged PD-L1 (20 nM):
   a. Reconstitute lyophilized PD-L1 (1.3 µg) in 100 µL H_2O to make 500 nM PD-L1
   b. Add 60 µL of 500 nM PD-L1 to 1440 µL 1X immunoassay buffer.

4) Preparation of 4X biotinylated PD-1 (20 nM):
   a. Reconstitute lyophilized PD-1 (2.13 µg) in 100 µL H_2O to make 500 nM PD-1
   b. Add 60 µL of 500 nM PD-1 to 1440 µL 1X immunoassay buffer.

5) Preparation of the mix of 4X Anti-6xHis AlphaLISA Acceptor beads (40 µg/mL) and 4X Streptavidin (SA) Donor beads (80 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 12 µL of 5 mg/mL Anti-6xHis AlphaLISA Acceptor beads and 24 µL of 5 mg/mL SA-Donor beads to 1464 µL of 1X AlphaLISA Immunoassay Buffer
   c. Prepare just before use.

6) In a white Optiplate (384 wells):

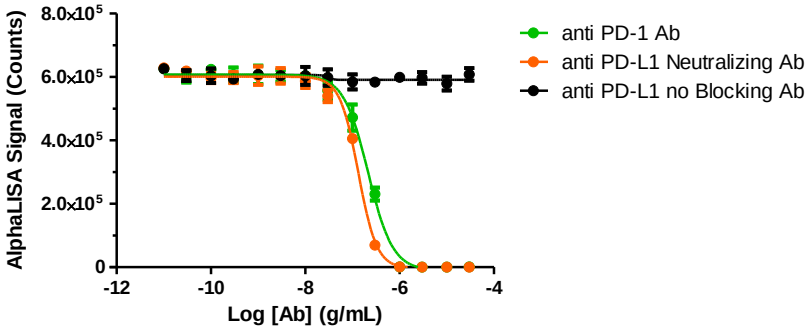


Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

If screening anti-PD-1 antibodies, add PD-1 first, then add PD-L1.

Typical competitive binding Data:

Antibodies competitive binding to PD-1:PD-L1(5nM:5nM)
40 µL total volume in OptiPlate



	anti PD-1 Ab	anti PD-L1 Neutralizing Ab
IC50	2.157e-007	1.334e-007

Figure 3. Antibody competition binding to PD-1/PD-L1. Black points showed anti-PD-L1 no Blocking antibody as a negative control. The IC_{50} values are 216 and 133 ng/mL for anti-PD-1 antibody (green points) and anti-PD-L1 neutralizing antibody (orange points) respectively and were calculated by using nonlinear regression fitting with GraphPad Prism 5.

Troubleshooting Guide

You will find below recommendations for common situations that you might encounter with your AlphaLISA Epigenetics detection assay. If further assistance is needed, do not hesitate to contact our technical support team for assistance.

Issue	Recommendations and Comments
High background signal	• Buffer is not freshly made. Make new. • Incubation time is longer than recommended range.
Low AlphaLISA signal	• Optimize EnVision with Plate format.
High variation between replicates or low Z' values	• Make sure that reagents are at the bottom of the well by tapping or swirling the plate gently on a smooth surface after each addition.

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at:

http://www.perkinelmer.com/in/resources/technicalresources/applicationsupportknowledgebase/alphalisa-alphascreen-no-washassays/alpha_troubleshoot.xhtml

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