MANUAL

AlphaLISA® PD-1 and PD-L2 (Human) Binding Kit

Product number: AL3128 C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed to assess inhibitors of human PD-1 and human PD-L2 binding, using a homogeneous AlphaLISA assay (no wash steps). This assay can facilitate the design and development of antibody therapeutics by using competitive binding to human PD-1/PD-L2 to complement Revvity's PD-1/PD-L1(Human) AlphaLISA Binding Kit (Cat# AL356).

Sensitivity: IC_{50}: 0.089 µg/mL (average, using anti hPD-L2 antibody as inhibitor, BioLegend Cat # 329610)

Signal to background ratio: 1500 using 3 nM hPD-1 and 3 nM hPD-L2

Kit contents: The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human PD-1, His tagged human PD-L2 and 10X Binding Assay Buffer.

Storage: The kit components must be stored at 4°C in the dark. Reconstituted proteins can be stored at -20°C for 3 months.

Stability: This kit is stable for at least 12 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Programmed cell death protein 1 (PD-1), also known as cluster of differentiation 279 (CD279), belongs to the immunoglobulin superfamily and is a transmembrane receptor protein. Programmed death ligand 2 (PD-L2), also known as B7-DC and CD273 is a member of the B7 family of proteins that provide signals for regulating T-cell activation and tolerance. PD-L2, together with PD-L1 (Programmed death ligand 1), are two ligands bound to PD-1 with comparable affinities. PD-L1 and PD-L2 compete for PD-1 binding and conversely, an antagonist PD-1 mAb blocks both PD-L1 and PD-L2 binding to PD-1 which strongly enhance T cell proliferation. Therefore, blocking PD-1 and PD-L2 or PD-L1 binding is considered as a promising therapeutic target for human autoimmune disease and malignant cancers.

Description of the AlphaLISA Assay

The AlphaLISA detection of human PD-1 and human PD-L2 binding uses anti-6xHis AlphaLISA® acceptor beads to capture the His tagged hPD-L2 and Streptavidin-coated donor beads to capture the biotinylated hPD-1. Donor beads and acceptor beads come into proximity through hPD-L2 binding to hPD-1. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp emission at 615 nm (Figure 1).

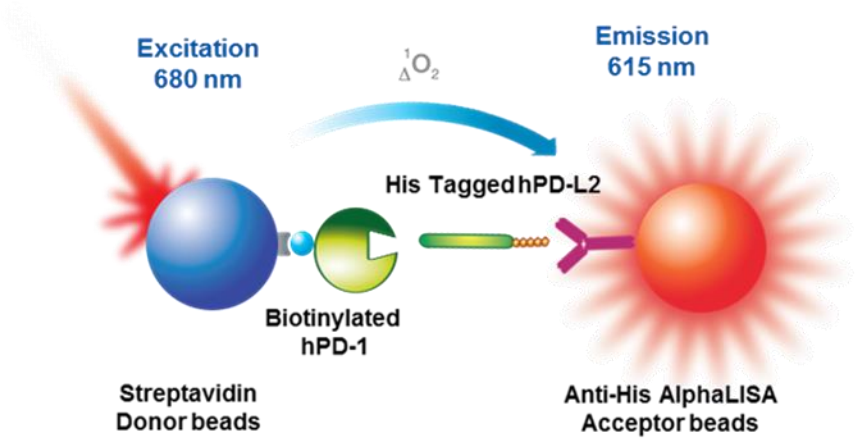


Figure 1. AlphaLISA Assay Principle.

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 proteins included in this kit is from a human source.

Kit Content: Reagents and Materials

Kit components	AL3128C*** (500 assay points)	AL3128F*** (5000 assay points)
Anti-6xHis AlphaLISA Acceptor beads stored in PBS, 0.05% Kathon CG/ICP, pH 7.2	20 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% Kathon CG/ICP, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	400 µL @ 5 mg/mL (1 brown tube, black cap)
Lyophilized hPD-1 (Biotinylated) *	1.33 µg, lyophilized (1 tube, clear cap)	1.33 µg, lyophilized (10 tubes, clear caps)
Lyophilized hPD-L2 (His tagged) *	0.702 µg, lyophilized (1 tube, clear cap)	0.702 µg, lyophilized (10 tubes, clear caps)
AlphaLISA 10X Binding Assay Buffer**	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute hPD-1 and hPD-L2 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 # AL018C: 10 mL, cat # AL018F: 100 mL).

*** The number of assay points is based on an assay volume of 20 µL in 384 well plates using the kit components at the recommended concentrations (3 nM).

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.

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	Revvity Inc.	6050185
AlphaPlate-384, Shallow Well (ProxiPlate)	Revvity Inc.	6008350 6008359
EnVision®-Alpha Reader	Revvity Inc.	-

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
Anti-human PD-L2 antibody	BioLegend	329610
mouse IgG2a k, control	BioLegend	401503
Anti-human PD-1 antibody	BioLegend	329926
mouse IgG1 k, control	BioLegend	400165
Anti-human PD-L1 antibody, control	R&D Systems	MAB1562
Human PD-L2	R&D Systems	1224-PL-100
Human PD-1	AcroBiosystems	PD1-H5257

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 proteins) 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 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 Plus 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 manual described below is an ***example*** for generating 500 assay points in a 20 µL final assay volume per point. If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

One Incubation Step Manual described as below:

1) Preparation of 1X Binding Assay Buffer
   Add 1 mL of 10X Binding Assay Buffer to 9 mL Milli-Q® grade H2O.

2) Serial dilutions of 4X anti hPD-1 or anti hPD-L2 antibody in 1X Assay Buffer as follows:

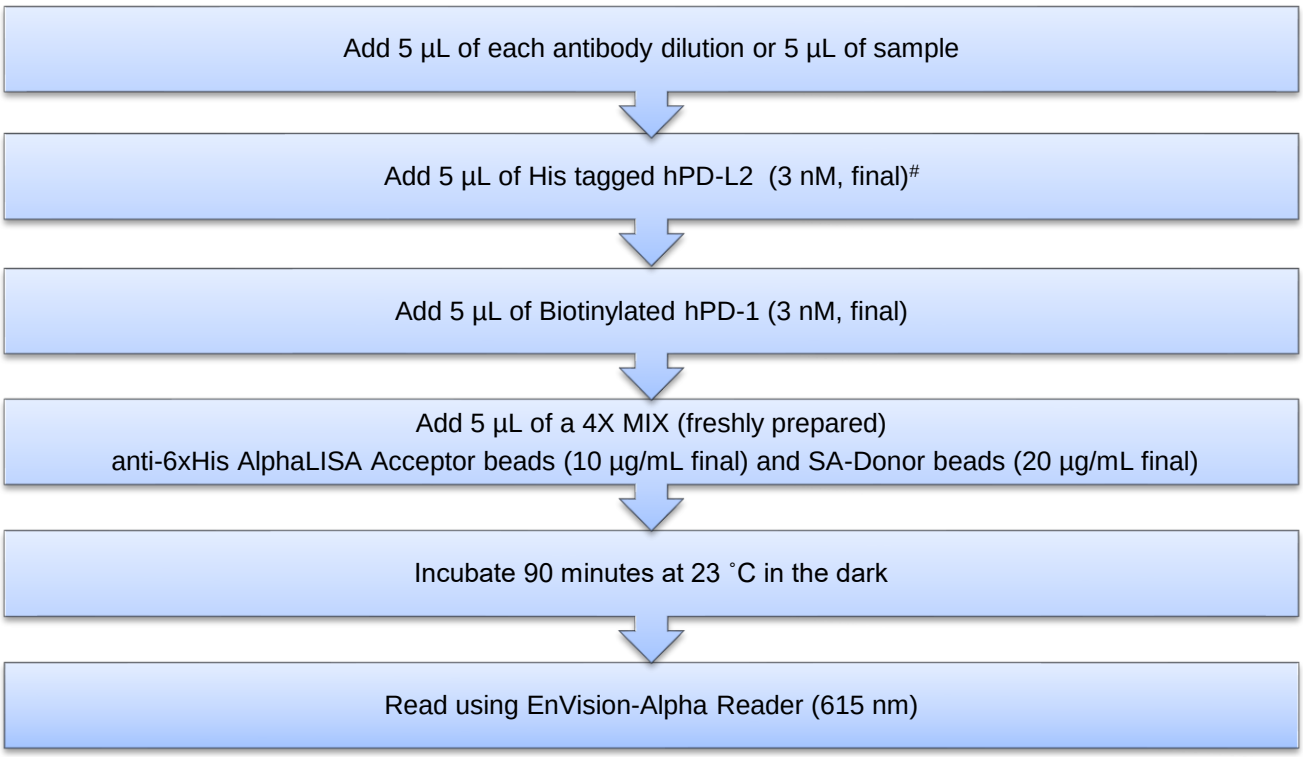
Tube	Volume of Antibody	Volume of 1X buffer	[Ab] (µg/mL) (4X)	[Ab] (µg/mL) (1X)
A	4µL of 1 mg/mL stock	96 µL	40	10
B	30 µL of tube A	70 µL	12	3
C	30 µL of tube B	60 µL	4	1
D	30 µL of tube C	70 µL	1.2	0.3
E	30 µL of tube D	60 µL	0.4	0.1
F	30 µL of tube E	70 µL	0.12	0.03
G	30 µL of tube F	60 µL	0.04	0.01
H	30 µL of tube G	70 µL	0.012	0.003
I	30 µL of tube H	60 µL	0.004	0.001
J	30 µL of tube I	70 µL	0.0012	0.0003
K	30 µL of tube J	60 µL	0.0004	0.0001
L	0	60 µL	0	0

3) Preparation of 4X His tagged hPD-L2 (12 nM):
   a. Reconstitute lyophilized hPD-L2 (0.702 µg) in 100 µL H_2O to make 300 nM hPD-L2.
   b. Add 100 µL of 300 nM hPD-L2 to 2400 µL 1X Binding Assay Buffer.
   c. Prepare just before use.

4) Preparation of 4X biotinylated hPD-1 (12 nM):
   a. Reconstitute lyophilized hPD-1 (1.33 µg) in 100 µL H_2O to make 300 nM hPD-1.
   b. Add 100 µL of 300 nM hPD-1 to 2400 µL 1X Binding Assay Buffer.
   c. Prepare just before use.

5) Preparation of 4X mix of Anti-6xHis AlphaLISA Acceptor beads (40 µg/mL) and Streptavidin (SA) Donor beads (80 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 20 µL of 5 mg/mL Anti-6xHis AlphaLISA Acceptor beads and 40 µL of 5 mg/mL SA-Donor beads to 2440 µL of 1X Binding Assay Buffer.
   c. Prepare just before use.

6) In a shallow ProxiPlate (384 wells):

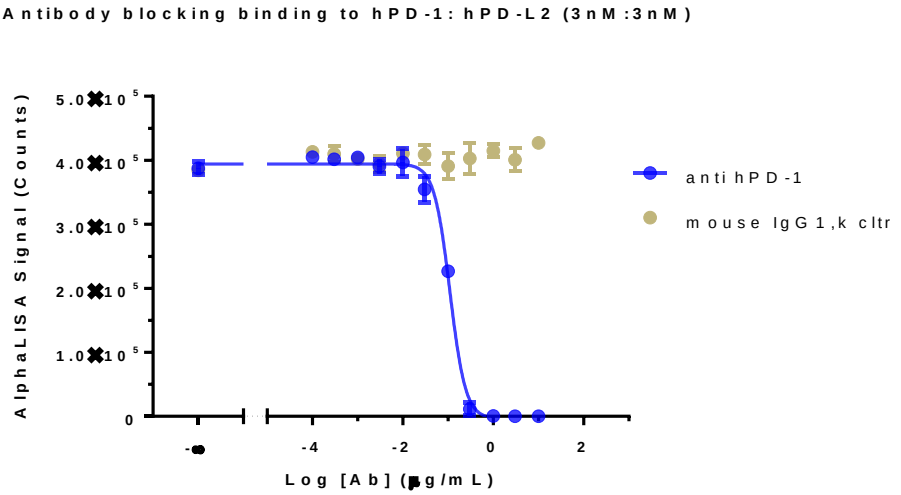


Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance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

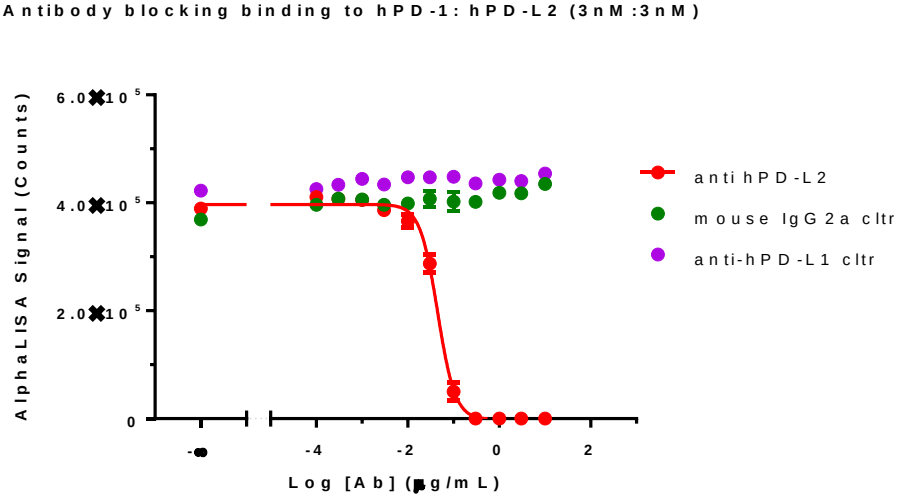
If screening anti-hPD-1 antibodies, add hPD-1 first, then add hPD-L2.

Typical competitive binding Data:

(A)



(B)



(C)

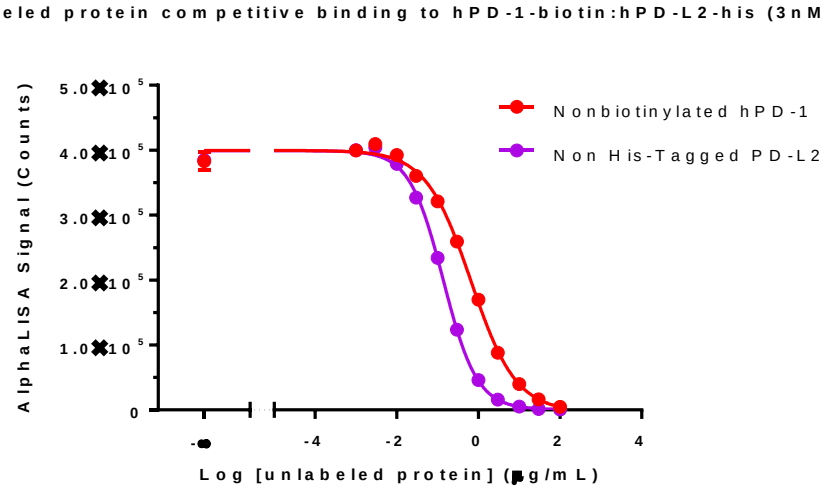


Figure 2. Competitive Binding: (A) Anti-hPD-1 antibody blocking hPD-1/hPD-L2 binding with IC_{50} = 0.11 µg/mL. Mouse IgG1, κ was measured as a negative control. (B) Anti-hPD-L2 antibody blocking hPD-1/hPD-L2 binding with IC_{50} = 0.045 µg/mL. Mouse IgG2a, κ was measured as a negative control. (C) Nonbiotinylated hPD-1 competitive binding to hPD-L2: the IC_{50} was 0.66 µg/mL (15.5 nM) and Non-His-tagged human PD-L2 competitive binding to hPD-1: the IC_{50} was 0.14 µg/mL (2.86 nM). All IC_{50} values were calculated by using nonlinear regression fitting with GraphPad Prism 7.

Troubleshooting Guide

You will find below recommendations for common situations that you might encounter with your AlphaLISA binding 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.

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

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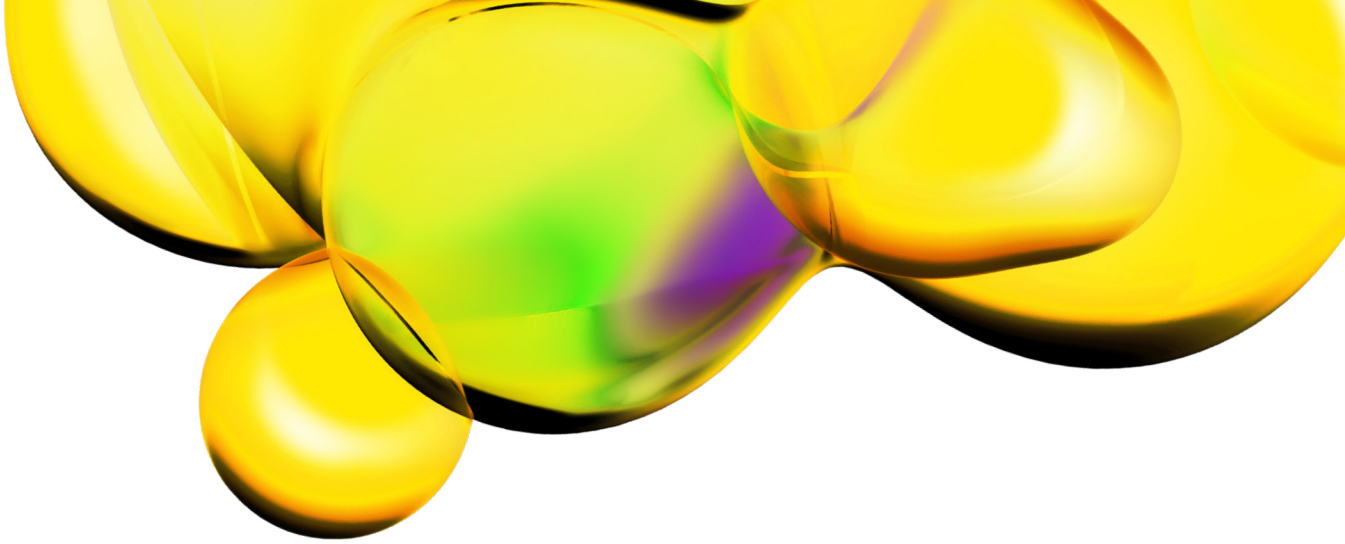

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