MANUAL

AlphaLISA® PD-1 and PD-L1 (Mouse) Binding Kit

Product number: AL580/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed to assess inhibitors of mouse PD-1 and mouse PD-L1 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 mouse PD-1/PD-L1 in combination with the human PD-1/PD-L1 kit, AL356.

Sensitivity: IC_{50}: 0.072 µg/mL (average, using anti mPD-1 antibody, BioLegend Cat # 135204)

Signal to background ratio: 816 using 6 nM mPD-1 and 6 nM mPD-L1

Kit contents: The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated mouse PD-L1, His tagged mouse PD-1 and AlphaLISA Immunoassay 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 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. Mouse PD-1 shares 65% with human PD-1 extracellular domain (ECD) and mouse PD-L1 shares 73% with human PD-L1 ECD. PD-L1 and 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 tumors 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 autoimmune disease and malignant cancers.

Description of the AlphaLISA Assay

The AlphaLISA detection of mouse PD-1 and mouse PD-L1 binding uses anti-6xHis AlphaLISA® acceptor beads to capture the His tagged mPD-1 and Streptavidin-coated donor beads to capture the biotinylated mPD-L1. Donor beads and acceptor beads come into proximity through mPD-L1 binding to mPD-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 peak of light emission at 615 nm (Figure 1).

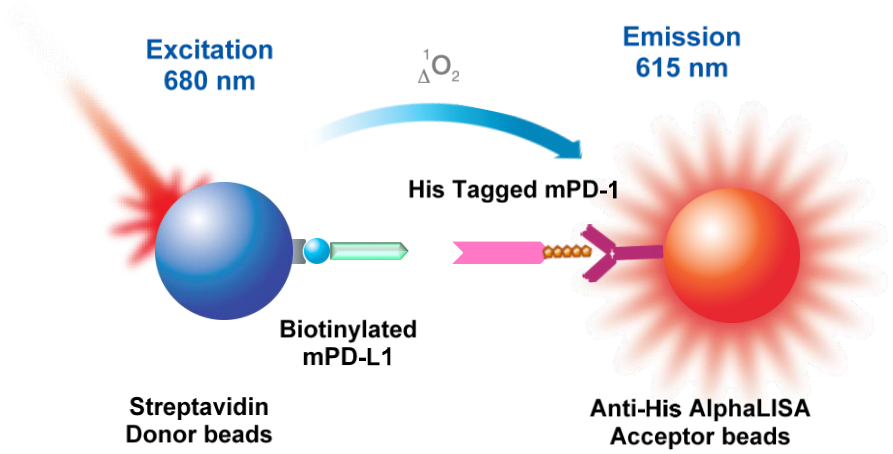


Figure 1. AlphaLISA Assay Principle.

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 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.

Kit Content: Reagents and Materials

Kit components	AL580C*** (500 assay points)	AL580F*** (5000 assay points)
Anti-6xHis AlphaLISA Acceptor beads stored in PBS, 0.05% Kathon, 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, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	400 µL @ 5 mg/mL (1 brown tube, black cap)
Lyophilized Mouse PD-L1(Biotinylated)*	3.08 µg, lyophilized (1 tube, clear cap)	3.08 µg, lyophilized (10 tubes, clear caps)
Lyophilized Mouse PD-1(His tagged)*	1.02 µg, lyophilized (1 tube, clear cap)	1.02 µg, lyophilized (10 tubes, clear caps)
AlphaLISA Immunoassay Buffer (10X)**	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute mPD-1 and mPD-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 20 µL in 384 well plates 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.

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-mouse PD-1 antibody	BioLegend	135204
Anti-mouse PD-L1 antibody	BioLegend	124304
Rat IgG2a k, control	BioLegend	400515
Rat IgG2b k, control	BioLegend	400621
Mouse PD-L1	R&D Systems	1019-B7

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 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 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 six inhibition curves in a 20 µL final assay volume (216 wells, triplicate determinations). 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 AlphaLISA Immunoassay Buffer (for 10 mL):
   Add 1 mL of 10X AlphaLISA Immunoassay Buffer to 9 mL H_2O.

2) Preparation of 40 µg/mL stock antibody in AlphaLISA immunoassay buffer

3) Preparation of serial dilutions of 4X anti mPD-1 or anti mPD-L1 antibody in 1X AlphaLISA immunoassay buffer as follows:

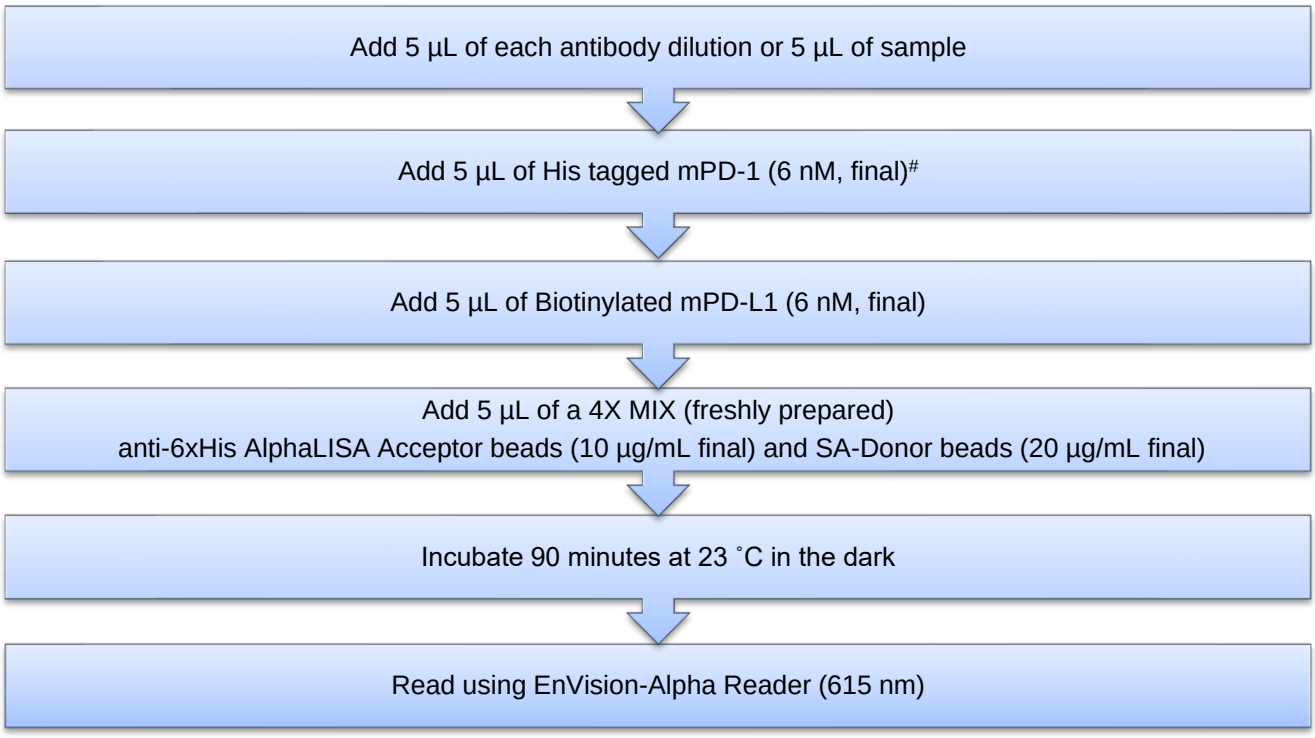
Tube	Volume of Antibody	Volume of 1X buffer	[Ab] (g/mL) (4X)	[Ab] (g/mL) (1X)
A	100 µL of 40 µg/mL stock	0	4.00E-05	1.00E-05
B	30 µL of tube A	70 µL	1.20E-05	3.00E-06
C	30 µL of tube B	60 µL	4.00E-06	1.00E-06
D	30 µL of tube C	70 µL	1.20E-06	3.00E-07
E	30 µL of tube D	60 µL	4.00E-07	1.00E-07
F	30 µL 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	0	70 µL	0	0

4) Preparation of 4X His tagged mPD-1 (24 nM):
   a. Reconstitute lyophilized mPD-1 (1.02 µg) in 100 µL H_2O to make 600 nM mPD-1.
   b. Add 48 µL of 600 nM mPD-1 to 1152 µL 1X AlphaLISA immunoassay buffer.
   c. Prepare just before use.

5) Preparation of 4X biotinylated mPD-L1 (24 nM):
   a. Reconstitute lyophilized mPD-L1 (3.08 µg) in 100 µL H_2O to make 600 nM mPD-L1.
   b. Add 48 µL of 600 nM mPD-L1 to 1152 µL 1X AlphaLISA immunoassay buffer.
   c. Prepare just before use.

6) 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 10 µL of 5 mg/mL Anti-6xHis AlphaLISA Acceptor beads and 20 □L of 5 mg/mL SA-Donor beads to 1220 µL of 1X AlphaLISA Immunoassay Buffer.
   c. Prepare just before use.

7) In a ProxiPlate (384 wells):

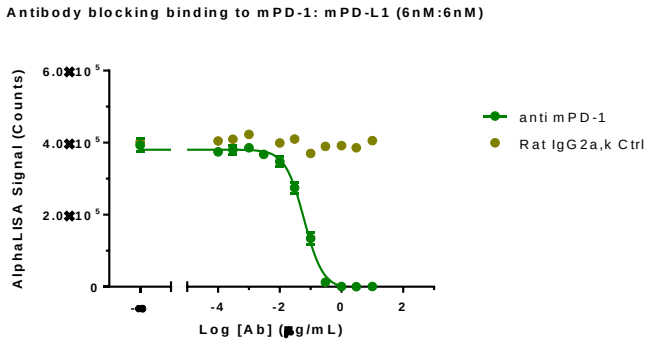


Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance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

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

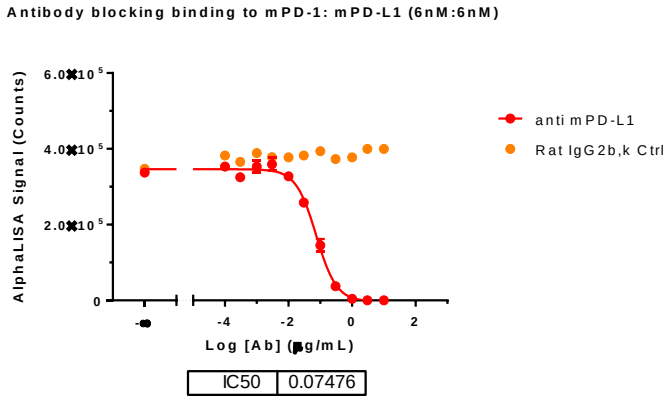
Typical competitive binding Data:

(A)



IC50	0.06152

(B)



(C)

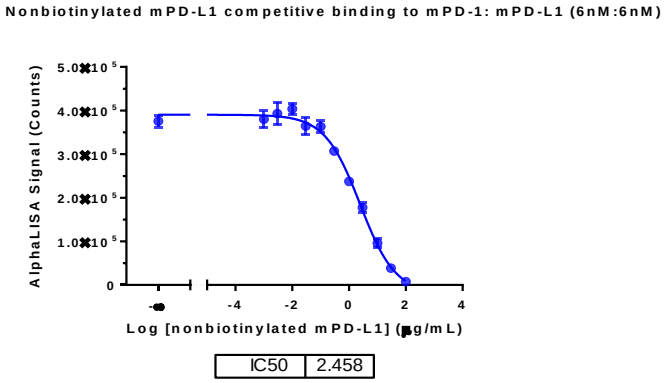


Figure 2. Competitive Binding: (A) anti-mPD-1 antibody blocking mPD-1/mPD-L1 binding with IC_{50} = 0.06 µg/mL. Rat IgG2a, κ was measured as a negative control. (B) anti-mPD-L1 antibody blocking mPD-1/mPD-L1 binding with IC_{50} = 0.07 µg/mL. Rat IgG2b, κ was measured as a negative control. (C) Nonbiotinylated mPD-L1 competitive binding to mPD-1.The IC_{50} was 2.46 µg/mL (48 nM). All IC_{50} values were calculated by using nonlinear regression fitting with GraphPad Prism 7.

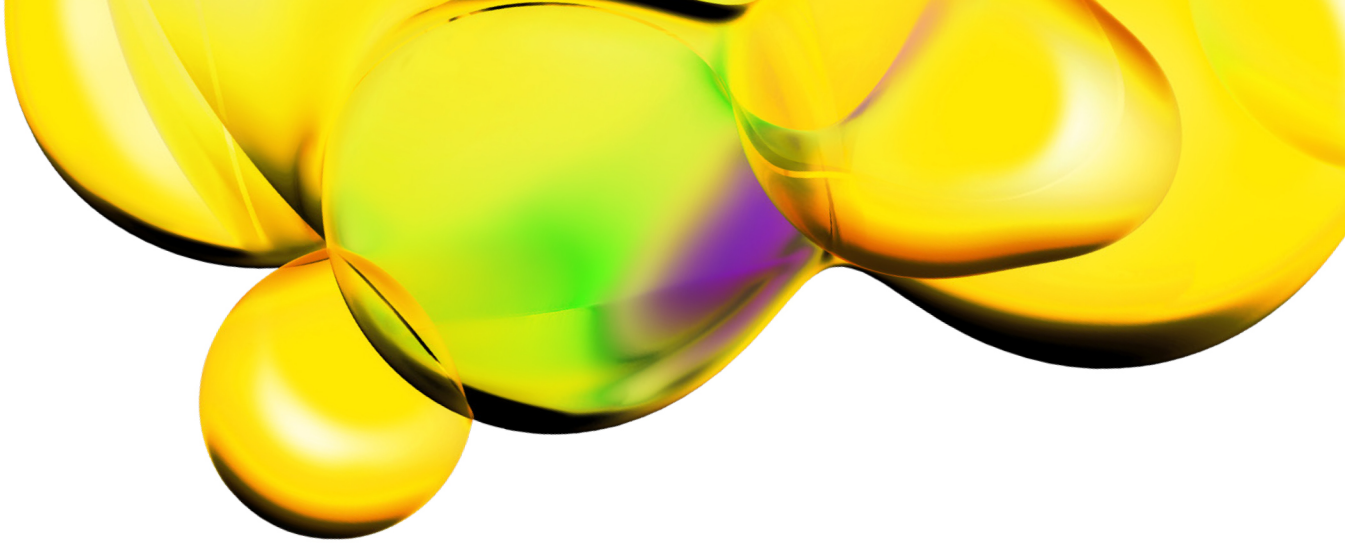
Troubleshooting Guide

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

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.



www.revvity.com

revvity

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com