TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

CTLA-4 and CD86 (Human) Binding AlphaLISA Kit

Product number: AL3047

Caution: Research Use Only. Not for use in diagnostic procedures

Contents

Product Information

Application: This kit is designed for the detection of binding activity between Human CTLA-4 and CD86, using a homogeneous AlphaLISA assay (no wash steps). This assay can facilitate the design and development of antibody therapetics by using competitive binding to CTLA-4/CD86.

Sensitivity: $K_{d(app):}$ 1.02 nM (average) using 2 nM CD86

Signal to background ratio: 209 (average) using 2 nM CD86

Kit contents: The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human CD86, His tagged human CTLA-4 and 5X AlphaLISA Universal 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.

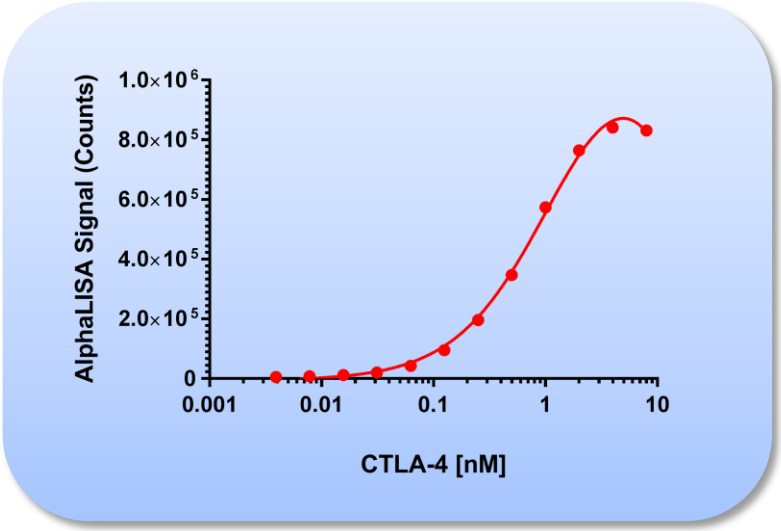


Figure 1. Typical Binding curve (2 nM of CD86) in AlphaLISA Universal Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

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

Human Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), also known as CD152 (cluster of differentiation 152), is a cell membrane receptor and a member of immunoglobulin superfamily. CTLA-4 is expressed once a T cell becomes active and modulates T cell signals by blocking the CD80 (B7.1) and CD86 (B7.2) ligands from binding to CD28. CTLA-4, functioning as an immune checkpoint, downregulates T cell immune responses. Because of its profound inhibitory role blocking CTLA-4 and CD80 or CD86 binding has been considered as promising therapeutic target for human autoimmune disease and 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 CD86 binds to the Streptavidin-coated Alpha Donor beads, while His tagged CTLA-4 is captured by Anti-His AlphaLISA Acceptor beads. When CD86 binding to CTLA-4 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).

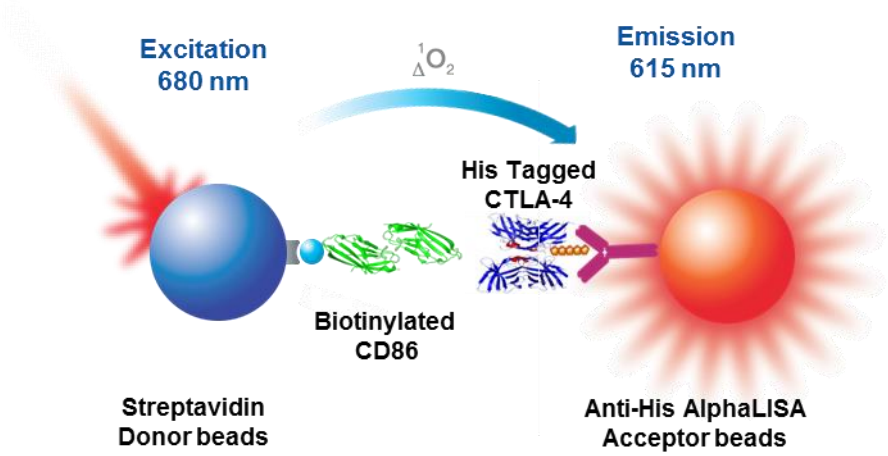


Figure 2. AlphaLISA Assay Principle.

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.
- The Biotinylated sample contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3047C (500 assay points)***	AL3047F (5000 assay points)***
Anti-6xHis AlphaLISA Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	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)	200 µL @ 5 mg/mL (1 brown tube, black cap)
Lyophilized Human CTLA-4 (His tagged)*	1.28 µg, lyophilized (2 tubes, clear caps)	1.28 µg, lyophilized (7 tubes, clear caps)
Lyophilized Human CD86 (Biotinylated)*	2.595 µg, lyophilized (1 tubes, clear cap)	2.595 µg, lyophilized (4 tubes, clear caps)
AlphaLISA Universal Buffer (5X)**	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute CTLA-4 and CD86 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 # AL001C: 10 mL, cat # AL001F: 100 mL).

*** The number of assay points is based on an assay volume of 40 µL in 96- or 384-well assay plates or 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. 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 CTLA-4 neutralizing antibody	BPS Biosystems	71212
Anti-human CD80 antibody	R&D Systems	MAB140
Anti-human CD86 antibody	R&D Systems	MAB141

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 5X AlphaLISA Universal 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 by briefly centrifuging the plate (1000g, 10-15 sec) after adding samples.
- 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.

Antibody Inhibitory 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 (117 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**	**His Tag CTLA-4**	**Biotinylated CD86**	**Mix of SA-Donor beads and anti His Acceptor beads**	**Plate recommendation**
AL3047C	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)
AL3047F	5 000	20 µL	5 µL	5 µL	5 µL	5 µL	ProxiPlate-384 Plus (cat # 6008280)
	10 000	10 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

One Incubation Step Protocol described as below:

1) Preparation of 1X AlphaLISA Universal Buffer (for 10 mL):
   Add 2 mL of 10X AlphaLISA Universal Buffer to 8 mL H_2O.

2) Preparation of serial dilutions of 4X antibody in 1x Universal buffer as follows:

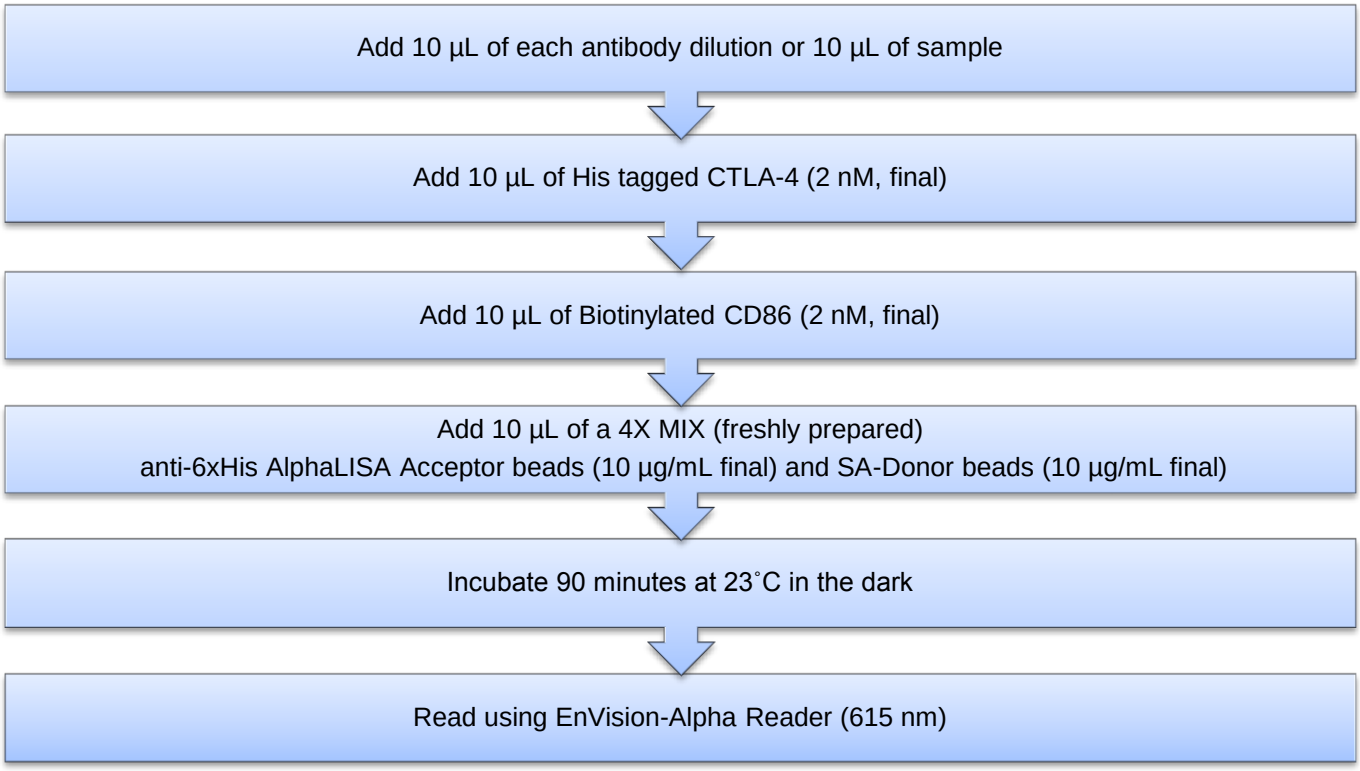
Tube	Volume of Antibody	Volume of 1X buffer	[Ab] (g/mL) (4X)	[Ab] (µg/mL) (1X)
A	0.12 mg/mL stock	0	1.20E-04	30
B	30 µL of tube A	60 µL	4.00E-05	10
C	30 µL of tube B	70 µL	1.20E-05	3
D	30 µL of tube C	60 µL	4.00E-06	1
E	30 µL of tube D	70 µL	1.20E-06	0.3
F	30 µL of tube E	60 µL	4.00E-07	0.1
G	30 µL of tube F	70 µL	1.20E-07	0.03
H	30 µL of tube G	60 µL	4.00E-08	0.01
I	30 µL of tube H	70 µL	1.20E-08	0.003
J	30 µL of tube I	60 µL	4.00E-09	0.001
K	30 µL of tube J	70 µL	1.20E-09	0.0003
L	30 µL of tube K	60 µL	4.00E-10	0.0001
M	0	100 µL	0	0

3) Preparation of 4X His tagged CTLA-4 (8 nM):
   a. Reconstitute lyophilized CTLA-4 (1.28 µg) in 100 µL H_2O to make 320 nM CTLA-4
   b. Add 30 µL of 320 nM CTLA-4 to 1170 µL 1X Universal buffer.

4) Preparation of 4X biotinylated CD86 (8 nM):
   a. Reconstitute lyophilized CD86 (2.595 µg) in 100 µL H_2O to make 500 nM CD86
   b. Add 19.2 µL of 500 nM CD86 to 1180.8 µL 1X Universal buffer.

5) Preparation of the mix of 4X Anti-6xHis AlphaLISA Acceptor beads (40 µg/mL) and 4X Streptavidin (SA) Donor beads (40 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 9.6 µL of 5 mg/mL Anti-6xHis AlphaLISA Acceptor beads and 9.6 µL of 5 mg/mL SA-Donor beads to 1180.8 µL of 1X AlphaLISA Universal 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).

Typical Inhibition Data:

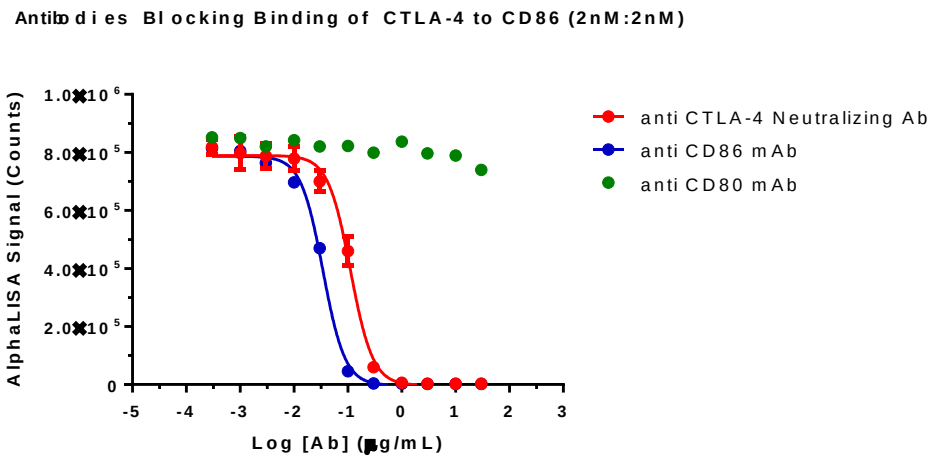


Figure 3. Antibodies blocking binding of CTLA-4 to CD86. Green points showed anti-CD80 antibody as a negative control. The IC_{50} values are 112 and 34.6 ng/mL for anti-CTLA-4 neutralizing antibody (red points) and anti-CD86 monoclonal antibody (blue points) respectively and were calculated by using 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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