TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Research Use Only. Not for use in diagnostic procedures.

CD28 and CD86 (Human) Binding AlphaLISA Kit

Product No.: AL3131C/F

Contents

Product Information

Application: This kit is designed to assess inhibitors of human CD28 and human CD86 binding, using a homogeneous AlphaLISA assay (no wash steps). This assay can facilitate the design and development of antibody therapetics by using competitive binding to human CD28/CD86 to complement PerkinElmer's CTLA-4/CD86 (Human) AlphaLISA Binding kit (Cat# AL3047)

Sensitivity: $IC_{50:}$ 0.07 µg/mL (average, using anti-hCD28 antibody, ThermoFisher Cat # MA1-10166)

Signal to background ratio: 723 using 5 nM human CD28 and 5 nM human CD86

Kit contents: The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human CD86, His tagged human CD28 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 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals 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 and minimum counts may vary between bead lots and the instrument used**.**

Analyte of Interest

CD28 (Cluster of Differentiation 28) is a member of the immunoglobulin gene superfamily. It is the receptor for CD80 (Cluster of Differentiation 80, also call B7.1) and CD86 (Cluster of Differentiation 86, also call B7.2) proteins. CD28 is predominantly expressed on all naive T cells and is stimulated by CD80 and CD86. CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) competes with CD28 for binding to CD80 and CD86. CD28 is a highly expressed but low-affinity receptor and an enhancer for T-cell activation. CD80 and CD86 are found on professional antigen-presenting cells and are also involved in innate immune responses. Therefore, blocking CD28 and CD86 (or CD80) binding is considered as a promising therapeutic target for autoimmune disease and malignant cancers.

Description of the AlphaLISA Assay

The AlphaLISA detection of human CD28/CD86 binding uses anti-6xHis AlphaLISA® acceptor beads to capture the His tagged CD28 and Streptavidin-coated donor beads to capture the biotinylated CD86. Donor beads and acceptor beads come into proximity through CD86 binding to CD28. 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).

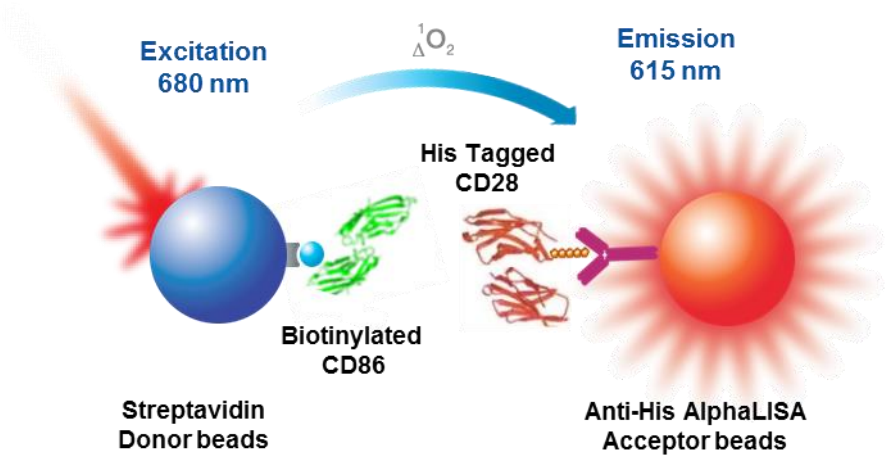


Figure 1. AlphaLISA Assay Principle.

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 are from a human source.

Kit Content: Reagents and Materials

Kit components	AL3131C*** (500 assay points)	AL3131F*** (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	20 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)
Lyophilized CD86 (Biotinylated) *	2.69 µg, lyophilized (1 tube, clear cap)	2.69 µg, lyophilized (10 tubes, clear caps)
Lyophilized CD28 (His tagged) *	2.12 µg, lyophilized (1 tube, clear cap)	2.12 µg, lyophilized (10 tubes, clear caps)
AlphaLISA 10X Binding Assay Buffer**	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute human CD28 and human CD86 in 100 µL Milli-Q® grade H_2O respectively. The reconstituted proteins should be used freshly 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 (5 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	PerkinElmer Inc.	6050185
AlphaPlate-384, Shallow Well (ProxiPlate)	PerkinElmer Inc.	6008350 6008359
EnVision®-Alpha Reader	PerkinElmer Inc.	-

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
Human CD28 Monoclonal Antibody	ThermoFisher	MA1-10166
mouse IgG1 k, control	BioLegend	400165
Human B7-2/CD86 Antibody	R&D Systems	MAB141-100
Human CTLA-4, Fc Chimera	R&D Systems	7268-CT-100
Human CD28 Fc, CF	R&D Systems	342-CD-200
Human B7-2 / CD86 Protein, Fc Tag	Acro Biosystems	CD6-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 volume 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 protocol 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 protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

One Incubation Step Protocol described as below:

1) Preparation of 1X Binding Assay Buffer
Add 1 mL of 10X Binding Assay Buffer to 9 mL Milli-Q® grade H_2O.

2) Serial dilutions of 4X anti-hCD28 or anti-hCD86 antibody in 1X Binding Assay Buffer as follows:

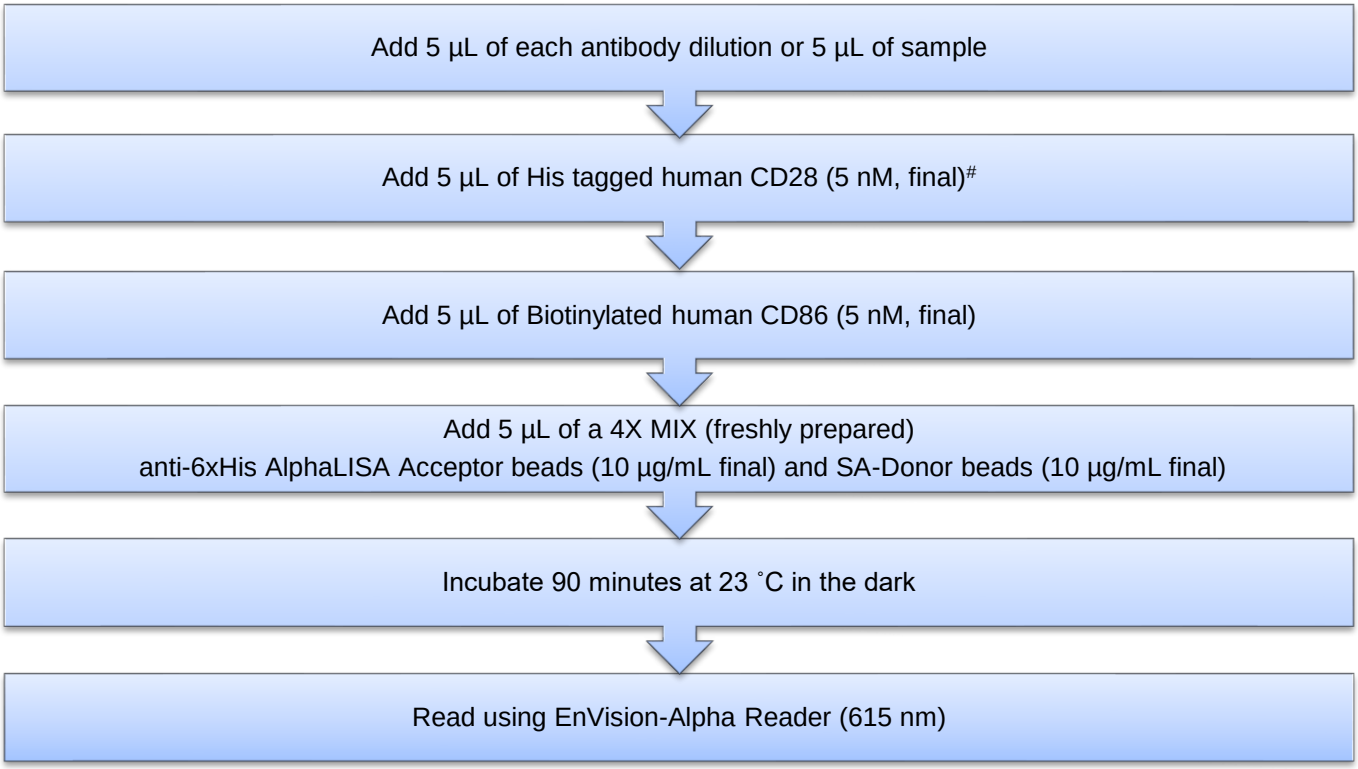
Tube	Volume of Antibody	Volume of buffer	[Ab] (g/mL) (4X)	[Ab] (g/mL) (1X)
A	4 µL of 1 mg/mL stock	96 µL	4.00E-05	1.00E-05
B	30 µL of tube A	70 µL	1.20E-05	3.00E-06
C	30 µL of tube B	60 µL	4.00E-06	1.00E-06
D	30 µL of tube C	70 µL	1.20E-06	3.00E-07
E	30 µL of tube D	60 µL	4.00E-07	1.00E-07
F	30 µL of 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

3) Preparation of 4X His tagged human CD28 (20 nM):
   a. Reconstitute lyophilized human CD28 (2.12 µg) in 100 µL H_2O to make 500 nM human CD28.
   b. Add 100 µL of 500 nM human CD28 to 2400 µL 1X Binding Assay Buffer.
   c. Prepare just before use and diluted CD28 should be added to the assay plate in 10 minutes

4) Preparation of 4X biotinylated human CD86 (20 nM):
   a. Reconstitute lyophilized human CD86 (2.69 µg) in 100 µL H_2O to make 500 nM human CD86.
   b. Add 100 µL of 500 nM human CD86 to 2400 µL 1X Binding Assay Buffer.
   c. Prepare just before use and diluted CD86 should be added to the assay plate in 10 minutes

5) Preparation of 4X mix of Anti-6xHis AlphaLISA Acceptor beads (40 µg/mL) and Streptavidin (SA) Donor beads (40 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 20 µL of 5 mg/mL Anti-6xHis AlphaLISA Acceptor beads and 20 µL of 5 mg/mL SA-Donor beads to 2460 µL of 1X Binding Assay Buffer.
   c. Prepare just before use.

6) In a Shallow ProxiPlate (384 wells):

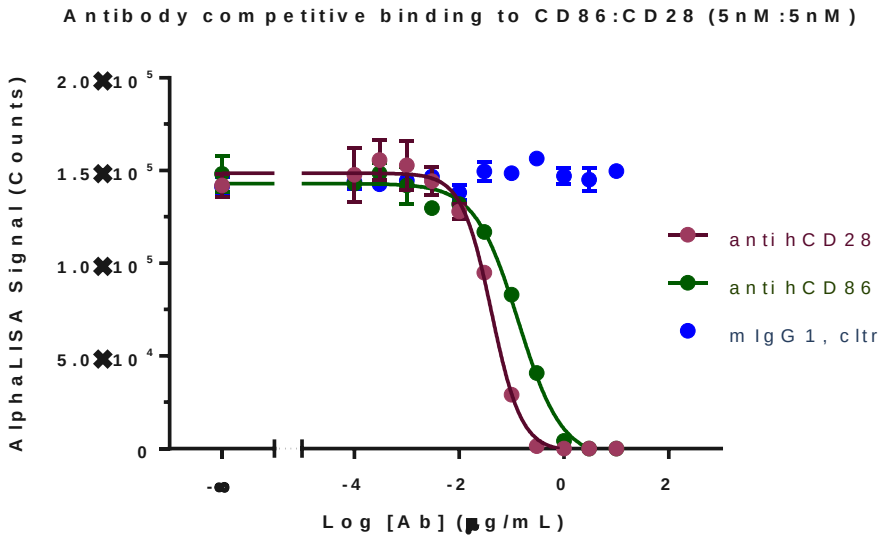


Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance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

If screening anti-hCD86 antibodies, add human CD86 first, then add human CD28.

Typical competitive binding Data:

(A)



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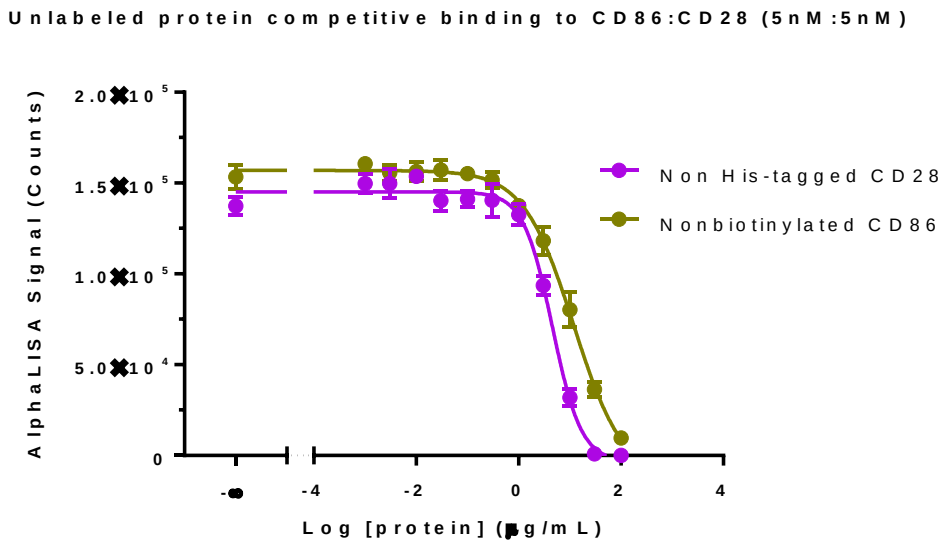

(C)

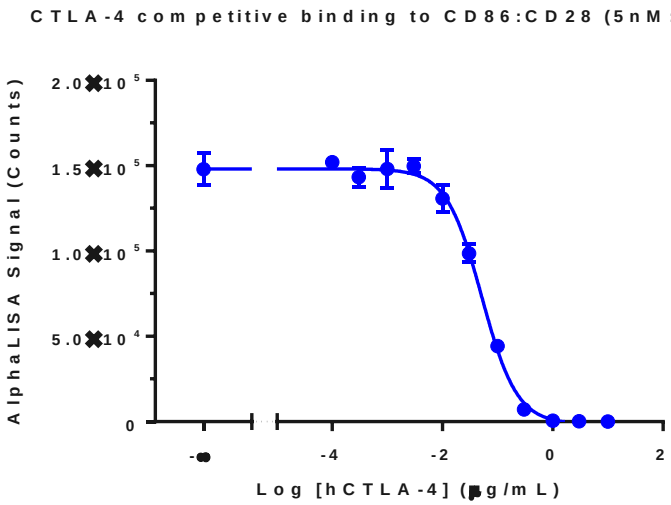

Figure 2. Competitive Binding: (A) Anti hCD28 body blocking human CD28/CD86 binding with IC_{50} = 0.04 µg/m. Anti-hCD86 antibody block human CD28/CD86 binding with IC_{50} = 0.14 µg/m. Mouse IgG1, κ was measured as a negative control. (B) Non His-tagged human CD28 competitive binding to human CD86. The IC_{50} was 4.58 µg/mL (109 nM). Nonbiotinylated human CD86 competitive binding to human CD28. The IC_{50} was 11.9 µg/mL (231 nM). (C) Human CTLA-4 competitive binding to human CD86. The IC_{50} was 0.051 µg/mL (1.28 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:

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

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