TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Research Use Only. Not for use in diagnostic procedures.

HLA DRA (alpha chain) and LAG-3 (Human) Binding AlphaLISA Kit

Product No.: AL3066

Contents

Product Information

Application: This kit is designed for the detection of binding activity between Human HLA DRA and LAG-3, using a homogeneous AlphaLISA assay (no wash step). This assay can facilitate the design and development of antibody therapeutics by using competitive binding to HLA DRA and LAG-3.

IC_{50}: 19 nM (average) using 10 nM:10 nM (HLA DRA: LAG-3) with unlabeled LAG-3

Signal to background ratio: 16 (average) using 10 nM:10 nM (HLA DRA: LAG-3) with unlabeled LAG-3

Kit contents: The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human LAG-3, His tagged human HLA DRA and PPI buffer (5X).

Storage: The beads must be stored at 4 ˚C in the dark. The target proteins must be stored at -20 °C. The 5X PPI buffer must be stored at 4 ˚C.

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.

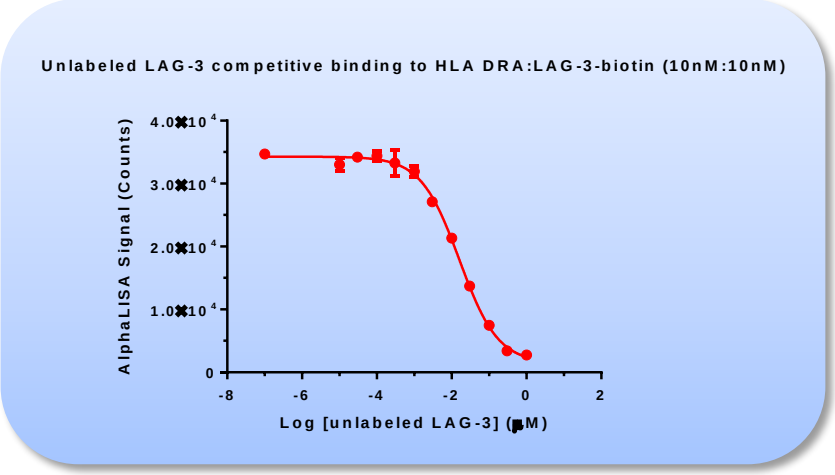

Figure 1. Typical inhibition curve in AlphaLISA 1X PPI Assay Buffer. The data was generated using a Proxiplate™-384-SW microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals and the apparent binding dissociation constant IC_{50} 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 IC_{50} measurement.

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Analyte of Interest

Human Leukocyte Antigen (HLA) is an MHC (major histocompatibility complex) class II cell surface receptor. HLA DRA is one of Antigen D related HLA class II (HLA DR) alpha chain paralogues. This class II molecule is a heterodimer consisting of an alpha and a beta chain, both anchored in the membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Lymphocyte activation gene-3 (LAG-3), also known as CD223, is a member of the immunoglobulin superfamily. LAG-3 binds to MHC class II with higher affinity, providing negative regulation of T cell receptor signaling. Binding of a homodimerized LAG-3/Ig fusion protein to MHC class II molecules induces maturation of immature dendritic cells and secretion of cytokines. Deletion of LAG-3 facilitates anti-cancer immune response, also blocks self-tolerance and increases susceptibility to autoimmune diseases. Because of its profound inhibitory role, blocking HLA DR and LAG-3 binding has been considered as promising therapeutic target for human autoimmune disease and cancers.

Description of the AlphaLISA Assay

AlphaLISA technology allows detecting the binding of target proteins in a highly sensitive, quantitative, reproducible and user-friendly mode. In this AlphaLISA assay, a biotinylated LAG-3 binds to the Streptavidin-coated Alpha Donor beads, while His-tagged HLA DRA is captured by Anti-His AlphaLISA Acceptor beads. When LAG-3 binding to HLA DRA 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).

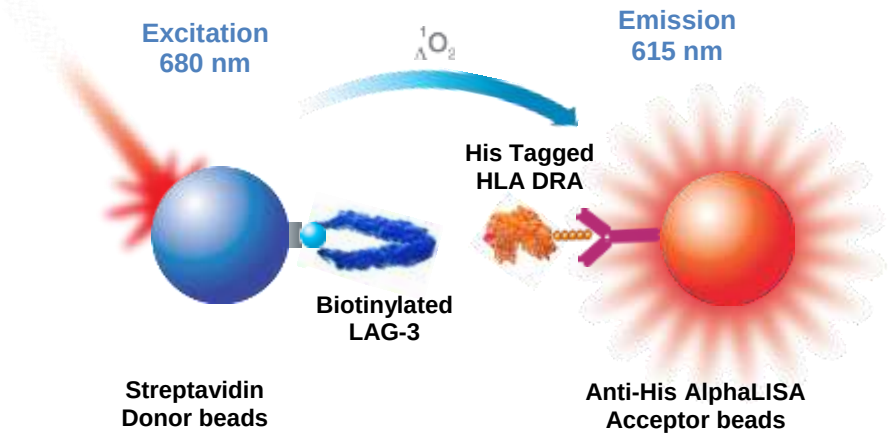

Figure 2. AlphaLISA Assay Principle.

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.

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Kit Content: Reagents and Materials

Kit components	AL3066C (500 assay points)**	AL3066F (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)	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	80 µL @ 5 mg/mL (1 brown tube, black cap)	800 µL @ 5 mg/mL (1 brown tube, black cap)
Human LAG-3 (Biotinylated), 5 µM	20 µL (1 tube, clear cap)	200 µL (1 tube, clear cap)
Human HLA DRA (His tagged), 0.5 mg/mL	10 µL (1 tube, clear cap)	100 µL (1 tube, clear cap)
PPI Buffer (5X)*	10 mL, 1 small bottle	100 mL, 1 large bottle

* Extra buffer can be ordered separately (cat # AL015C: 10 mL, cat # AL015F: 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.

Specific additional required reagents and materials:

The following material is recommended:

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

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
Human LAG-3 /CD223	AcroBioSystems	LA3-H5255
Human LAG-3 antibody	R&D Systems	AF2319
Goat IgG	R&D Systems	AB-108-C

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 before use to improve recovery of content (2000g, 10-15 sec).
  Re-suspend the beads by vortexing before use. Do not vortex the 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 the microplate with a lid or a plate sealing film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaScreen standard settings for Proxiplates (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 20 µL final assay volume (500 wells). 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 HLA DRA**	**Biotinylated LAG-3**	**Mix of SA-Donor beads and anti His Acceptor beads**	**Plate recommendation**
AL306 6C	250	40 µL	10 µL	10 µL	10 µL	10 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290)
	500	20 µL	5 µL	5 µL	5 µL	5 µL	ProxiPlate™-384 SW (cat # 6008350) ProxiPlate™-384 HS (cat # 6008270)
	1 000	10 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	ProxiPlate™-384 SW (cat # 6008350)

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AL306 6F	5 000	20 µL	5 µL	5 µL	5 µL	5 µL	ProxiPlate™-384 SW (cat # 6008350)
	10 000	10 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

Protocol for anti LAG-3 antibody inhibition of HLA DRA and LAG-3 Binding AlphaLISA Assay

1) Preparation of 1X PPI Buffer:

   a. Add 2 mL of PPI Buffer (5X) to 8 mL H2O to make 10 mL of 1X PPI Buffer

2) Preparation of 4X anti LAG-3 antibody concentration:

   a. Measure human LAG-3 antibody concentration with NanoDrop (for example 1 mg/mL)
   b. Prepare standard dilutions as follows in 1X PPI Buffer (change tip between each standard dilution):

Tube	Vol. of Antibody	Vol. of diluent (µL) *	[Ab] in standard curve	
			4 X (g/mL)	1X (µg/mL)
A	20 µL of 1 mg/mL antibody	30	4.0E-04	100
B	15 µL of tube A	35	1.2E-04	30
C	15 µL of tube B	30	4.0E-05	10
D	15 µL of tube C	35	1.2E-05	3
E	15 µL of tube D	30	4.0E-06	1
F	15 µL of tube E	35	1.2E-06	0.3
G	15 µL of tube F	30	4.0E-07	0.1
H	15 µL of tube G	35	1.2E-07	0.03
I	15 µL of tube H	30	4.0E-08	0.01
J	15 µL of tube I	35	1.2E-08	0.003
K	15 µL of tube J	30	4.0E-09	0.001
M (background)	0	50	0	0

* Dilute standards in diluent (e.g. 1X PPI Buffer).
At low concentrations of the protein, a significant amount of the protein can bind to the vial. Therefore, load the standard dilutions in the assay microplate within 60 minutes of preparation.

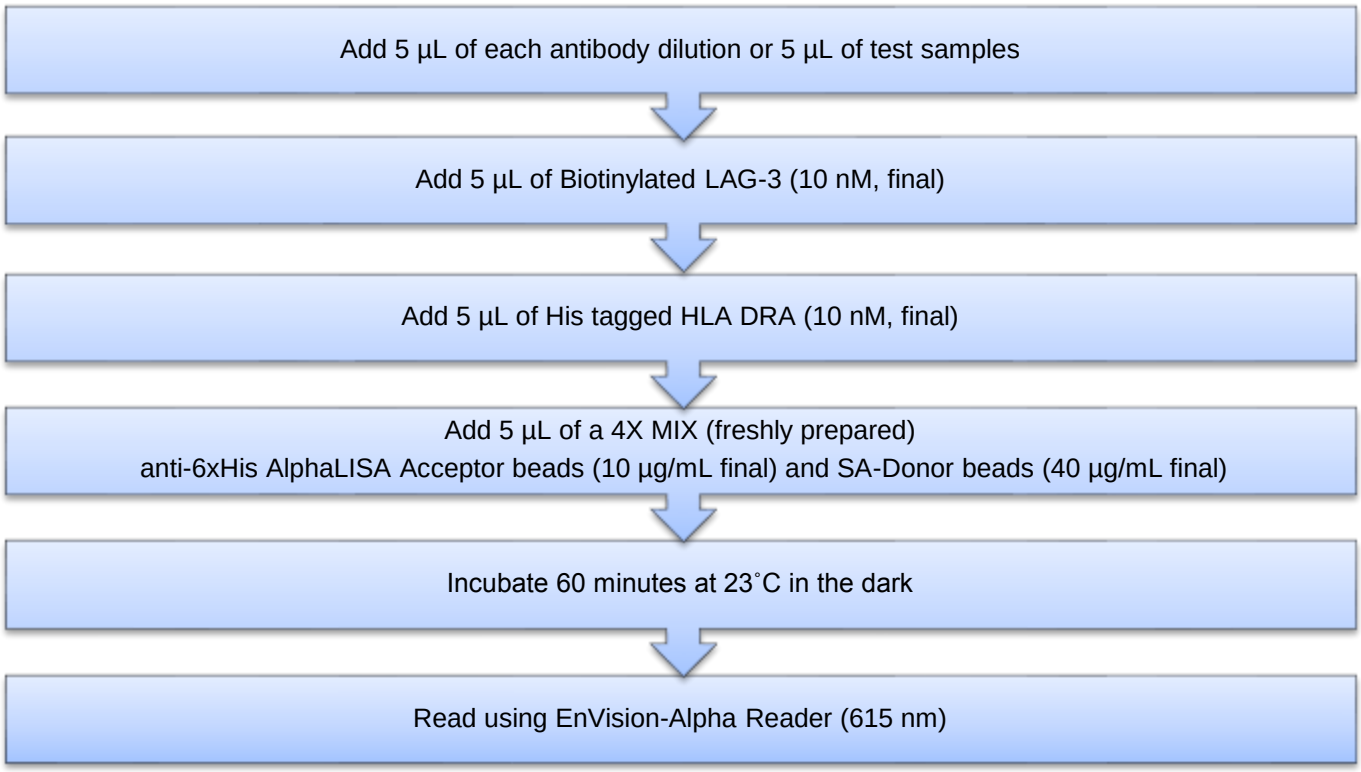
3) Preparation of 4X biotinylated human LAG-3 (40 nM):
   a. Add 20 µL of 5 µM biotinylated LAG-3 to 2480 µL of 1X PPI Buffer.
   b. Prepare just before use.

4) Preparation of 4X human His -HLA DRA (40 nM):
   a. Add 4.9 µL of 0.5 mg/mL (20.58 µM) His-HLA DRA to 2495 µL of 1X PPI Buffer
   b. Prepare just before use

5) Preparation of the mix of 4X AlphaLISA anti-His Acceptor beads (40 µg/mL) and 4X Streptavidin (SA) Donor beads (160 µg/mL):

a. Keep the beads under subdued laboratory lighting.
b. Add 20 µL of 5 mg/mL AlphaLISA Anti-His Acceptor beads and 80 µL of 5 mg/mL SA-Donor beads to 2400 µL of 1X PPI Buffer.
c. Prepare just before use.

6) In a ProxiPlate (384 wells)



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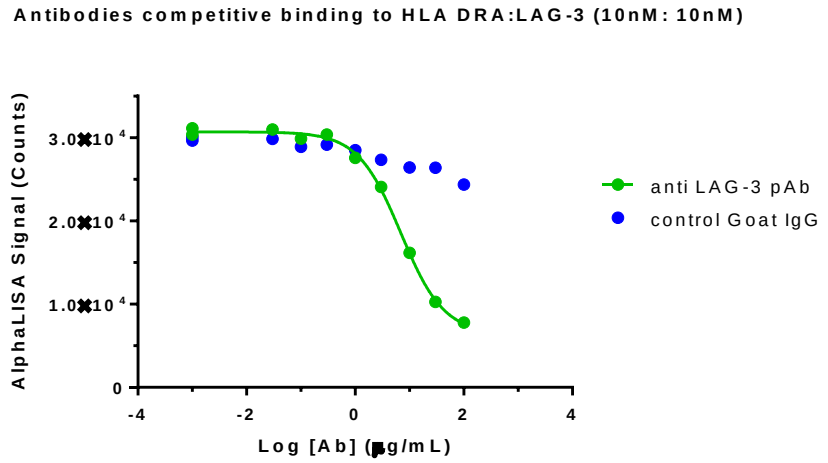
Typical Inhibition Data:

(a)



(b)

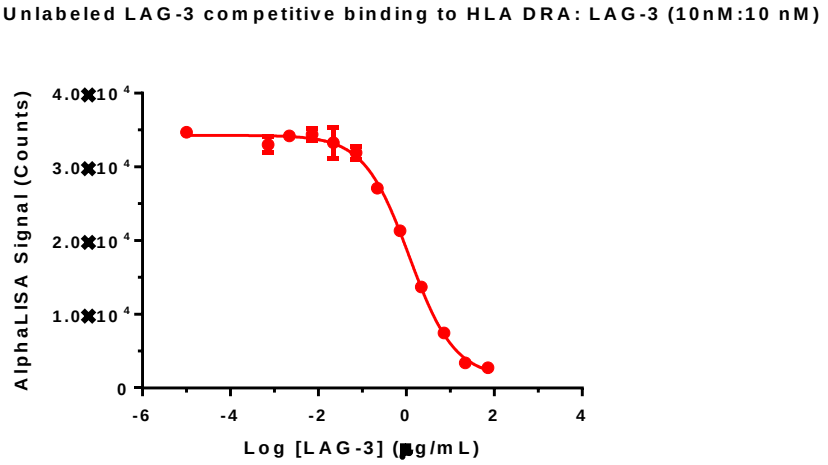


Figure 3. (a) Antibodies blocking binding of HLA DRA to LAG-3. Goat IgG showed as a negative control (blue points). The IC_{50} value is 6.9 µg/mL for human LAG-3 polyclonal antibody (green points). (b) Unlabeled LAG-3 competitive binding to HLA DRA. The IC_{50} is 1.2 µg/mL (16.6 nM). The IC_{50} values were calculated by using nonlinear regression fitting with GraphPad Prism 6.

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 ProxiPlate 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/lab-products-and-services/application-support-knowledgebase/alphalisa-alphascreen-no-wash-assays/alpha-troubleshooting.html

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