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## 4-1BB and 4-1BBL (Human) Binding AlphaLISA Kit

Product No.: AL3113C/F

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## Product Information

- Application:** This kit is designed to assess inhibitors of human 4-1BB and human 4-1BBL 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 4-1BB/4-1BBL.
- Sensitivity:**  $IC_{50}$ : 79 nM (average, using anti 4-1BB antibody, R&D Systems Cat # AF838)
- Signal to background ratio:** 104 using 5 nM 4-1BB and 5 nM 4-1BBL
- Kit contents:** The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human 4-1BBL, His tagged human 4-1BB, and AlphaLISA Immunoassay buffer.
- Storage:** Anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, and AlphaLISA Immunoassay buffer must be stored at 4 °C in the dark. Biotinylated human 4-1BBL-Fc and His tagged human 4-1BB-Fc must be stored at -20 °C. Thawed and aliquoted biotinylated human 4-1BBL-Fc and his-tagged human 4-1BB-Fc proteins need to be stored at -20 °C for up to 3 months. Avoid multiple freeze-thaw cycle of aliquoted proteins.
- Stability:** This kit is stable for at least 3 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

## Quality Control

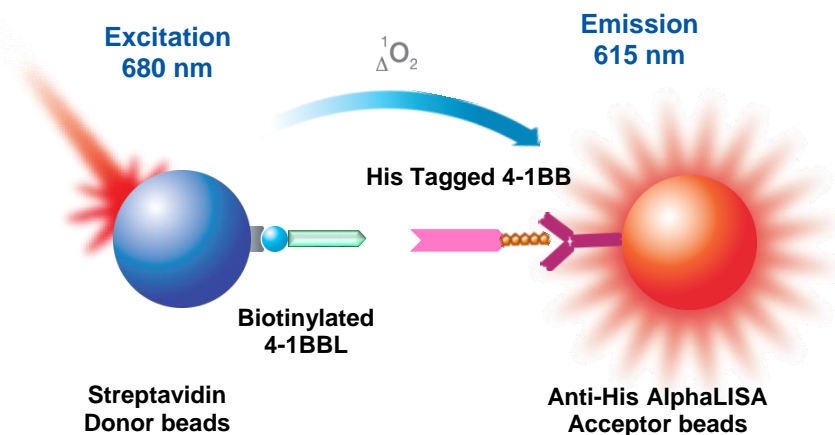
Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals were measured on the EnVision Multilabel Plate Reader with Alpha option using the protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum and minimum counts may vary between bead lots and the instrument used.

## Analyte of Interest

4-1BB is a member of the TNF superfamily. It is a type 2 transmembrane glycoprotein receptor with approximate molecular weight of 30 kDa and expressed mostly on activated T Lymphocytes. The expression is also reported on dendritic cells, B cells, follicular dendritic cells, natural killer cells, granulocytes and cells of blood vessel walls at sites of inflammation. It has many different names such as CD137 and TNFRSF9. 4-1BB binds with high affinity to the transmembrane 4-1BB Ligand/TNFSF9 which is expressed on antigen presenting cells and myeloid progenitor cells. This interaction co-stimulates the proliferation, activation, and/or survival of the 4-1BB expressing cells. 4-1BB activation enhances CD8+ T cell and NK cell mediated anti-tumor immunity. Soluble forms of 4-1BB and 4-1BB Ligand circulate at elevated levels in the serum of rheumatoid arthritis and hematologic cancer patients, respectively.

## Description of the AlphaLISA Assay

The AlphaLISA human 4-1BB and human 4-1BBL binding assay uses anti-6xHis AlphaLISA® acceptor beads to capture the His tagged 4-1BB and Streptavidin-coated donor beads to capture the biotinylated 4-1BBL. Donor beads and acceptor beads come into proximity through 4-1BB binding to 4-1BBL. 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 4-1BB and 4-1BBL binding 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	AL3113C*** (500 assay points)	AL3113F*** (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, <u>white</u> cap)	200 µL @ 5 mg/mL (1 brown tube, <u>white</u> 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, <u>black</u> cap)	400 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)
Biotinylated 4-1BBL-Fc stored at -20°C*	100 µL@ 1.7 µM (1 tube, <u>clear</u> cap)	100 µL@ 1.7 µM (10 tubes, <u>clear</u> caps)
His tagged 4-1BB-Fc stored at -20°C*	100 µL@ 1.7 µM (1 tube, <u>clear</u> cap)	100 µL@ 1.7 µM (10 tubes, <u>clear</u> caps)
AlphaLISA Immunoassay Buffer (10X)**	10 mL, 1 small bottle	100 mL, 1 large bottle

\* The Biotinylated 4-1BBL-Fc and His tagged 4-1BB-Fc should be stored at -20°C upon receive. After the first thawing, the proteins should be used within 60 minutes. These proteins can tolerate one more freeze-thaw cycle. The remaining proteins can be aliquoted into screw-capped 0.5 mL polypropylene vials and stored at -20°C for future experiments. The aliquoted proteins at -20°C is stable up to 90 days. Avoid additional 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 50 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	PerkinElmer Inc.	6050185
White OptiPlate-384 Well plate	PerkinElmer Inc.	6007290
EnVision®-Alpha Reader	PerkinElmer Inc.	-

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
Anti-human 4-1BB antibody	R&D Systems	AF838
Anti-human 4-1BBL antibody	R&D Systems	AF2295
Normal Goat IgG Control	R&D Systems	Ab-108-C
Un-labeled Human 4-1BB (no His-Tag)	Acro Biosystems	41B-H5258
Un-labeled Human 4-1BBL (no-biotin)	Acro Biosystems	41L-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 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<sub>2</sub>O 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 protocol described below is an **example** for generating three inhibition curves using anti-4-1BB antibody in a 50 µL final assay volume (144 wells, triplicate determinations). If a different number 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.
- If generating the inhibition curves using anti-4-1BB antibody, anti-4-1BBL antibody, un-labeled 4-1BB (no His-Tag) protein, or un-labeled 4-1BBL (not biotin-labeled), the concentrations of inhibitors (antibodies or un-labeled proteins) may be modified and adding **sequences of reagents** to the wells must be modified. Please see "**Typical competitive binding Data**" for more details.

**Two Incubation Step Protocol described below is using Anti-4-1BB Antibody as Inhibitor:**

1. Preparation of 1X AlphaLISA Immunoassay Buffer (for 10 mL)

Add 1 mL of 10X AlphaLISA Immunoassay Buffer and 9 mL of MilliQ water.

2. Preparation of serial dilution of Anti-4-1BB Antibody (R&D Systems, Cat No. AF838, this antibody is not provided with the kit)

- a. Reconstitute 100 µg lyophilized anti 4-1BB antibody with 100 µL PBS to make 1 mg/mL (6.9 µM).
- b. Prepare serial dilutions of Anti-4-1BB Antibody in 1x AlphaLISA Immunoassay Buffer as follows, change tips between each dilution:

Tube	Vol. of Anti-4-1BB Antibody (µL)	Vol. of 1x buffer (µL) *	Anti-4-1BB Antibody
			(M in 10 µL)
A	29 µL of 1 mg/mL (6.9 µM) Anti-4-1BB Antibody	171	1.00E-06
B	100 µL of tube A	100	5.00E-07
C	100 µL of tube B	100	2.50E-07
D	100 µL of tube C	100	1.25E-07
E	100 µL of tube D	100	6.25E-08
F	100 µL of tube E	100	3.13E-08
G	100 µL of tube F	100	1.56E-08
H	100µL of tube G	100	7.81E-09
I	100 µL of tube H	100	3.91E-09
J	100 µL of tube I	100	1.95E-09
K	100 µL of tube J	100	9.77E-10
L	0	100	0

3. Preparation of 5x His-tagged 4-1BB-Fc (25 nM):

- a. Thaw 1 tube of His-tagged 4-1BB-Fc, 75 µg/mL (1.7 µM).
- b. Add 22.1 µL of 1.7 µM His-tagged 4-1BB-Fc to 1477.9 µL 1X AlphaLISA immunoassay buffer.
- c. Vertex briefly.
- d. The remaining His-tagged 4-1BB-Fc should be aliquoted immediately and stored at -20°C for future assays (see page 4 for more details).

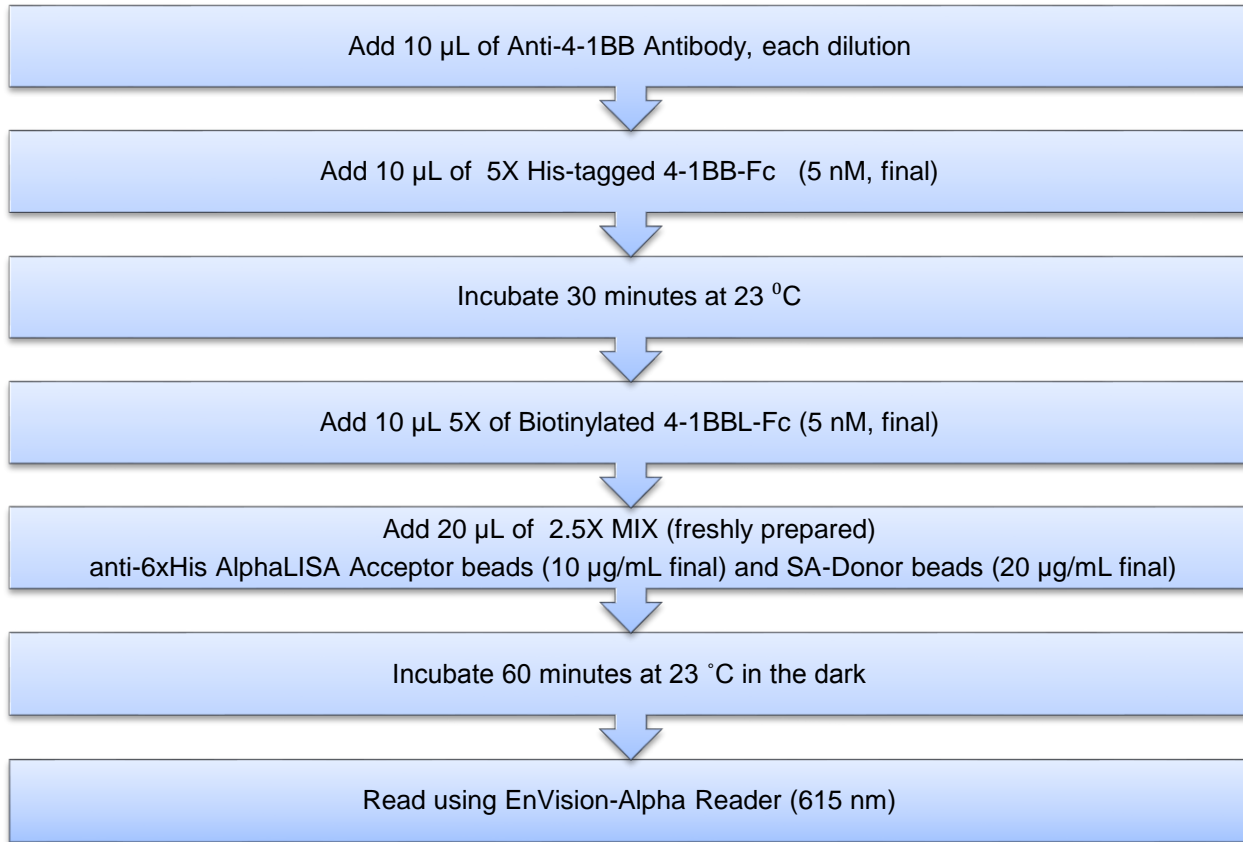
4. Preparation of 5x biotinylated 4-1BBL-Fc (25 nM):

- a. Thaw 1 tube biotinylated 4-1BBL-Fc, 83.3 µg/mL (1.7 µM).
- b. Add 22.1 µL of 1.7 µM biotinylated 4-1BBL-Fc to 1477.9 µL 1X AlphaLISA immunoassay buffer.
- c. Vertex briefly.
- d. The remaining biotinylated 4-1BBL-Fc should be aliquoted immediately and stored at -20°C for future assays (see page 4 for more details).

5. Preparation of 2.5x mix of anti-6xHis AlphaLISA Acceptor beads (25 µg/mL) and 4X Streptavidin Donor beads (SA-DB, 50 µg/mL):

- a. Keep the SA-DB beads under subdued laboratory lighting.
- b. Add 15 µL of 5 mg/mL Anti-6xHis AlphaLISA Acceptor beads and 30 µL of 5 mg/mL SA-Donor beads to 2955 µL of 1X AlphaLISA Immunoassay Buffer
- c. Prepare just before use. Vertex briefly.

6) In a white Optiplate (384 wells):



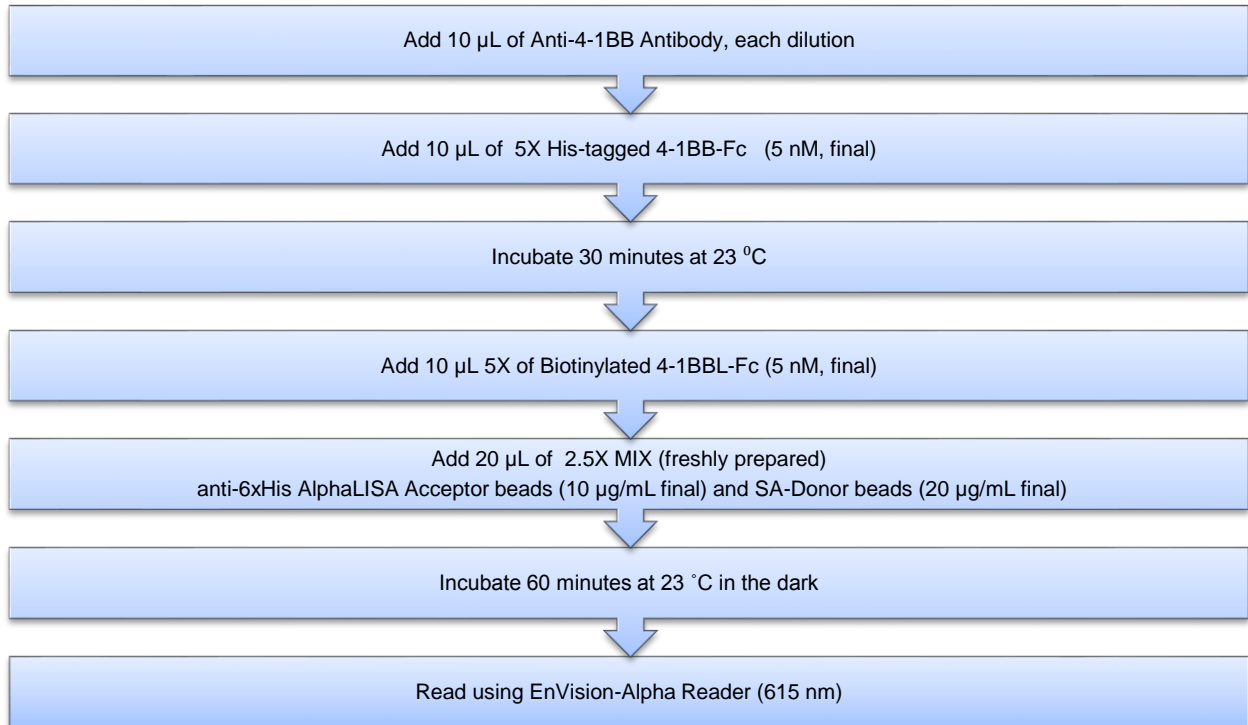
**Read Settings:** AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance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

## Typical competitive binding Data:

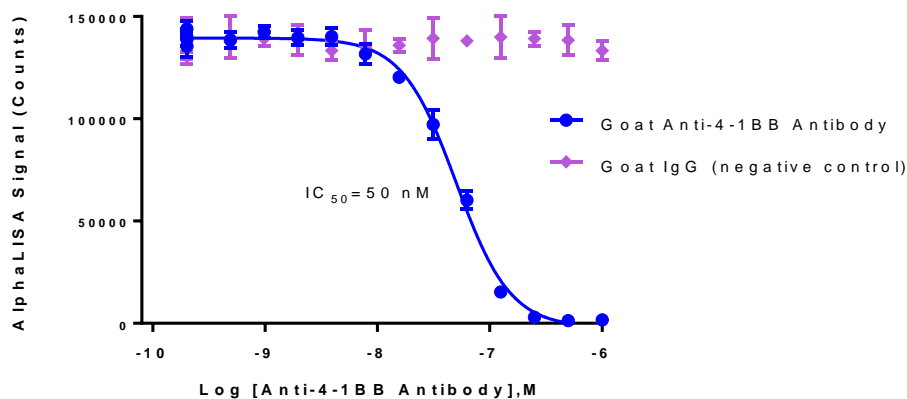
Sequence of adding reagents to the wells and the incubation times **vary** when using different antibodies or different un-labeled proteins as blocking agents.

### Blocking using anti-4-1BB antibody (Figure 2)

Sequence of adding flow-chart (Same as one page 7)



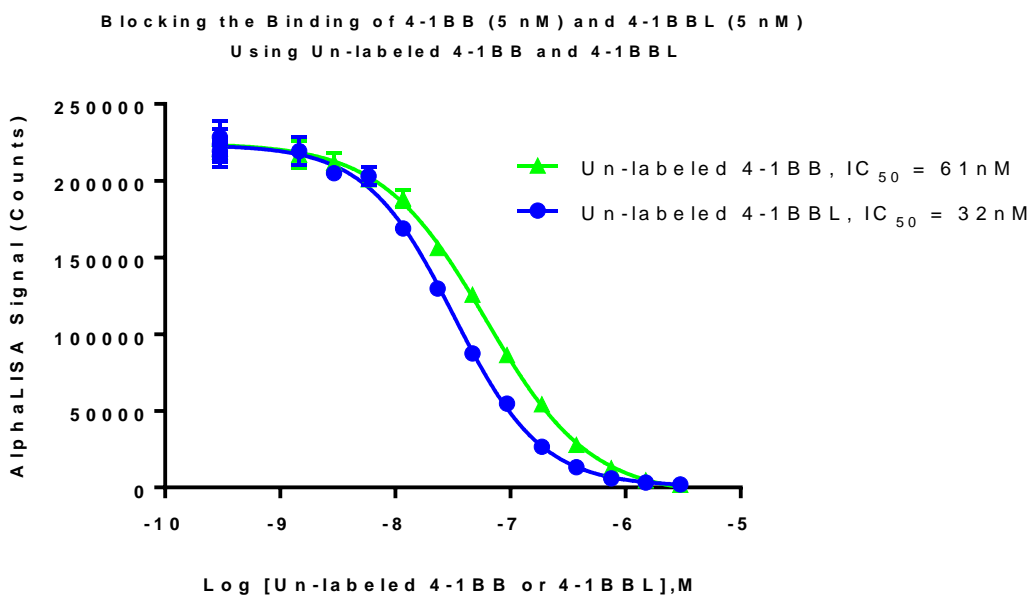
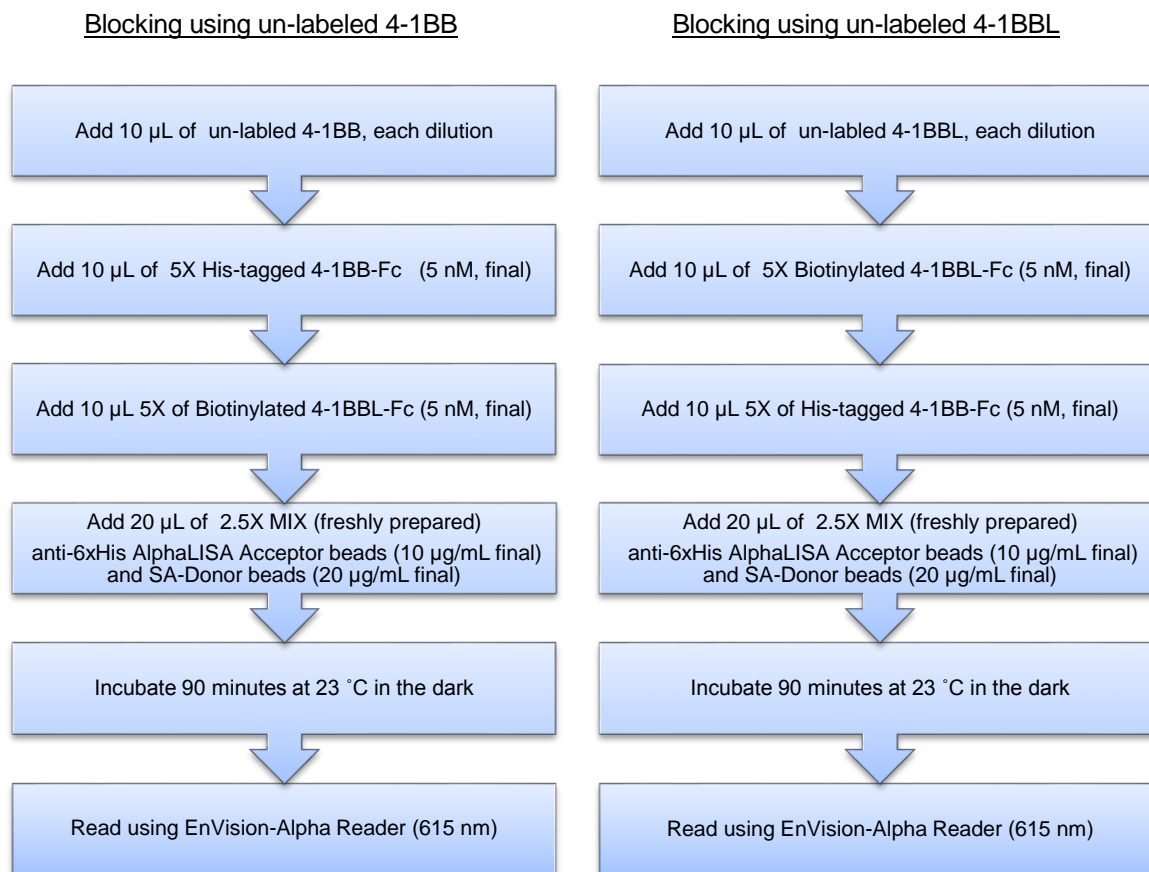
Blocking the Binding of 4-1BB (5 nM) and 4-1BBL (5 nM)  
Using Un-labeled 4-1BB and 4-1BBL



**Figure 2.** Blocking the binding of 4-1BB/4-1BBL with anti-4-1BB antibody ( $IC_{50} = 50$  nM). Goat IgG was used as a negative control. All  $IC_{50}$  values were calculated by using nonlinear regression fitting with GraphPad Prism 7.



### Blocking using un-labeled 4-1BB or 4-1BBL (Figure 3)



**Figure 3.** Blocking the binding of 4-1BB/4-1BBL with Un-labeled 4-1BB and 4-1BBL ( $IC_{50} = 61$  nM and  $IC_{50} = 32$  nM, respectively). 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	<ul style="list-style-type: none"><li>• Buffer is not freshly made. Make new.</li><li>• Incubation time is longer than recommended range.</li></ul>
Low AlphaLISA signal	<ul style="list-style-type: none"><li>• Optimize EnVision with Plate format.</li></ul>
High variation between replicates or low Z' values	<ul style="list-style-type: none"><li>• 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.</li></ul>

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

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