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Human 4-1BBL AlphaLISA Detection Kit

Product No.: AL3108C/F

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Product Information

Application:	This kit is designed for the quantitative determination of 4-1BBL in buffer, cell culture media, cell supernatants and cell lysates using a homogeneous AlphaLISA assay (no wash steps).
Sensitivity:	Lower Detection Limit (LDL): 12.6 pg/mL Lower Limit of Quantification (LLOQ): 41.3 pg/mL EC ₅₀ : 33.1 ng/mL
Dynamic range:	12.6 – 100 000 pg/mL

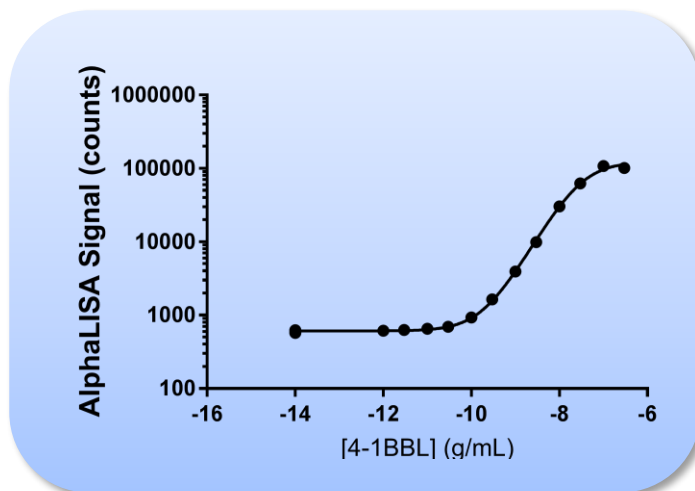


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white OptiplatTM-384 microplate and the EnVision[®] Multilabel Plate Reader 2102 with Alpha option.

Storage:	Store kit in the dark at 4°C. For reconstituted analyte aliquot and store at -20 °C. Avoid freeze-thaw cycles.
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, EC₅₀ and LDL 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 LDL measurement.

Analyte of Interest

4-1BBL, also known as CD137L or TNFSF9, is a type II transmembrane receptor protein expressed on activated B cells, monocytes, neutrophils, and neurons. It binds to 4-1BB on activated CD4⁺ and CD8⁺ T cells. Ligation of 4-1BBL results in survival of T cells during infection. A soluble form of 4-1BBL has been shown to be cleaved from the surface of activated B cells. There are many drug targets centered on interrupting the binding of 4-1BB to 4-1BBL. These are focused on stimulating immune response to cancers.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, cell supernatants, and cell lysates in a highly sensitive, quantitative, reproducible and user-friendly mode. In this AlphaLISA assay, a biotinylated Anti-4-1BBL Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-4-1BBL Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the 4-1BBL, the 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).

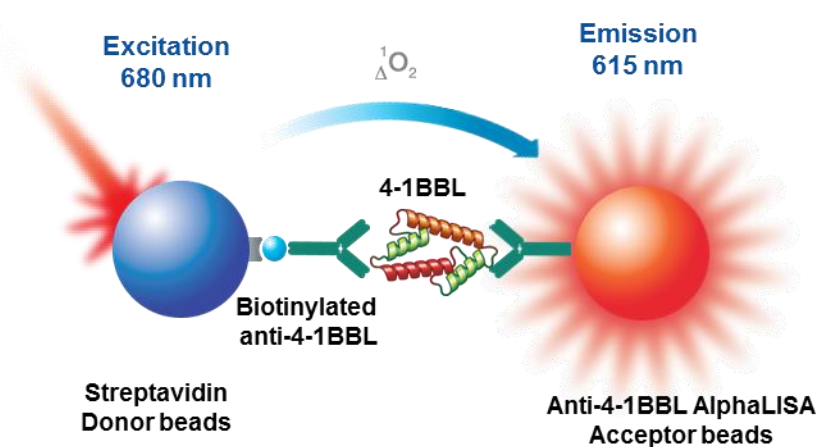


Figure 2. AlphaLISA 4-1BBL Assay Principle.

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.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3108HV (100 assay points ^{***})	AL3108C (500 assay points ^{***})	AL3108F (5000 assay points ^{***})
AlphaLISA Anti-4-1BBL Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)	50 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)	500 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	80 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	200 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, <u>black</u> caps)
Biotinylated Anti-4-1BBL Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN ₃ , pH 7.4	20 µL @ 500 nM (1 tube, <u>black</u> cap)	50 µL @ 500 nM (1 tube, <u>black</u> cap)	500 µL @ 500 nM (1 tube, <u>black</u> cap)
Lyophilized Recombinant 4-1BBL*	0.3 µg (1 tube, <u>clear</u> cap)	0.3 µg (1 tube, <u>clear</u> cap)	0.3 µg (1 tube, <u>clear</u> cap)
AlphaLISA Immunoassay Buffer (10X)**	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Reconstitute lyophilized analyte in 100 µL Milli-Q® grade H₂O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped 0.5 mL polypropylene vials and stored at -20°C for future experiments. The aliquoted analyte stored at -20°C is stable up to 30 days. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3108S).

** 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 100 µL in HV size kits or 50 µL in C/F size kits 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 Plus Adhesive Sealing Film	PerkinElmer Inc.	6050185
EnVision®-Alpha Reader	PerkinElmer Inc.	-

Recommendations

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- 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 all reagents by vortexing before use.
- Use Milli-Q® grade H₂O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- 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 Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multilabel Plate 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: D6 as, 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.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment.

Assay Procedure

- The protocol described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The protocols also include testing samples in 452 wells. If 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 standard dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

Format	# of data points	Volume					Plate recommendation
		Final	Sample	AlphaLISA Acceptor Beads	Biotinylated Antibody	SA-Donor beads	
AL3108HV	100	100 μ L	10 μ L	20 μ L	20 μ L	50 μ L	White OptiPlate-96 (cat # 6005290) White 1/2 AreaPlate-96 (cat # 6005560)
AL3108C	250	100 μ L	10 μ L	20 μ L	20 μ L	50 μ L	White OptiPlate-96 (cat # 6005290) White 1/2 AreaPlate-96 (cat # 6005560)
	500	50 μ L	5 μ L	10 μ L	10 μ L	25 μ L	White 1/2 AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 μ L	2 μ L	4 μ L	4 μ L	10 μ L	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 μ L	1 μ L	2 μ L	2 μ L	5 μ L	Light gray AlphaPlate-1536 (cat # 6004350)
AL3108F	5 000	50 μ L	5 μ L	10 μ L	10 μ L	25 μ L	White 1/2 AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 μ L	2 μ L	4 μ L	4 μ L	10 μ L	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 μ L	1 μ L	2 μ L	2 μ L	5 μ L	Light gray AlphaPlate-1536 (cat # 6004350)

3 Step Protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). If different amounts of samples are tested, the volumes of all reagents have to be adjusted accordingly.

1) Preparation of 1X AlphaLISA Immunoassay Buffer:

Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL Milli-Q® grade H₂O.

2) Preparation of 4-1BBL analyte standard dilutions:

- a. Reconstitute lyophilized 4-1BBL (0.3 µg) in 100 µL Milli-Q® grade H₂O. The remaining reconstituted analyte should be aliquoted immediately and stored at -20°C for future assays (see page 4 for more details).
- b. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of 4-1BBL (µL)	Vol. of diluent (µL)*	[4-1BBL] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted 4-1BBL	90	3.00E-07	300 000
B	60 µL of tube A	120	1.00E-07	100 000
C	60 µL of tube B	140	3.00E-08	30 000
D	60 µL of tube C	120	1.00E-08	10 000
E	60 µL of tube D	140	3.00E-09	3 000
F	60 µL of tube E	120	1.00E-09	1 000
G	60 µL of tube F	140	3.00E-10	300
H	60 µL of tube G	120	1.00E-10	100
I	60 µL of tube H	140	3.00E-11	30
J	60 µL of tube I	120	1.00E-11	10
K	60 µL of tube J	140	3.00E-12	3
L	60 µL of tube K	120	1.00E-12	1
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer, cell culture media, lysis buffer, or serum). The diluent used to dilute standards should match the sample type as closely as possible.

At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X Anti-4-1BBL AlphaLISA Acceptor beads (50 µg/mL):

- a. Prepare just before use.
- b. Add 50 µL Anti-4-1BBL Acceptor beads to 4950 µl of 1X AlphaLISA Immunoassay Buffer.

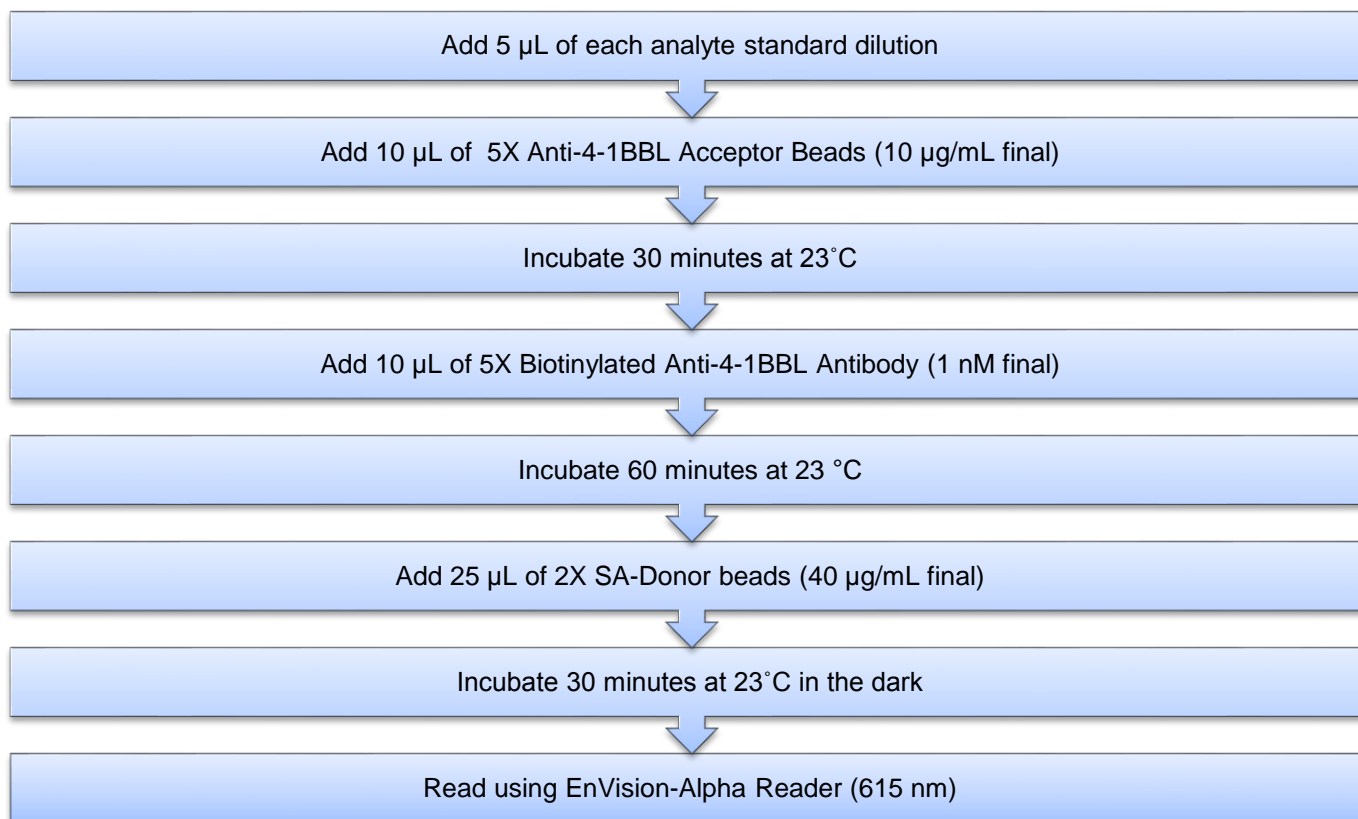
4) Preparation of 5X biotinylated Anti-4-1BBL antibody (5 nM):

- a. Prepare just before use.
- b. Add 50 µL 500 nM Biotinylated Anti-4-1BBL Antibody to 4950 µl of 1X AlphaLISA Immunoassay Buffer.

5) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):

- a. Prepare just before use.
- b. Keep the beads under subdued laboratory lighting.
- c. Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA Immunoassay Buffer.

6) In a white Optiplate (384 wells):



Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the 3 step protocol using AlphaLISA Immunoassay Buffer (IAB) as assay buffer. The analytes (standards) were prepared in IAB, DMEM + 10% FBS, 18% RPMI + 2% FBS in IAB, or AlphaLISA Lysis Buffer and all other components were prepared in IAB.

- Assay Sensitivity:

The LDL was calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL sample using the recommended assay conditions.

LDL (pg/mL)*	(Analyte diluent)	# of experiments
13	IAB	6
37	DMEM + 10% FBS	6
150	RPMI + 10% FBS*	6
20	18% RPMI + 2% FBS in IAB	6
16	AlphaLISA Lysis Buffer	6
216	100% FBS*	6

* It is advised to avoid using this assay to quantify 4-1BBL levels in serum samples, or samples containing undiluted RPMI. These sample matrices can result in a loss of total signal and sensitivity.

- Assay Precision:

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the analytes were prepared in IAB, DMEM + 10% FBS, 18% RPMI + 2% FBS in IAB, or AlphaLISA Lysis Buffer. All other components were prepared in IAB. Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well format.

- Intra-assay precision:

The intra-assay precision was determined using a total of 16 independent determinations in triplicate. Shown as CV%.

4-1BBL	IAB	DMEM + 10% FBS	18% RPMI + 2% FBS in IAB	ALB
CV (%)	5	6	7	4

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 9 measurements for 10 ng/mL sample. Shown as CV%.

4-1BBL	IAB	DMEM + 10% FBS	18% RPMI + 2% FBS in IAB	ALB
CV (%)	6	6	9	6

- Spike Recovery:

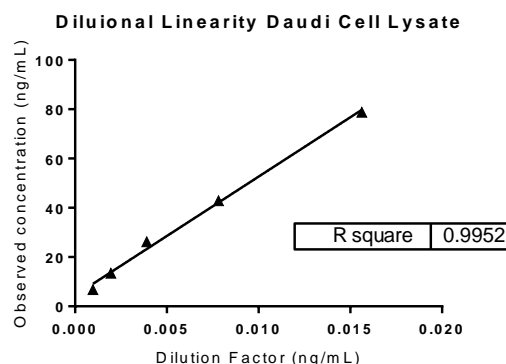
Three known concentrations of analyte were spiked into IAB, DMEM + 10% FBS, 18% RPMI + 2% FBS in IAB, or ALB. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in IAB, DMEM + 10% FBS, 18% RPMI + 2% FBS in IAB, or ALB. All other assay components were diluted in IAB.

Spiked 4-1BBL (ng/mL)	% Recovery			
	IAB	DMEM + 10% FBS	18% RPMI + 2% FBS in IAB	ALB
10	102	106	98	101
3	94	99	97	96
1	105	109	96	104

- Lysate Experiments

To validate the assay kit, 500K per mL of Daudi cells were lysed with AlphaLISA Lysis Buffer. The samples with unknown concentrations of 4-1BBL were tested. The standard was prepared in ALB and lysate samples were diluted with ALB at the dilution fold listed below. All other reagents were prepared in IAB. In the samples, 6.3 ng/mL 4-1BBL was detected and excellent dilution linearity ($R^2 = 0.995$) was achieved when lysate was diluted ≥ 64 fold. The results are summarized from 3 experiments and shown in table and figure below.

Cell Lysate Dilution Fold (DF)	4-1BBL detected in Positive Cell Lysate (pg/mL)	4-1BBL Positive Cell Lysate (ng/mL x DF)
64	78.8	5.0
128	42.9	5.5
256	26.2	6.7
512	13.5	6.9
Average \pm SD	N/A	6.3 \pm 0.9



- Supernatant Experiments

Supernatant was collected from Daudi cells cultured in RPMI supplement with 10% FBS with unknown concentrations of 4-1BBL. Cell Supernatant (RPMI + 10% FBS) was diluted 5X in IAB and interpolated against a standard curve of 18% RPMI + 2% FBS in IAB (Culture media diluted 5X in IAB) and was tested using this assay kit. The results of three independent assays were averaged and levels of 4-1BBL detected in these samples was 1016 pg/mL.

Troubleshooting Guide

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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