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Human Cluster of Differentiation 40 (CD40) AlphaLISA Detection Kit

Product No.: AL3102C/F

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Product Information

Application:	This kit is designed for the quantitative determination of CD40 in buffer, cell culture media, serum, and cell lysates using a homogeneous AlphaLISA assay (no wash steps).
Sensitivity:	Lower Detection Limit (LDL): 4.5 pg/mL Lower Limit of Quantification (LLOQ): 18.2 pg/mL EC ₅₀ : 32.1 ng/mL
Dynamic range:	4.5 – 300 000 pg/mL

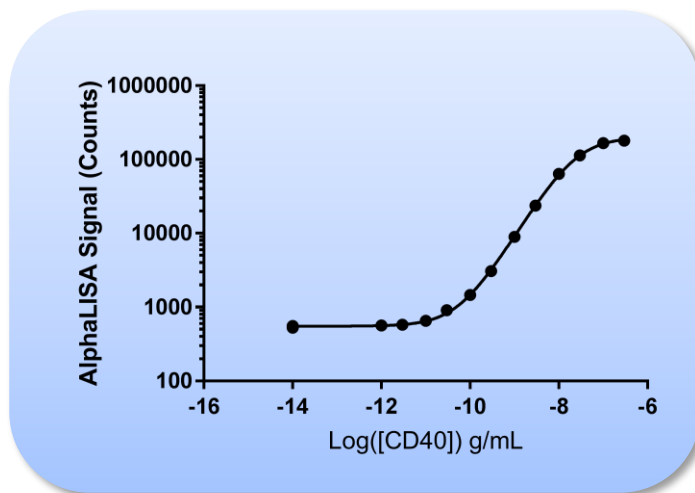


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

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

Cluster of differentiation 40 (CD40), also known as TNFRSF5, is a type II transmembrane receptor protein expressed on B cells, dendritic cells, activated monocytes, and others. It binds to CD40L. Ligation of CD40 results in the aggregation of CD40, which in turn initiates signaling pathways and stimulates B-cell proliferation. CD40 expression has been shown to be upregulated in a wide range of tumor cells including Non-Hodgkin's and Hodgkin's Lymphomas.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum, and cell lysates in a highly sensitive, quantitative, reproducible and user-friendly mode. In this AlphaLISA assay, a biotinylated Anti-CD40 Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-CD40 Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the CD40, 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).

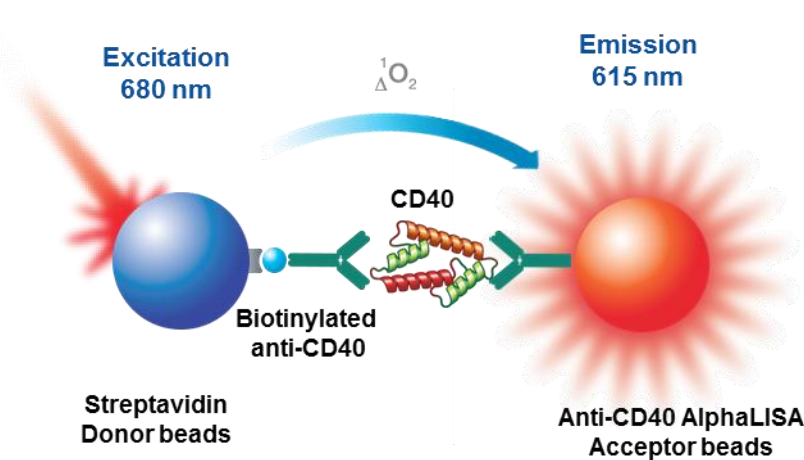


Figure 2. AlphaLISA CD40 Assay Principle.

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	AL3102HV (100 assay points ^{***})	AL3102C (500 assay points ^{***})	AL3102F (5000 assay points ^{***})
AlphaLISA Anti-CD40 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-CD40 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 CD40*	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 # AL3102S).

** 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: 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.
- 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 AccBeads + bAb MIX	SA-Donor beads	
AL3102HV	100	100 μ L	10 μ L	40 μ L	50 μ L	White OptiPlate-96 (cat # 6005290) White 1/2 AreaPlate-96 (cat # 6005560)
AL3102C	250	100 μ L	10 μ L	40 μ L	50 μ L	White OptiPlate-96 (cat # 6005290) White 1/2 AreaPlate-96 (cat # 6005560)
	500	50 μ L	5 μ L	20 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	8 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	4 μ L	5 μ L	Light gray AlphaPlate-1536 (cat # 6004350)
AL3102F	5 000	50 μ L	5 μ L	20 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	8 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	4 μ L	5 μ L	Light gray AlphaPlate-1536 (cat # 6004350)

2 Step Protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). If different amount 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 CD40 analyte standard dilutions:

- a. Reconstitute lyophilized CD40 (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 CD40 (µL)	Vol. of diluent (µL)*	[CD40] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted CD40	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.

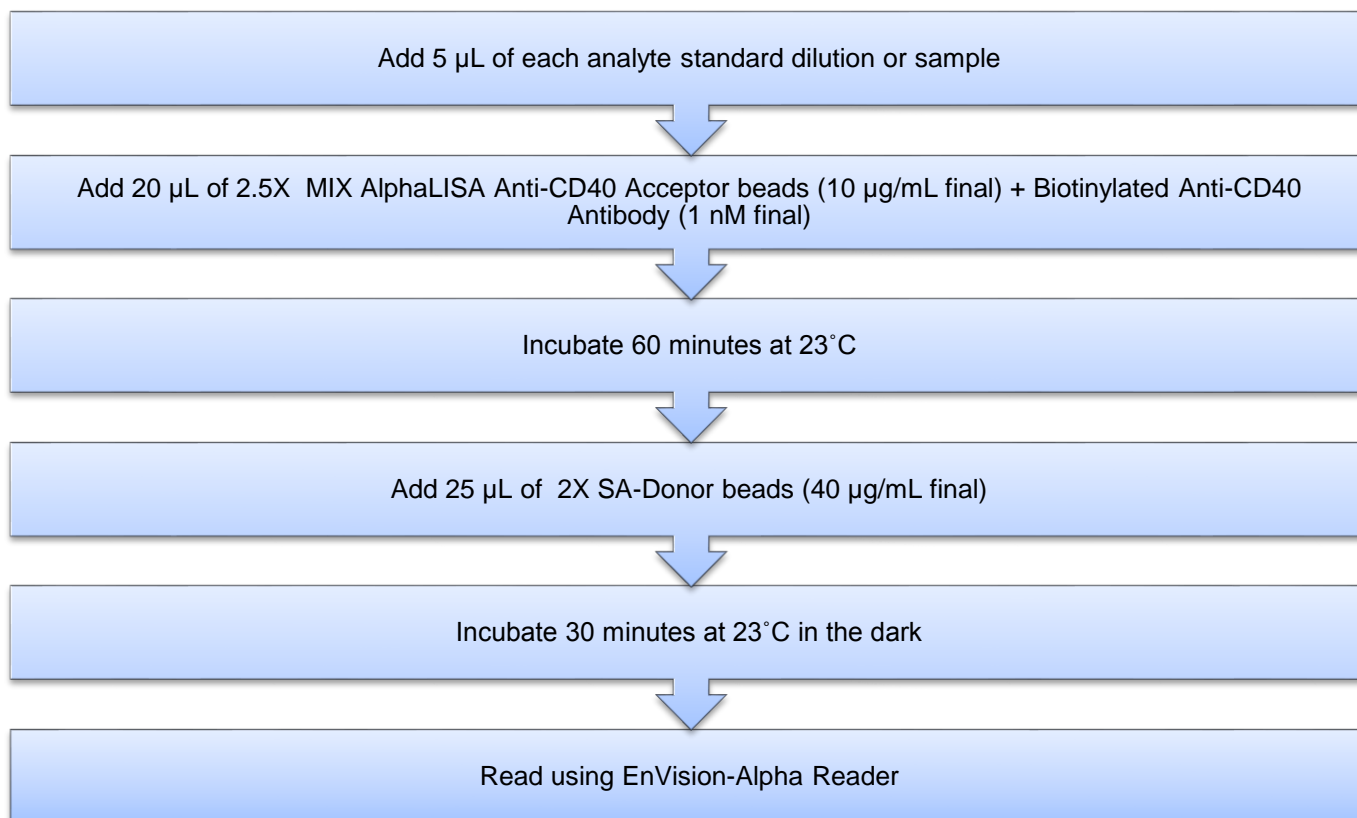
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** 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 2.5X MIX Anti-CD40 AlphaLISA Acceptor beads (25 µg/mL) + biotinylated Anti-CD40 antibody (2.5 nM):

- a. Prepare just before use.
- b. Add 50 µL Anti-CD40 Acceptor beads + 50 µL 500 nM Biotinylated Anti-CD40 Antibody to 9900 µL of 1X AlphaLISA Immunoassay Buffer.

- 4) 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.
- 5) 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 2 step protocol using AlphaLISA Immunoassay Buffer (IAB) as assay buffer. The analytes (standards) were prepared in IAB, DMEM + 10% FBS, RPMI + 10% FBS, 100% FBS, or RIPA 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 μ L sample using the recommended assay conditions.

LDL (pg/mL)*	(Analyte diluent)	# of experiments
4.5	IAB	6
2.9	DMEM + 10% FBS	6
22.3	RPMI + 10% FBS	6
5.8	100% FBS	6
8.2	RIPA	6

- 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, RPMI + 10% FBS, 100% FBS, or RIPA 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%.

CD40	IAB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS	RIPA
CV (%)	5	4	6	6	5

- 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%.

CD40	IAB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS	RIPA
CV (%)	6	5	9	6	6

- Spike Recovery:

Three known concentrations of analyte were spiked into IAB, DMEM + 10% FBS, RPMI + 10% FBS, 100% FBS, or RIPA buffer. 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, RPMI + 10% FBS, 100% FBS, or RIPA buffer. All other assay components were diluted in IAB.

Spiked CD40 (ng/mL)	% Recovery				
	IAB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS	RIPA
10	103	101	92	91	93
3	99	97	95	85	90
1	101	99	89	95	96

- Specificity:

Cross-reactivity of the CD40 AlphaLISA Kit was tested using the following proteins at 100 ng/mL in IAB.

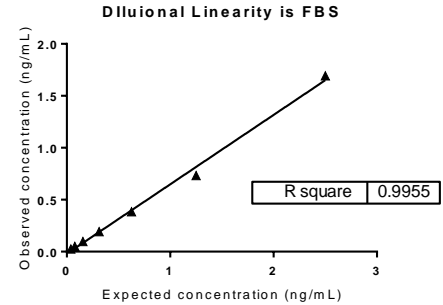
Tested Proteins	% Cross Reactivity
Mouse CD40	<1
Human 4-1BB	<0.1

- Serum Experiments

- Dilution Linearity

Neat Normal Human Serum and CD40-spiked (5 ng/mL) Normal Human Serum samples were diluted with 100% FBS and the assay was performed along with a standard curve using the analyte prepared in 100% FBS. Concentrations of CD40 in diluted human serum were determined by interpolating to the standard curve. In normal human serum, 0.52 ng/mL CD40 was detected when the samples were diluted ≥ 2 fold. Excellent dilution linearity ($R^2 > 0.995$) was achieved in the CD40-spiked human serum samples that were diluted ≥ 2 fold. The results are shown in table and figure below.

Dilution Factor (x)	Expected CD40 (ng/mL)	Observed CD40 (ng/mL)
2	2.50	1.69
4	1.25	0.73
8	0.63	0.38
16	0.31	0.19
32	0.16	0.10



- Spike Recovery

Three known amounts of CD40 were spiked into Normal Human Serum (30, 10, and 3 ng/mL CD40 in spiked samples) and then the samples were diluted 2-fold into 100% FBS. The standard was prepared in 100% FBS and all other reagents were prepared in IAB. The spike recoveries of CD40 were determined and the results are shown in table below. Recoveries were calculated after the endogenous CD40 level was subtracted (in this case, 0.52 ng/mL in normal human serum).

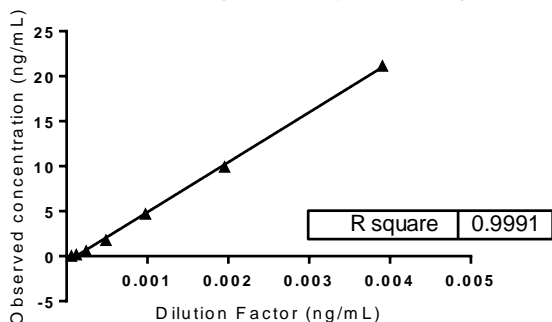
	Diluent: 100% FBS	
	Spiked sample (Normal Human Serum)	
Spike (ng/mL)	Concentration (ng/mL)	Recovery (%)
No spike	0.52	N/A
30	23.7	79
10	7.1	71
3	2.4	81

- Lysate Experiments

To validate the assay kit, commercially available cell lysate samples with unknown concentrations of CD40 were tested. The cell lysates include CD40 positive and negative samples. The standard was prepared in RIPA buffer and lysate samples were diluted with RIPA buffer at the dilution fold listed below. All other reagents were prepared in IAB. CD40 was not detected in negative samples. In the positive samples, 4.3 µg/mL CD40 was detected and excellent dilution linearity ($R^2 = 0.999$) was achieved when lysate was diluted ≥ 256 fold. The results are summarized from 3 experiments and shown in table and figure below.

Cell Lysate Dilution Fold (DF)	CD40 detected in Positive Cell Lysate (ng/mL)	CD40 Positive Cell Lysate (µg/mL x DF)	CD40 Negative Cell Lysate (ng/mL)*
256	21.2	5.4	0
512	9.9	5.1	0
1024	4.7	4.8	0
2048	1.8	3.7	0
5096	0.59	3.0	0
Average \pm SD	N/A	4.3 \pm 1.2	0

Dilutional Linearity OverExpressed Lysate



* Counts for negative cell lysate (regardless of dilution) sample are below or equal to the background counts (RIPA buffer only).

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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