Research Use Only. Not for use in diagnostic procedures.

Lymphocyte-Activation Gene 3/CD223 (LAG-3) AlphaLISA Detection Kit

Product No.: AL3058C/F

Contents

Product Information

Application: This kit is designed for the quantitative determination of Human LAG-3 in serum, cell lysates and cell culture media using a homogeneous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 74.1 pg/mL

Lower Limit of Quantification (LLOQ): 331.1 pg/mL

EC_{50}: 562.3 ng/mL
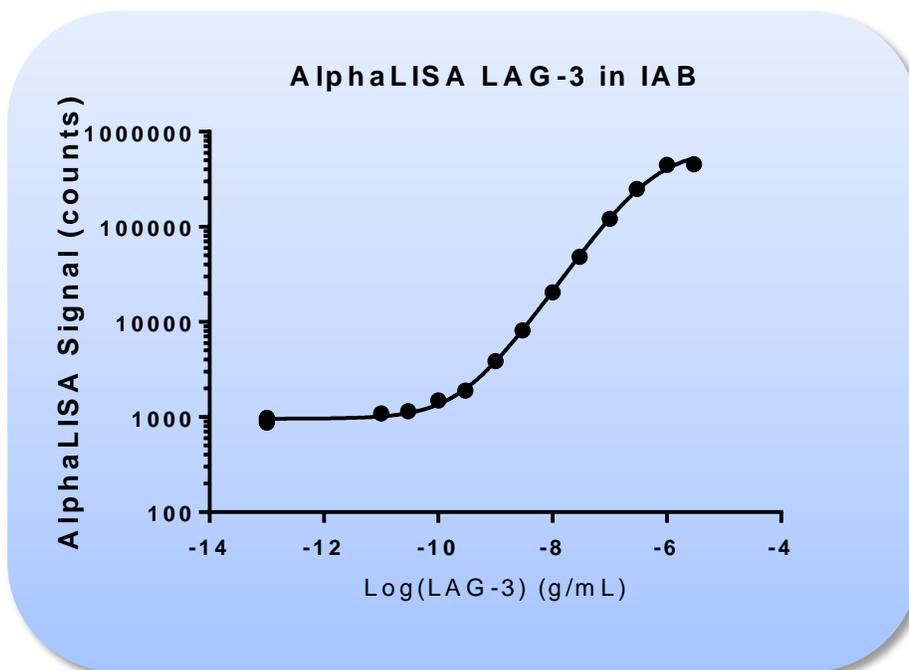
Dynamic range: 74.1 – 1 000 000 pg/mL

Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate[TM]-384 microplate and the EnVision[®] Multilabel Plate Reader 2103 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, EC_{50} 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

Lymphocyte-activation gene 3 (LAG-3), also known as CD223, is a cell surface protein that affects T-cell function in many ways. LAG-3 is expressed on natural killer cells, plasmacytoid dendritic cells, B-cells, and activated T-cells. LAG-3 works in similar fashion to CTLA-4 and PD-1 in that it negatively regulates cellular proliferation and Treg suppression. Maturation and activation of dendritic cells is also known to be affected by LAG-3 expression. Soluble forms are in clinical development to activate dendritic cells. Anti-LAG-3 antibodies are undergoing studies for anti-cancer and autoimmune response.

Description of the AlphaLISA Assay

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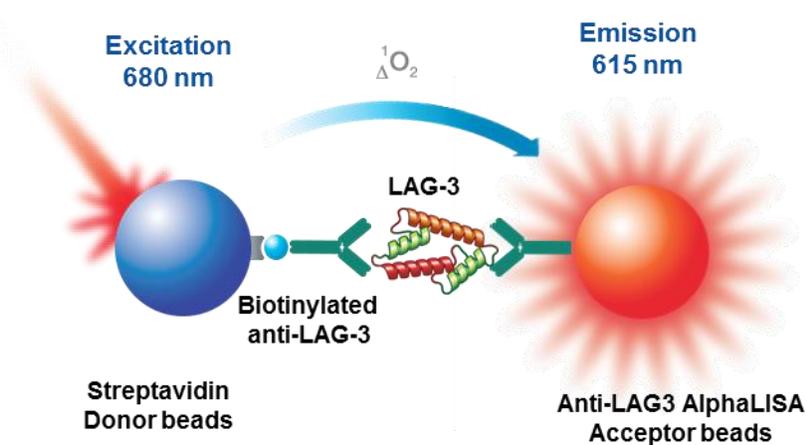

Figure 2. AlphaLISA Assay Principle.

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	AL3058HV (100 assay points***)	AL3058C (500 assay points***)	AL3058F (5000 assay points***)
AlphaLISA Anti-LAG-3 Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	40 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)	100 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)	1 mL @ 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)	100 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	1 mL @ 5 mg/mL (1 brown tube, <u>black</u> cap)
Biotinylated Anti-LAG-3 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 Human LAG-3*	3 µg (1 tube, <u>clear</u> cap)	3 µg (1 tube, <u>clear</u> cap)	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 H$_2$O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20°C for future experiments. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3058S).

** 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 96-well plates or 50 µL in 96- or 384-well assay 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. 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

- 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 H_2O (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.

- 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.

- 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

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- 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 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 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.

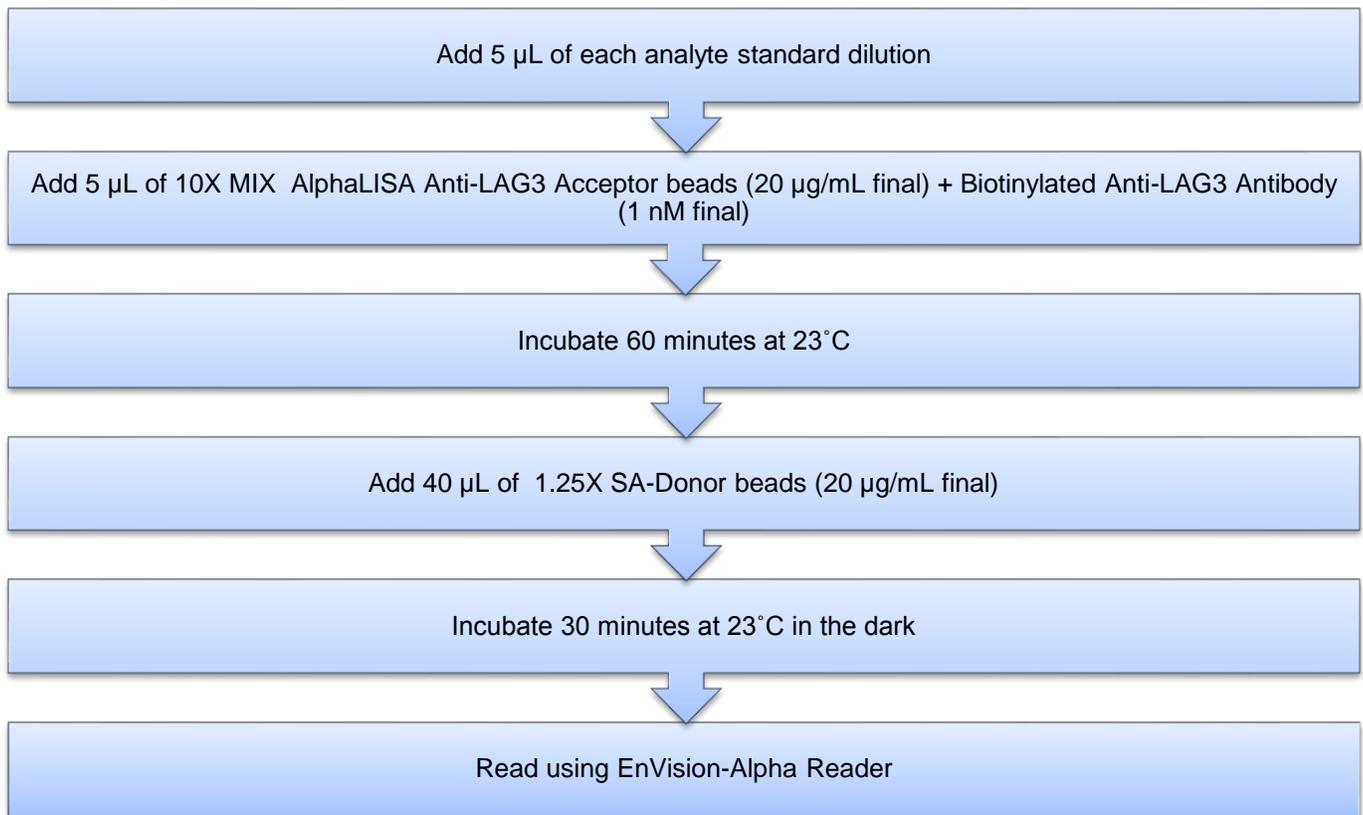| Format	# of data points	Volume				Plate recommendation
		Final	Sample	MIX AlphaLISA AccBeads + biotinylated Ab	SA-Donor beads	
AL3058HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3058C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3058F	5 000	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	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 a 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 H_2O.

2) Preparation of LAG-3 analyte standard dilutions:
   a. Reconstitute lyophilized LAG-3 (3 µg) in 100 µL Milli-Q® grade H_2O.

b. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of LAG-3 (µL)	Vol. of diluent (µL) *	[LAG-3] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted LAG-3	90	3.00E-06	3 000 000
B	60 µL of tube A	120	1.00E-06	1 000 000
C	60 µL of tube B	140	3.00E-07	300 000
D	60 µL of tube C	120	1.00E-07	100 000
E	60 µL of tube D	140	3.00E-08	30 000
F	60 µL of tube E	120	1.00E-08	10 000
G	60 µL of tube F	140	3.00E-09	3 000
H	60 µL of tube G	120	1.00E-09	1 000
I	60 µL of tube H	140	3.00E-10	300
J	60 µL of tube I	120	1.00E-10	100
K	60 µL of tube J	140	3.00E-11	30
L	60 µL of tube K	120	1.00E-11	10
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).
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 10X MIX Anti-LAG-3 AlphaLISA Acceptor beads (200 µg/mL) + biotinylated Anti-LAG-3 antibody (10 nM):
    a. Prepare just before use.
    b. Add 100 µL Anti-LAG-3 Acceptor beads and 50 µL of 500 nM biotinylated Anti-LAG-3 antibody to 2 350 µl of 1X AlphaLISA Immunoassay Buffer

4) Preparation of 2X Streptavidin (SA) Donor beads (25 µg/mL):
    a. Prepare just before use.
    b. Keep the beads under subdued laboratory lighting.
    c. Add 100 µL of 5 mg/mL SA-Donor beads to 19 900 µL of 1X AlphaLISA Immunoassay Buffer.

5) In a white Optiplate (384 wells):

Add 5 µL of each analyte standard dilution

⬇

Add 5 µL of 10X MIX AlphaLISA Anti-LAG3 Acceptor beads (20 µg/mL final) + Biotinylated Anti-LAG3 Antibody (1 nM final)

⬇

Incubate 60 minutes at 23˚C

⬇

Add 40 µL of 1.25X SA-Donor beads (20 µg/mL final)

⬇

Incubate 30 minutes at 23˚C in the dark

⬇

Read using EnVision-Alpha Reader

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, 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 µL sample using the recommended assay conditions.

LDL (pg/mL)*	(Analyte diluent)	# of experiments
74	IAB	6
92	DMEM + 10% FBS	6
514	RPMI + 10% FBS**	6
109	100% FBS	6
51	RIPA Buffer	6

* Note that LDL can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 µL of analyte in a final assay volume of 50 µL).
** Assay Performance is severely affected by RPMI. Use is not recommended

- 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, 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%.

LAG-3	IAB	DMEM+ 10% FBS	100% FBS	RIPA Buffer
CV (%)	7	10	9	8

- Inter-assay precision:

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

LAG-3	IAB	DMEM+ 10% FBS	100% FBS	RIPA Buffer
CV (%)	13	14	10	11

- Spike Recovery:

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

Spiked LAG-3 (ng/mL)	% Recovery			
	IAB	DMEM+ 10% FBS	100% FBS	RIPA Buffer
10	92	103	89	105
3	91	109	93	109
1	91	115	91	107

Human Serum Experiments

- Dilution Linearity

Neat Normal Human Serum and LAG-3-spiked (30 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 LAG-3 in diluted human serum were determined by interpolating to the standard curve. Excellent dilution linearity R^2 = 0.997)) was achieved in the LAG-3-spiked human serum samples that was diluted ≥ 2 fold. The results are shown in table and figure below.

Dilution Factor (x)	Expected LAG-3 (ng/mL)	Observed LAG-3 (ng/mL)
1	30	35.3
2	15	14.6
4	7.5	7.5
8	3.75	3.6
16	1.875	1.77
32	0.938	0.90
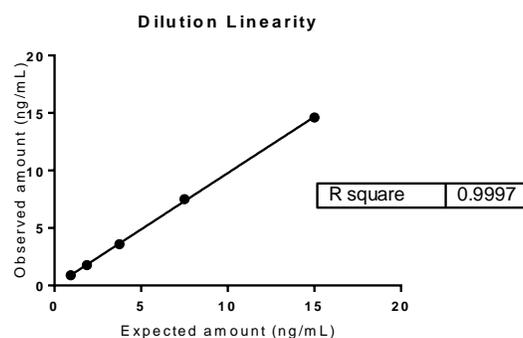
- Spike Recovery

Three known amounts of LAG-3 were spiked into Normal Human Serum (30, 10, and 3 ng/mL LAG-3 in spiked samples) and then the samples were diluted 2-fold into 100% FBS. The standard was prepared in FBS and all other reagents were prepared in IAB. The spike recoveries of LAG-3 were determined and the results are shown in table below. *Recoveries were calculated after the endogenous LAG-3 level was subtracted (in this case, 3.2 ng/mL in Normal Human Serum). Excellent recovery was achieved for all three spikes tested.
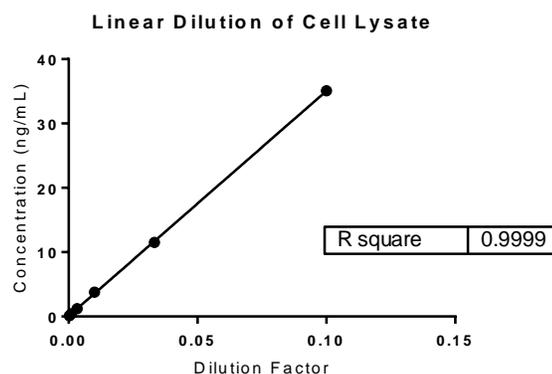
	Diluent: 100%FBS	
	Spiked sample (Normal Human Serum)	
Spike (ng/mL)	Concentration (ng/mL)*	Recovery (%)
No spike	3.2	N/A
30	28.1	94
10	9.8	98
3	2.7	90

Cell Lysate Experiments

- Dilution Linearity

To validate the assay kit, commercially available cell lysate samples with unknown concentrations of LAG-3 were tested. The cell lysates include LAG-3 positive and negative samples. The standard was prepared in the RIPA and lysate samples were diluted with RIPA. All other reagents were prepared in IAB. LAG-3 was not detected in negative samples. In the positive samples, 372 ng/mL LAG-3 was detected and excellent dilution linearity (R^2 = 0.999) was achieved when lysate was diluted ≥ 10 fold. The results are summarized from 3 experiments and shown in table and figure below.

Cell Lysate Dilution Fold (DF)	LAG-3 detected in Positive Cell Lysate (ng/mL)	LAG-3 Positive Cell Lysate (ng/mL x DF)	LAG-3 Negative Cell Lysate (ng/mL)*
10	35.1	351	0
30	11.5	345	0
100	3.8	380	0
300	1.2	360	0
1000	0.41	410	0
3000	0.13	390	0
Average ± SD	N/A	372 ± 25	0

Linear Dilution of Cell Lysate

| R square | 0.9999 |

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