MANUAL

AlphaLISA® Human IgG Fc Detection Kit

Product number: AL3129HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of hIgG Fc in cell culture media and serum using a homogeneous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 6 ng/mL

Lower Limit of Quantification (LLOQ): 35 ng/mL

EC_{50}: 42 ng/mL

Dynamic range: 6 – 100 000 ng/mL

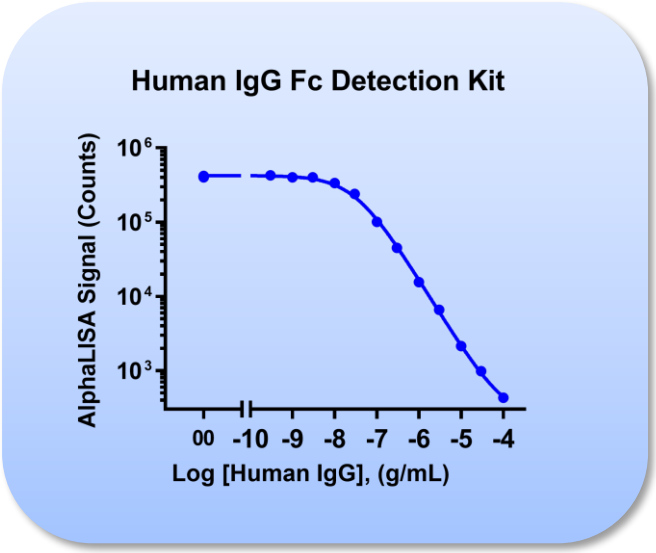


Figure 1. Typical sensitivity curve in AlphaLISA HiBlock Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

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 12 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Human Immunoglobulin G (hIgG) is one of the antibodies that is produced by plasma B-cells and protects the body from infections and toxifications such as viruses, bacteria, fungi and toxins. Human IgG is divided into 4 subtypes: IgG1, IgG2, IgG3, and IgG4. The molecular structure of human IgG is well known as a Y-like shape that contains Fab and Fc regions. The Fc portion of IgG is widely used in Fc fusion protein therapeutics for protein or antibody drug discovery and development. There are more than a dozen Fc-fusion protein drugs are available on the market to treat various human diseases. The Human IgG Fc AlphaLISA Detection Kit specifically detects human IgG Fc portion of all IgG subtypes (IgG1, IgG2, IgG3, and IgG4).

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA competition assay, a Biotinylated analog (hIgG) of the analyte of interest, the probe, binds to the Streptavidin-coated Alpha Donor beads, while the Anti-Analyte (hIgG Fc) Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of low analyte (IgG), 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). In the presence of high analyte (IgG), the beads are separated resulting in lower emission.

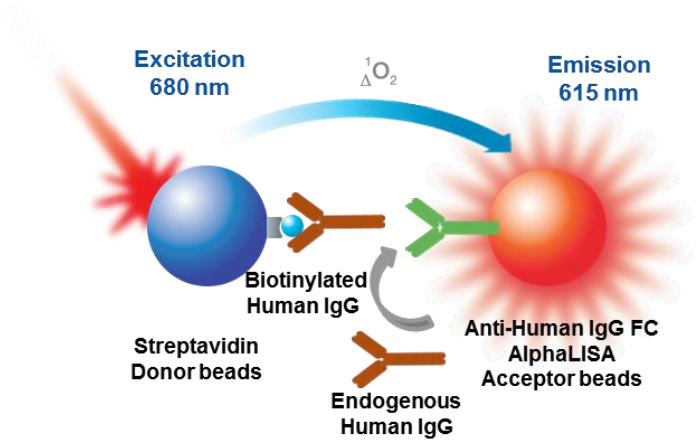


Figure 2. AlphaLISA hIgG Fc Detection (competition) Assay Principle.

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 hIgG probe contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3129HV (100 assay points***)	AL3129C (500 assay points***)	AL3129F (5000 assay points***)
AlphaLISA Anti-hIgG Fc Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	40 µL @ 5 mg/mL (1 brown tube, white cap)	100 µL @ 5 mg/mL (1 brown tube, white cap)	1 mL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4	80 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated hIgG Probe stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Lyophilized hIgG	200 µg (1 tube, clear cap)	200 µg (1 tube, clear cap)	200 µg (1 tube, clear cap)
AlphaLISA HiBlock Buffer (10X)	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Reconstitute lyophilized analyte in 200 µL Milli-Q® grade H_2O. 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 at -20°C is stable up to 90 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 # AL3129S).

** Extra buffer can be ordered separately (cat # AL004C: 10 mL, cat # AL004F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates or 50 µL in 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 hIgG probe 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	Revvity Inc.	6050185
EnVision®-Alpha Reader	Revvity 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 H_2O (18 MΩ•cm) to dilute 10X AlphaLISA HiBlock Buffer and to reconstitute the lyophilizedáanalyte.
- 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 manual described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The manuals 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 manual 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.

		Volume					
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA Acceptor Beads**	**Biotinylated hIgG Probe**	**SA-Donor beads**	**Plate recommendation**
AL3129HV	100	100 µL	40 µL	20 µL	20 µL	20 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3129C	250	100 µL	40 µL	20 µL	20 µL	20 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	20 µL	10 µL	10 µL	10 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	8 µL	4 µL	4 µL	4 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	4 µL	2 µL	2 µL	2 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3129F	5 000	50 µL	20 µL	10 µL	10 µL	10 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	8 µL	4 µL	4 µL	4 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	4 µL	2 µL	2 µL	2 µL	Light gray AlphaPlate-1536 (cat # 6004350)

3 Step Manual 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 HiBlock Buffer:
Add 5 mL of 10X AlphaLISA HiBlock Buffer to 45 mL Milli-Q® grade H_2O.

2) Preparation of hIgG analyte standard dilutions:

a. Reconstitute lyophilized hIgG (200 µg) in 200 µL Milli-Q® grade H_2O. The remaining reconstituted analyte should be aliquoted immediately and stored at -20 ºC for future assays (see page 4 for more details).
b. Prepare standard dilutions as follows in 1X AlphaLISA HiBlock Buffer (change tip between each standard dilution):

Tube	Vol. of hIgG (µL)	Vol. of diluent (µL)*	[hIgG] in standard curve	
			(g/mL in 20 µL)	(ng/mL in 20 µL)
A	20 µL of reconstituted hIgG	180	1.0E-04	100 000
B	60 µL of tube A	140	3.0E-05	30 000
C	60 µL of tube B	120	1.0E-05	10 000
D	60 µL of tube C	140	3.0E-06	3000
E	60 µL of tube D	120	1.0E-06	1000
F	60 µL of tube E	140	3.0E-07	300
G	60 µL of tube F	120	1.0E-07	100
H	60 µL of tube G	140	3.0E-08	30
I	60 µL of tube H	120	1.0E-08	10
J	60 µL of tube I	140	3.0E-09	3
K	60 µL of tube J	120	1.0E-09	1
L	60 µL of tube K	140	3.0E-10	0.3
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 HiBlock 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.

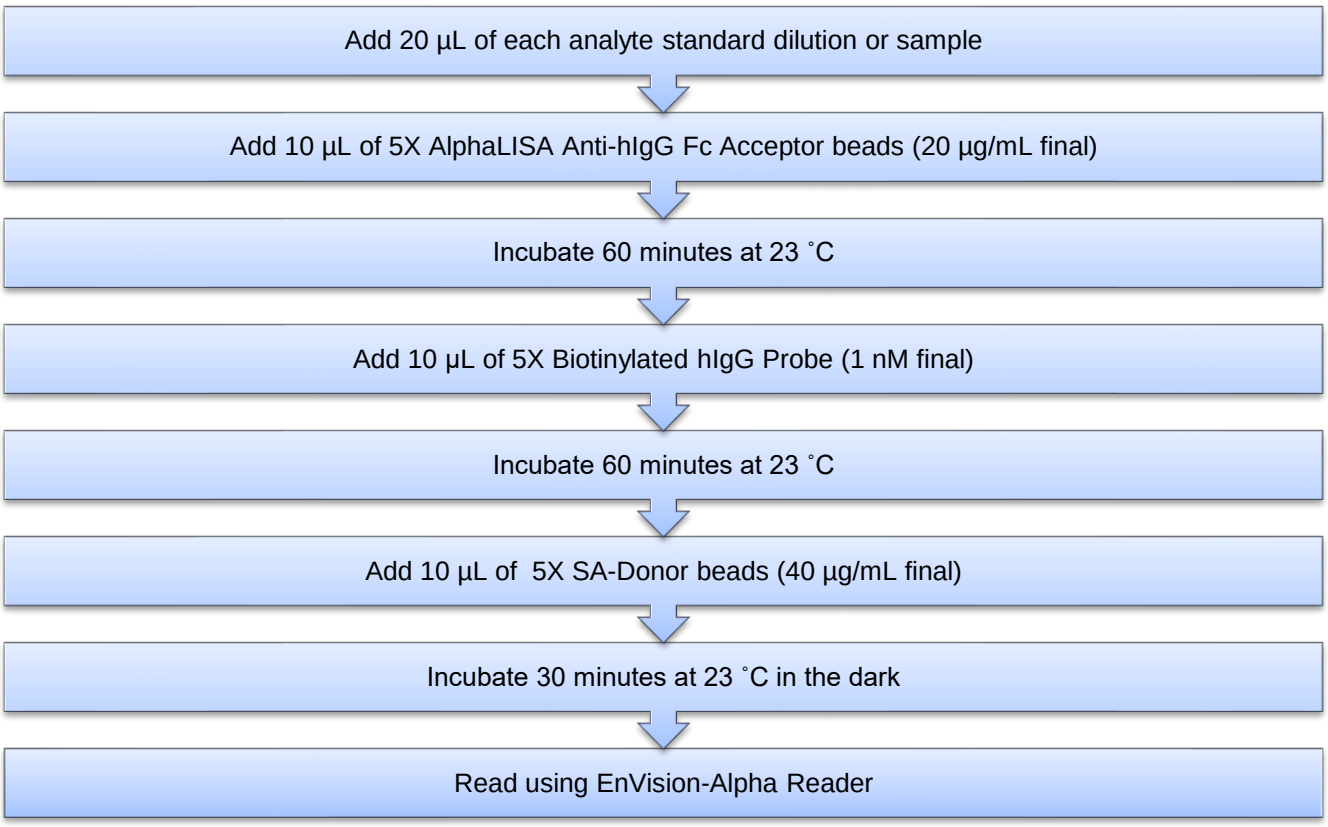
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X Anti-hIgG AlphaLISA Acceptor beads (100 µg/mL):
a. Prepare just before use.
b. Add 100 µL Anti-hIgG Acceptor beads to 4900 µl of 1X AlphaLISA HiBlock Buffer.

4) Preparation of 5X biotinylated Anti-hIgG antibody (5 nM):
a. Prepare just before use.
b. Add 50 µL 500 nM Biotinylated Anti-hIgG Antibody to 4950 µl of 1X AlphaLISA HiBlock Buffer.

5) Preparation of 5X Streptavidin (SA) Donor beads (200 µg/mL):
a. Prepare just before use.
b. Keep the beads under subdued laboratory lighting.
c. Add 500 µL of 5 mg/mL SA-Donor beads to 12 000 µL of 1X AlphaLISA HiBlock 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 a 3-step manual using AlphaLISA HiBlock Buffer (HB) as assay buffer. The analytes (standards) were prepared in HB, DMEM + 10% FBS, RPMI + 10% FBS, or 100% FBS and all other components were prepared in HB.

- 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 (ng/mL)	(Analyte diluent)	# of experiments
6	HB	6
9	DMEM + 10% FBS	6
7	RPMI + 10% FBS	6
11	100% FBS	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 HB, DMEM + 10% FBS, RPMI + 10% FBS, or 100% FBS. All other components were prepared in HB. 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%.

hIgG	HB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS
CV (%)	6	4	9	7

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 9 measurements for 30 ng/mL sample. Shown as CV%.

hIgG	HB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS
CV (%)	7	6	11	10

- Spike Recovery:

Three known concentrations of analyte were spiked into HB, DMEM + 10% FBS, RPMI + 10% FBS, or 100% FBS. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in HB, DMEM + 10% FBS, RPMI + 10% FBS, or 100% FBS. All other assay components were diluted in HB.

Spiked hIgG (µg/mL)	% Recovery			
	HB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS
10	86	95	98	88
3	95	101	87	87
0.1	80	89	76	90

- Specificity:

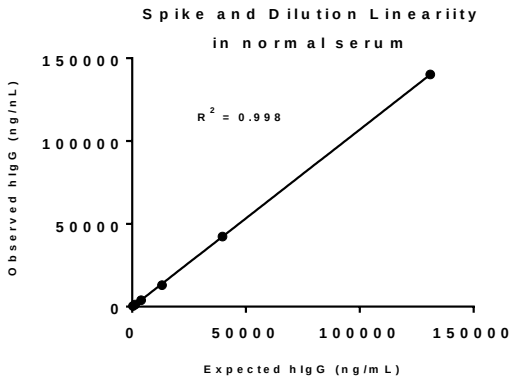
Cross-reactivity of the hIgG Fc AlphaLISA Detection Kit was tested using the following proteins at 100 ng/mL in HB. The cross reactivities were calculated using the counts of 100 ng/mL human IgG as 100%.

Tested Proteins	% Cross Reactivity
Human IgG Fab	0.2
Human IgG (Fab')2	0.2
Human IgA	3.6
Human IgD	5.1
Human IgM	3.4
Mouse IgG	5.5
Rat IgG	0.4
Monkey IgG	0.2
Donkey IgG	0.3
Rabbit	0.3
Bovine IgG	0.8
Goat IgG	0.3
Swine IgG	0.2

- Serum Experiments
    - Dilution Linearity

Neat Normal Human Serum and hIgG-spiked (100 µg/mL) Normal Human Serum samples were diluted with 100% FBS and the assay was performed along with a standard curve prepared in 100% FBS. Concentrations of hIgG in diluted human serum samples were determined by interpolating to the standard curve. In normal human serum, 13.1 mg/mL hIgG was detected when the samples were diluted 100 to 333333 folds with excellent dilution linearity ($R^2 > 0.999$). Excellent dilution linearity ($R^2 > 0.999$) was also achieved in the hIgG-spiked human serum samples that were diluted up to 333333 folds. The IgG levels are in spiked samples are almost same as the levels in the diluted neat normal human serum. This is due to the large amounts of endogenous IgG detected (13.1 mg/mL) in neat serum and the assay cannot differentiate the levels in spiked (spiking 0.1 mg/mL to 13.1 mg/mL = 13.2 mg/mL) vs. non-spiked (13.1 mg/mL). Therefore, the expected IgG in spiked serum samples was replaced with the endogenous levels plus the spiked amount (13.1 + 0.1 =13.2 mg/mL). The results are shown in table and figure below.

Dilution Factor (x)	Expected hIgG (ng/mL)	Observed hIgG (ng/mL)
100	132000	140234
333	40000	42290
1000	13200	12931
3333	3964	3927
10000	1320	1276
33333	396	409
100000	132	129
333333	40	32



Spike Recovery

Three known amounts of hIgG were spiked into Normal Human Serum pre-diluted with 100% FBS to 1000000-fold resulting in 10000, 1000, and 100 ng/mL hIgG in spiked samples. The samples were assayed along the standard prepared in 100% FBS. The spike recoveries of hIgG were determined and the results are shown in table below.

	Diluent: 100% FBS	
	Spiked sample (Normal Human Serum diluted to 1000000x)	
Spike (ng/mL)	Concentration (ng/mL)*	Recovery (%)
No spike	13.1	N/A
10000	9300	93
1000	888	88
100	89	89

* Recoveries were calculated after the no spike hIgG level was subtracted (in this case, 13.1 ng/mL in 1000000 x diluted Normal Human Serum). Excellent recovery was achieved for all three spikes tested.

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at: www.revvity.com

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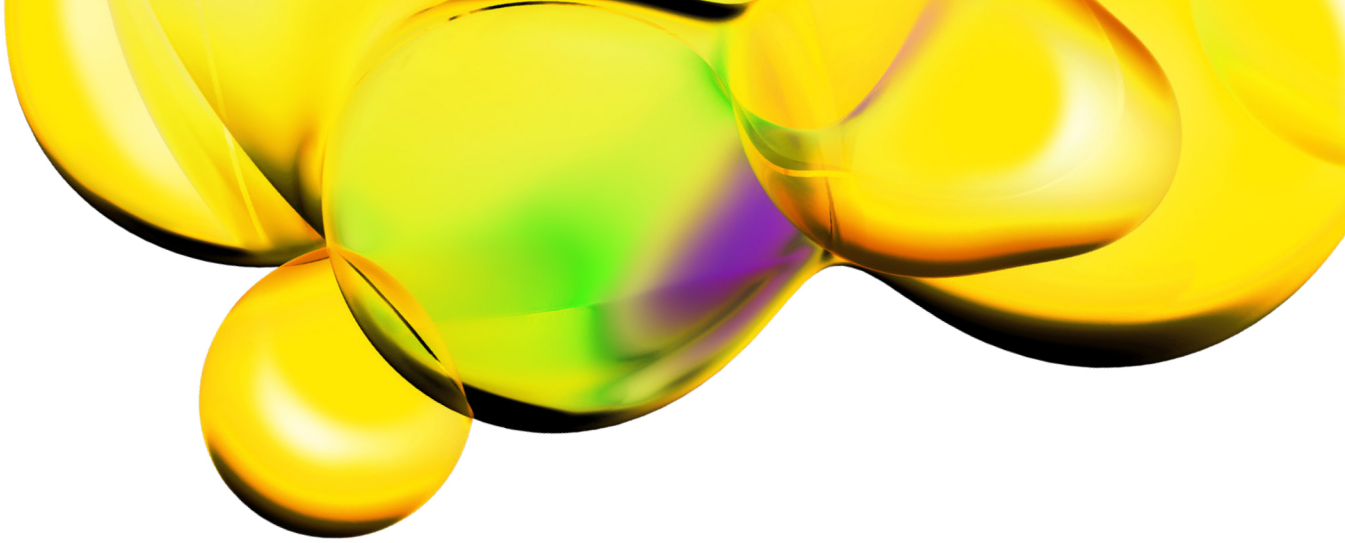

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