TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Research Use Only. Not for use in diagnostic procedures.

Porcine Interleukin 10 (pIL10) AlphaLISA Detection Kit

Product No.: AL574C/F

Contents

Product Information

Application: This kit is designed for the quantitative determination of porcine Interleukin 10 (pIL10) in serum and cell culture media using a homogeneous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 18.3 pg/mL

Lower Limit of Quantification (LLOQ): 58.5 pg/mL

EC_{50}: 18.5 ng/mL

Dynamic range: 18.3 – 100 000 pg/mL

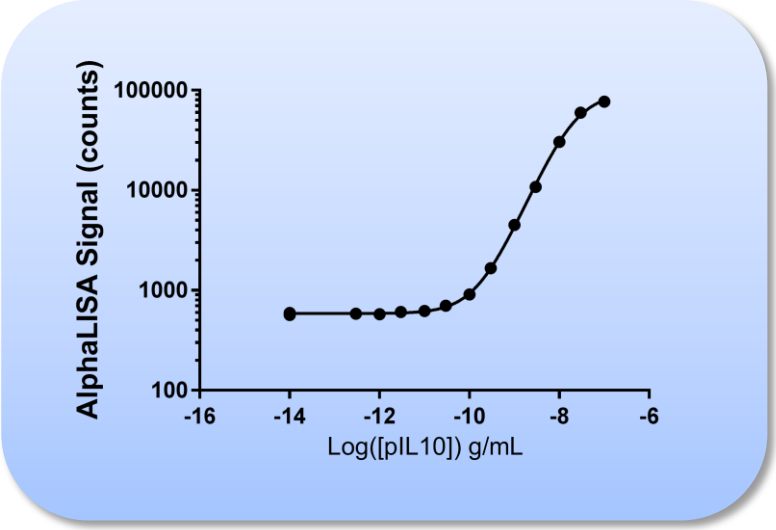

Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at +4˚C. For reconstituted analyte aliquot and store at -20 °C for up to 3 months. 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.

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.

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Analyte of Interest

Interleukin 10 (IL10) is a homodimer composed of two subunits of 18 kDa each. It is produced by various T cell populations, monocytes, macrophages, and different cell types in the liver when stimulated by endogenous or exogenous factors such as stress, exotoxins, tumor necrosis factor-a, and catecholamines. IL10 inhibits interferon-γ synthesis in Th1 cell clones, monocytes and macrophages. IL10 also inhibits antigen presentation to T cells and IL12 production by monocytes. It also impairs the proliferation and cytokine synthesis of CD4+ T cells, without having a direct inhibitory effect on CD8+ T cells. On the other hand, IL10 has a stimulatory effect on B cells, prevents apoptosis and enhances proliferation and differentiation of plasma cells, and inhibits the release of various chemokines by neutrophils. In general, the main biological function of IL10 is to limit and terminate the inflammatory responses, block proinflammatory cytokine secretion and regulate the differentiation and proliferation of several immune cells. IL10 activity is mediated by the heteromeric IL10 receptor (IL-10R), and signals through the tyrosine kinases Jak1 and Tyk2, and STATs.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, and serum in a highly sensitive, quantitative, reproducible and user-friendly mode. In this AlphaLISA assay, a Biotinylated Anti-pIL10 Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-pIL10 Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the pIL10, 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).

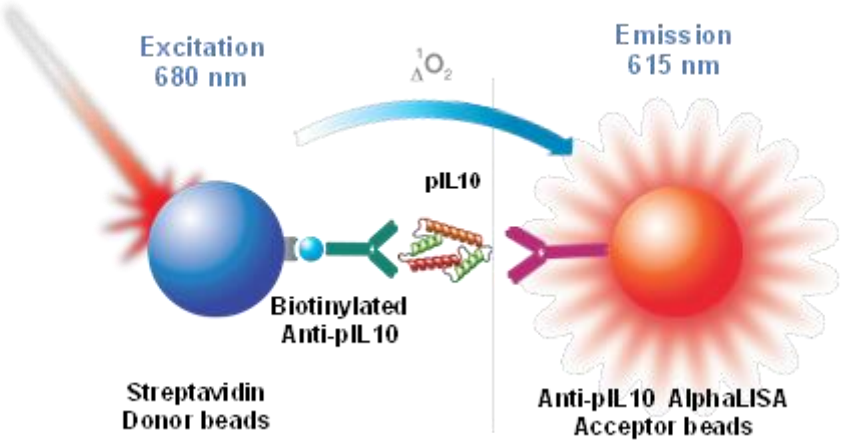

Figure 2. AlphaLISA Assay Principle.

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	AL574HV (100 assay points***)	AL574C (500 assay points***)	AL574F (5000 assay points***)
AlphaLISA Anti-pIL10 Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white 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, 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 Anti-pIL10 Antibody 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 Recombinant Porcine IL10*	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)	1 µg (1 tube, clear 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_2O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored up to 3 months 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 # AL574S).

** 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

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 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.

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		Volume				
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA AccBead + bAb MIX**	**SA-Donor beads**	**Plate recommendation**
AL574HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL574C	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)
AL574F	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 Hi Concentration 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 H_2O.

2) Preparation of pIL10 analyte standard dilutions:

a. Reconstitute lyophilized pIL10 (1 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 pIL10 (µL)	Vol. of diluent (µL) *	[pIL10] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	2 µL of reconstituted pIL10	198	1.00E-07	100 000
B	60 µL of tube A	140	3.00E-08	30 000
C	60 µL of tube B	120	1.00E-08	10 000
D	60 µL of tube C	140	3.00E-09	3 000
E	60 µL of tube D	120	1.00E-09	1 000
F	60 µL of tube E	140	3.00E-10	300
G	60 µL of tube F	120	1.00E-10	100
H	60 µL of tube G	140	3.00E-11	30
I	60 µL of tube H	120	1.00E-11	10
J	60 µL of tube I	140	3.00E-12	3
K	60 µL of tube J	120	1.00E-12	1
L	60 µL of tube K	140	3.00E-13	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 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).

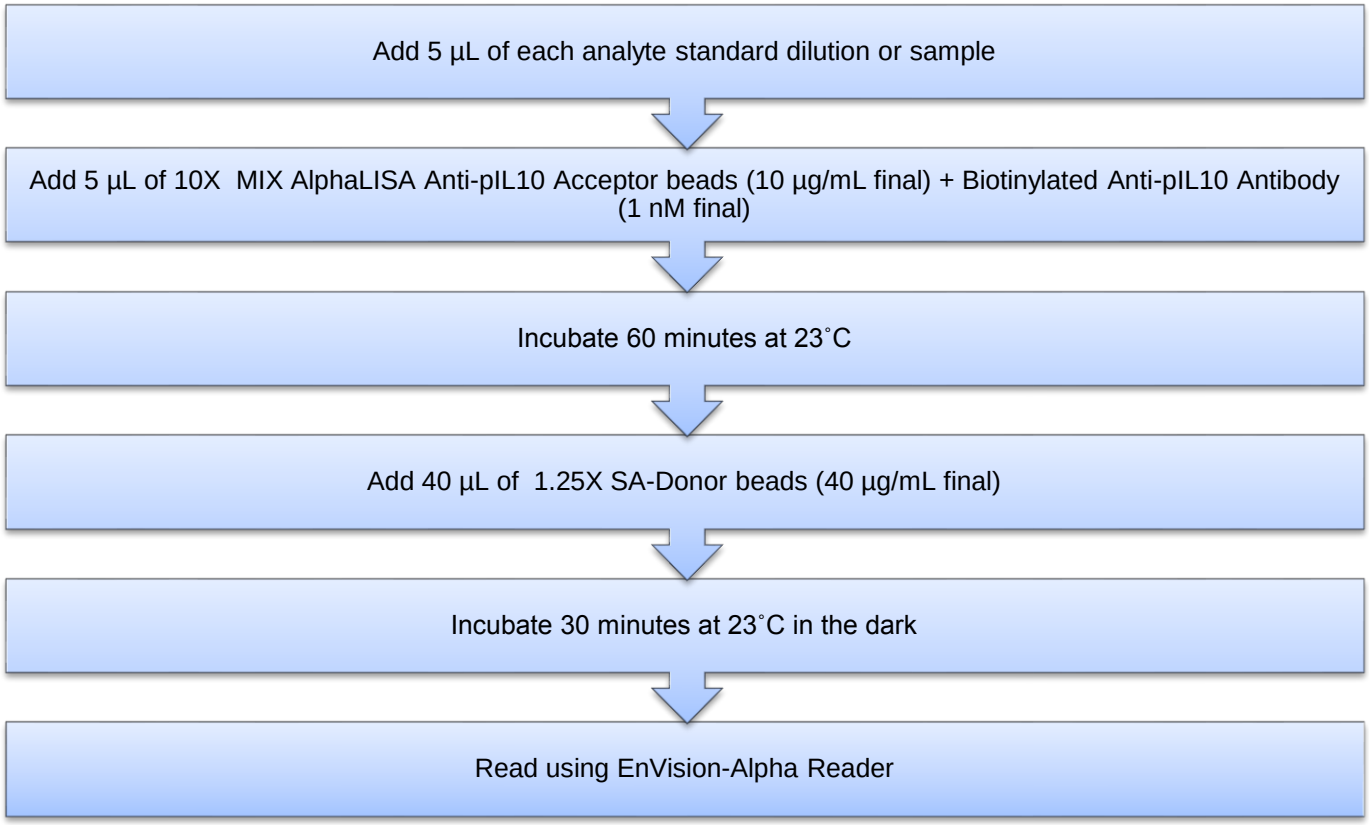
3) Preparation of 10X MIX Anti-pIL10 AlphaLISA Acceptor beads (100 µg/mL) + biotinylated Anti-pIL10 antibody (10 nM):

a. Prepare just before use.
b. Add 50 µL of 5 mg/mL Anti-pIL10 AlphaLISA Acceptor Beads and 50 µL of 500 nM Biotinylated Anti-pIL10 Antibody to 2400 µl of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 1.25X Streptavidin (SA) Donor beads (50 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 19 800 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 Hi-concentration protocol using AlphaLISA Immunoassay Buffer (IAB) as assay buffer. The analytes (standards) were prepared in IAB, DMEM + 10%

FBS, RPMI + 10% FBS, or 100% FBS and all other components were prepared in IAB. Standard curve in 100% FBS was started at 1 µg/mL for best curve fiting.

- 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
10.9	IAB	6
21.1	DMEM + 10% FBS	6
25.1	RPMI + 10% FBS	6
42.1	100% FBS	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 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 or 100% FBS. 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%.

pIL10	IAB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS
CV (%)	4	4	7	6

- 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%.

pIL10	IAB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS
CV (%)	8	9	12	8

- Spike Recovery:

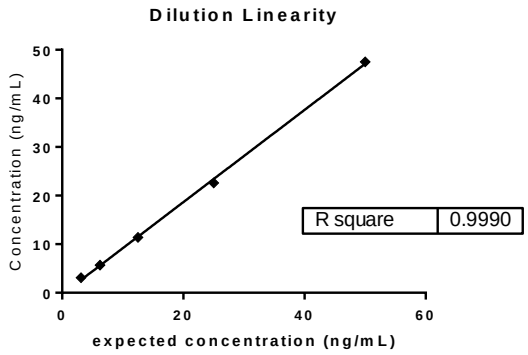
Three known concentrations of analyte were spiked into IAB, DMEM + 10% FBS, RPMI + 10% FBS, or 100% FBS. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in IAB, DMEM + 10% FBS, RPMI + 10% FBS, or 100% FBS.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

Spiked pIL10 (ng/mL)	% Recovery			
	IAB	DMEM + 10% FBS	RPMI + 10% FBS	100% FBS
1	99	105	92	92
0.3	105	104	97	88
0.1	92	109	89	87

- Porcine Serum Experiments
  - Dilution Linearity

It is recommended to start standard curves in 100% FBS at 1 µg/mL for best curve fitting. Neat Normal Porcine Serum and pIL10-spiked (100 ng/mL) Normal Porcine 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 pIL10 in diluted porcine serum were determined by interpolating to the standard curve. Excellent dilution linearity (R^2 > 0.9990) was achieved in the pIL10-spiked porcine serum samples that were diluted ≥ 2 fold. The results are shown in table and figure below.

Dilution Factor (x)	Expected pIL10 (ng/mL)	Observed pIL10 (ng/mL)
2	50.0	47.5
4	25.0	22.6
8	12.5	11.4
16	6.25	5.7
32	3.12	3.1



  - Spike Recovery

Three known amounts of pIL10 were spiked into Normal Porcine Serum (10, 3, and 1 ng/mL pIL10 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 pIL10 were determined and the results are shown in table below.

	Diluent: 100% FBS	
	Spiked sample (Normal Porcine Serum)	
Spike (ng/mL)	Concentration (ng/mL)	Recovery (%)

No spike	Below LDL	N/A
10	8.7	87
3	2.8	94
1	0.85	85

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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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com