MANUAL

# AlphaLISA® Human FAS Ligand (FASL) Detection Kit

**Product number:** AL3104 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

## Product Information

**Application:** This kit is designed for the quantitative determination of FASL in buffer, cell culture media, serum, plasma and cell supernatant using a homogeneous AlphaLISA assay (no wash steps).

**Sensitivity:** Lower Detection Limit (LDL): 1.3 pg/mL

Lower Limit of Quantification (LLOQ): 5.2 pg/mL

$EC_{50}$: 12.7 ng/mL

**Dynamic range:** 1.3 – 100 000 pg/mL

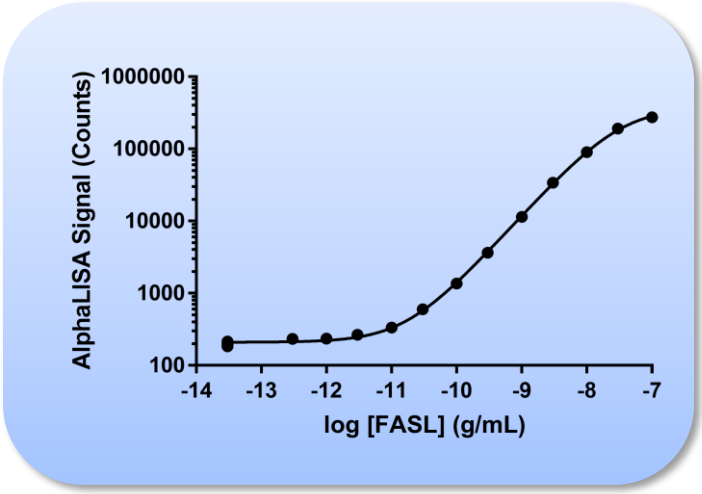



Figure 1. Typical sensitivity curve in AlphaLISA HiBlock Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

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

## Analyte of Interest

FAS Ligand (FASL), also known as CD95L is a transmembrane protein expressed on cytotoxic T cells. It binds to CD95 regulatory T-cells. Upon binding to its receptor, the protein forms trimers that initiate apoptosis of the cells. The protein is involved in regulation of cytotoxic T-cells levels, protection of self-immune effects and control of inflammation. However, some cancer cells can overexpress FASL, blocking the immune response from destroying the tumor.

## Description of the AlphaLISA Assay

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

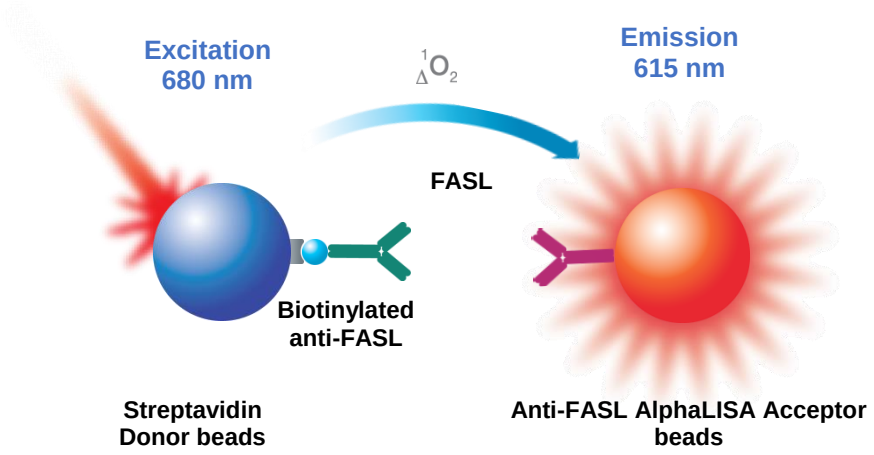



Figure 2. AlphaLISA FASL Assay Principle.

## 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 | AL3104HV (100 assay points***) | AL3104C (500 assay points***) | AL3104F (5000 assay points***) |
|---|---|---|---|
| AlphaLISA Anti-FASL 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-FASL 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 FASL* | 0.1 µg (1 tube, clear cap) | 0.1 µg (1 tube, clear cap) | 0.1 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 100 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 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 # AL3104S).

** 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 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 | 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 $H_2O$ to dilute 10X AlphaLISA HiBlock 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 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 Antibody** | **SA-Donor beads** | **Plate recommendation** |
| **AL3104HV** | 100 | 100 µL | 10 µL | 20 µL | 20 µL | 50 µL | White OptiPlate-96 (cat # 6005290)<br>White ½ AreaPlate-96 (cat # 6005560) |
| **AL3104C** | 250 | 100 µL | 10 µL | 20 µL | 20 µL | 50 µL | White OptiPlate-96 (cat # 6005290)<br>White ½ AreaPlate-96 (cat # 6005560) |
| | 500 | 50 µL | 5 µL | 10 µL | 10 µL | 25 µL | White ½ AreaPlate-96 (cat # 6005560)<br>White OptiPlate-384 (cat # 6007290)<br>Light gray AlphaPlate™-384 (cat # 6005350) |
| | 1 250 | 20 µL | 2 µL | 4 µL | 4 µL | 10 µL | Light gray AlphaPlate-384 (cat # 6005350)<br>ProxiPlate™-384 Plus (cat # 6008280)<br>White OptiPlate-384 (cat # 6007290) |
| | 2 500 | 10 µL | 1 µL | 2 µL | 2 µL | 5 µL | Light gray AlphaPlate-1536 (cat # 6004350) |
| **AL3104F** | 5 000 | 50 µL | 5 µL | 10 µL | 10 µL | 25 µL | White ½ AreaPlate-96 (cat # 6005560)<br>White OptiPlate-384 (cat # 6007290)<br>Light gray AlphaPlate-384 (cat # 6005350) |
| | 12 500 | 20 µL | 2 µL | 4 µL | 4 µL | 10 µL | Light gray AlphaPlate-384 (cat # 6005350)<br>ProxiPlate-384 Plus (cat # 6008280)<br>White OptiPlate-384 (cat # 6007290) |
| | 25 000 | 10 µL | 1 µL | 2 µL | 2 µL | 5 µL | Light gray AlphaPlate-1536 (cat # 6004350) |

**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 FASL analyte standard dilutions:

   a. Reconstitute lyophilized FASL (0.1 µg) in 100 µL Milli-Q® grade $H_2O$. The remaining reconstituted analyte should be aliquoted immediately and stored at -20ºC 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 FASL (µL) | Vol. of diluent (µL)* | [FASL] in standard curve | |
|---|---|---|---|---|
| | | | (g/mL in 5 µL) | (pg/mL in 5 µL) |
| A | 10 µL of reconstituted FASL | 90 | 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 HiBlock 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-FASL AlphaLISA Acceptor beads (50 µg/mL):

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

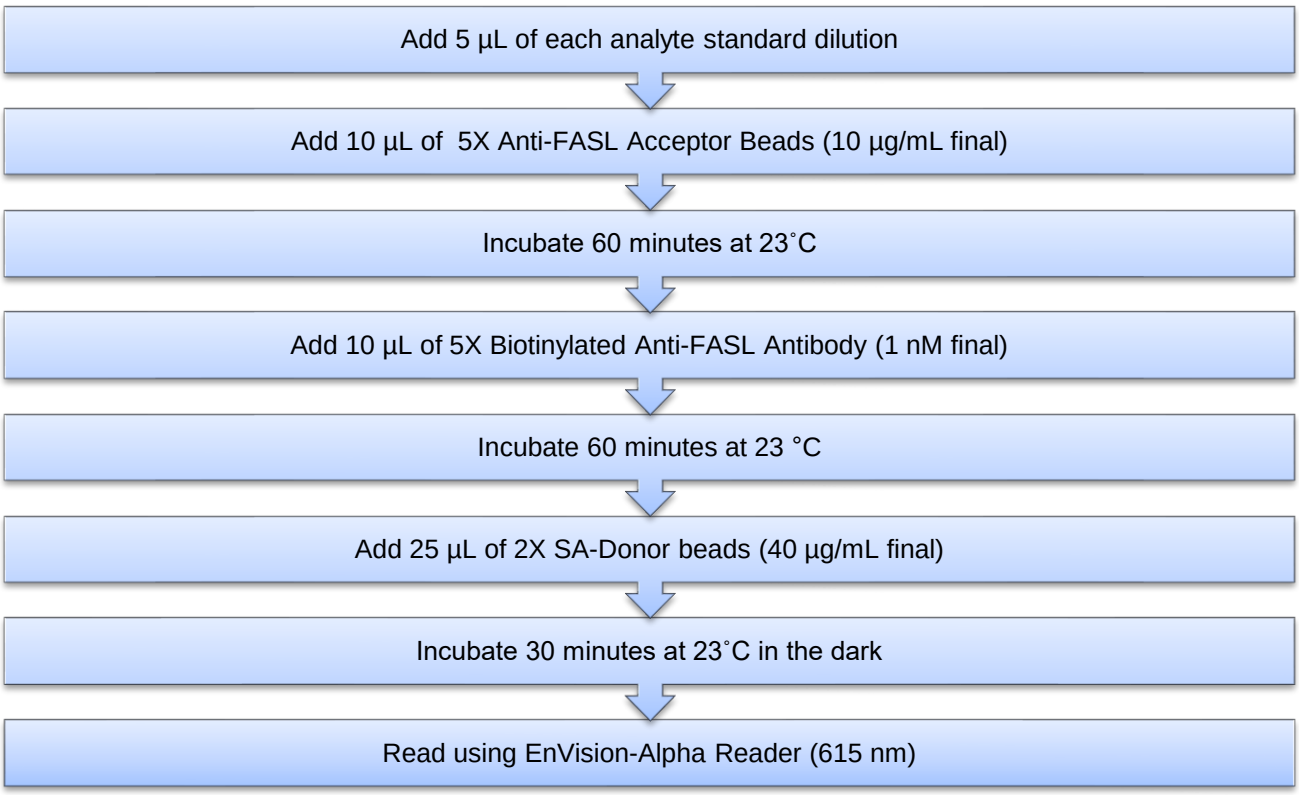
4) Preparation of 5X biotinylated Anti-FASL antibody (5 nM):

   a. Prepare just before use.
   b. Add 50 µL 500 nM Biotinylated Anti-FASL Antibody to 4950 µl of 1X AlphaLISA HiBlock 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 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 using the 3 step manual using AlphaLISA HiBlock Buffer as assay buffer. The analytes (standards) were prepared in HiBlock Buffer, DMEM, RPMI 1640, 100% FBS, 10% FBS in HiBlock Buffer, or AlphaLISA Lysis Buffer and all other components were prepared in HiBlock Buffer.

- 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 |
|---|---|---|
| 1.3 | HiBlock Buffer | 6 |
| 3.4 | DMEM | 6 |
| 11.3* | RPMI 1640 | 6 |
| 4.0 | 100% FBS | 6 |
| 1.7 | 10% FBS in HiBlock Buffer | 6 |
| 2.1 | AlphaLISA Lysis Buffer | 6 |

( * ) Diluting RPMI 1640 1:1 with HiBlock buffer reduces LDL to 1.4 pg/mL

- 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 HiBlock Buffer, DMEM, RPMI 1640, 100% FBS, 10% FBS in HiBlock Buffer, or AlphaLISA Lysis Buffer. All other components were prepared in HiBlock Buffer. 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%.

| FASL | HiBlock Buffer | DMEM | RPMI 1640 | 100% FBS | 10% FBS in HiBlock Buffer | AlphaLISA Lysis Buffer |
|---|---|---|---|---|---|---|
| **CV (%)** | 1.9 | 4.1 | 6.8 | 5.6 | 3.3 | 1.7 |

- 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%.

| FASL | HiBlock Buffer | DMEM | RPMI 1640 | 100% FBS | 10% FBS in HiBlock Buffer | AlphaLISA Lysis Buffer |
|---|---|---|---|---|---|---|
| **CV (%)** | 5.4 | 9.1 | 12.3 | 9.7 | 11.6 | 12.1 |

- Spike Recovery:

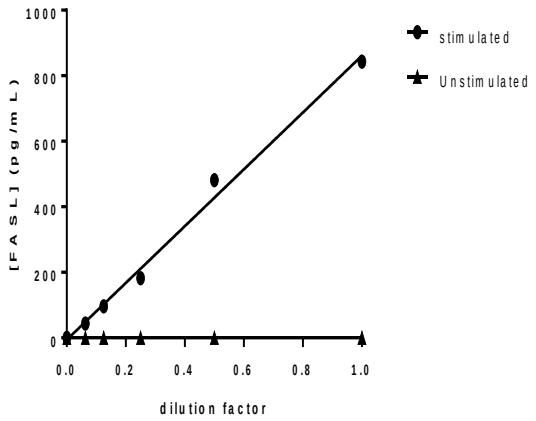
Three known concentrations of analyte were spiked into HiBlock Buffer, DMEM, RPMI 1640, 100% FBS, 10% FBS in HiBlock Buffer, or AlphaLISA Lysis Buffer. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in HiBlock Buffer, DMEM, RPMI 1640, 100% FBS, 10% FBS in HiBlock Buffer, or AlphaLISA Lysis Buffer. All other assay components were diluted in HiBlock Buffer.

| Spiked FASL (ng/mL) | % Recovery | | | | | |
|---|---|---|---|---|---|---|
| | HiBlock Buffer | DMEM | RPMI 1640 | 100% FBS | 10% FBS in HiBlock Buffer | AlphaLISA Lysis Buffer |
| 10 | 91 | 110 | 88 | 110 | 93 | 103 |
| 3 | 97 | 89 | 87 | 114 | 94 | 104 |
| 1 | 102 | 92 | 101 | 107 | 87 | 115 |

- **Cell Supernatant Experiments**

Peripheral monocytic blood cells (PBMC) were grown in RPMI 1640 + 10% FBS at a concentration of 500 000 cells per mL. One sample of cells was stimulated by incubation for 48 hours with CD3/CD28 Dynabeads (ThermoFisher 11131D) with 1 Dynabead per cells. After incubation, cells were counted and centrifuged. The supernatant was collected and tested against a standard curve of FASL in RPMI 1640 + 10% FBS. All supernatant dilutions were performed in RPMI 1640 + 10% FBS.

| Cell supernatant Dilution Fold (DF) | FASL detected in Stimulated Cell Supernatant (pg/mL) | Stimulated Cell Supernatant (pg/mL x DF) | FASL detected in Unstimulated Cell Supernatant (pg/mL)* |
|---|---|---|---|
| 1 | 843 | 843 | < LDL |
| 2 | 481 | 962 | < LDL |
| 4 | 182 | 728 | < LDL |
| 8 | 96 | 768 | < LDL |
| 16 | 44 | 704 | < LDL |
| Average | N/A | 801 | < LDL |

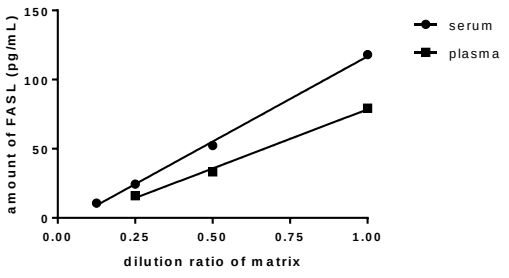



* Counts for unstimulated cell lysate (regardless of dilution) sample gave results below the LDL of the assay

- **Serum and Plasma assays**

Samples of human serum or human plasma were used as is or diluted 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 with fetal bovine serum. Results were compared vs a standard curve made with fetal bovine serum. Both human serum and plasma were filtered over 0.45 µm filters to remove lipid aggregates.

| Dilution factor | Human serum (pg/mL) | Human plasma (pg/mL) |
|---|---|---|
| raw | 118 | 79.3 |
| 2 | 52.3 | 33.3 |
| 4 | 24.4 | 16.0 |
| 8 | 10.7 | <LDL |
| No matrix | 0 | 0 |




$R^2$ fit serum 0.9981

$R^2$ fit plasma 0.9957

The kit can detect the protein with good linearity in raw matrix, and in matrix diluted up to 1:8 for serum and 1:4 for plasma.

A spike and recovery experiment was performed on the matrixes. Both human serum and plasma were spiked with 30, 10 or 3 ng/mL of recombinant FASL. A sample with no addition was used to measure basal levels and a sample of FBS was used as a control. All data were plotted against a standard curve performed in FBS

Serum

| Spike amount (ng/mL) | Observed amount (ng/mL) | Corrected amount (ng/mL) | % recovery |
|---|---|---|---|
| No spike | 0.12 | NA | NA |
| 30 | 35.0 | 34.9 | 116 |
| 10 | 12.1 | 12.0 | 120 |
| 3 | 2.72 | 2.6 | 87 |

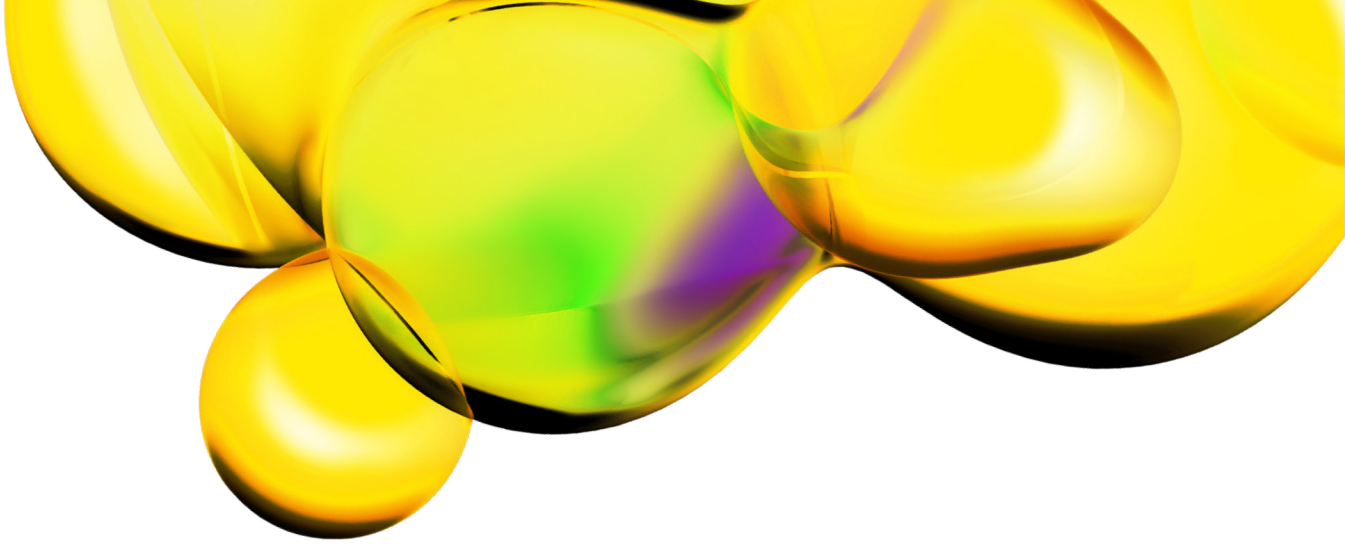
Plasma

| Spike amount (ng/mL) | Observed amount (ng/mL) | Corrected amount (ng/mL) | % recovery |
|---|---|---|---|
| No spike | 0.08 | NA | NA |
| 30 | 30.0 | 29.9 | 100 |
| 10 | 10.9 | 10.8 | 108 |
| 3 | 2.78 | 2.7 | 90 |

## Troubleshooting Guide

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

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**





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