



AlphaLISA[®] Human Tumor Necrosis Factor alpha (TNF α) kit

Product number: AL208 C/F

Research Use Only. Not for use in diagnostic procedures.

Material provided:

Format: AL208C: 500 assay points AL208F: 5 000 assay points

The number of assay points is based on an assay volume of 50 μ L in 96- or 384-well assay plates using the kit components at the recommended concentrations.

Document version: 1

Product Information

Kit content: The kit contains 5 components: AlphaLISA Acceptor beads coated with an Anti-Analyte Antibody, Streptavidin-coated Donor beads, Biotinylated Anti-Analyte Antibody, lyophilized analyte and 10X AlphaLISA Immunoassay Buffer. Assay microplates (96-, 384- or 1536-well plates) must be purchased separately (see page 3 for more details).

Storage: Store kit in the dark at +4°C. Store reconstituted analyte at -20°C.

Stability: This product is stable for at least 12 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the human TNF α analyte is stable for at least 45 days at -20°C (see page 2: Reagents and Materials).

Application: This kit is designed for the quantitative determination of human TNF α in serum, buffered solution or cell culture medium using a homogenous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 2.2 pg/mL (see page 9: Assay Performance Characteristics).

Dynamic range: 2.2 – 30 000 pg/mL (see page 9: Assay Performance Characteristics).

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

Precautions

- Only the AlphaScreen® 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.
- All blood components and biological materials should be handled as potentially hazardous. Some analytes are from human source.
- Some analytes are present in saliva. 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.

Reagents and Materials

The reagents provided in the AlphaLISA kit are listed in the table below:

Kit components	AL208C (500 assay points)	AL208F (5 000 assay points)
AlphaLISA Anti-TNF α Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	50 μ L @ 5 mg/mL (1 brown tube, <u>white</u> cap)	500 μ L @ 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% Kathon, pH 7.4	200 μ L @ 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 Antibody Anti-TNF α stored in PBS, 0.1% Tween-20, 0.05% NaN ₃ , pH 7.4	50 μ L @ 500 nM (1 tube, <u>black</u> cap)	500 μ L @ 500 nM (1 tube, <u>black</u> cap)
AlphaLISA human TNF α (0.1 μ g), lyophilized analyte *	1 tube, <u>clear</u> cap	1 tube, <u>clear</u> cap
AlphaLISA Immunoassay Buffer (10X) **	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute human TNF α in 100 μ L Milli-Q® grade H₂O. The reconstituted analyte should be used within 60 minutes, if possible, or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted human TNF α is stable for at least 45 days at -20°C. One vial contains an amount of human TNF α sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL208S).

** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100 and 0.5% Kathon. Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).
Note: 10X buffer might be slightly yellow. However, this does not affect the assay results.

Once diluted, 1X AlphaLISA Immunoassay Buffer contains 25 mM HEPES, pH 7.4, 0.1% Casein, 1 mg/mL Dextran-500, 0.5% Triton X-100 and 0.05% Kathon.

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 Adhesive Sealing Film	Revvity Inc.	6050195
EnVision®-Alpha Reader	Revvity Inc.	-

Manuals have been optimized for 50 µL assays in white OptiPlate™-384 microplates. Other assay volumes can be used with similar manuals and identical final AlphaLISA reagent concentrations:

Format	# of data points	Total assay volume	Sample volume	AlphaLISA beads / Biotin Antibody MIX volume*	SA-Donor beads volume*	Plate recommendation
AL208C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	5 µL	20 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL208F	5 000	50 µL	5 µL	20 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

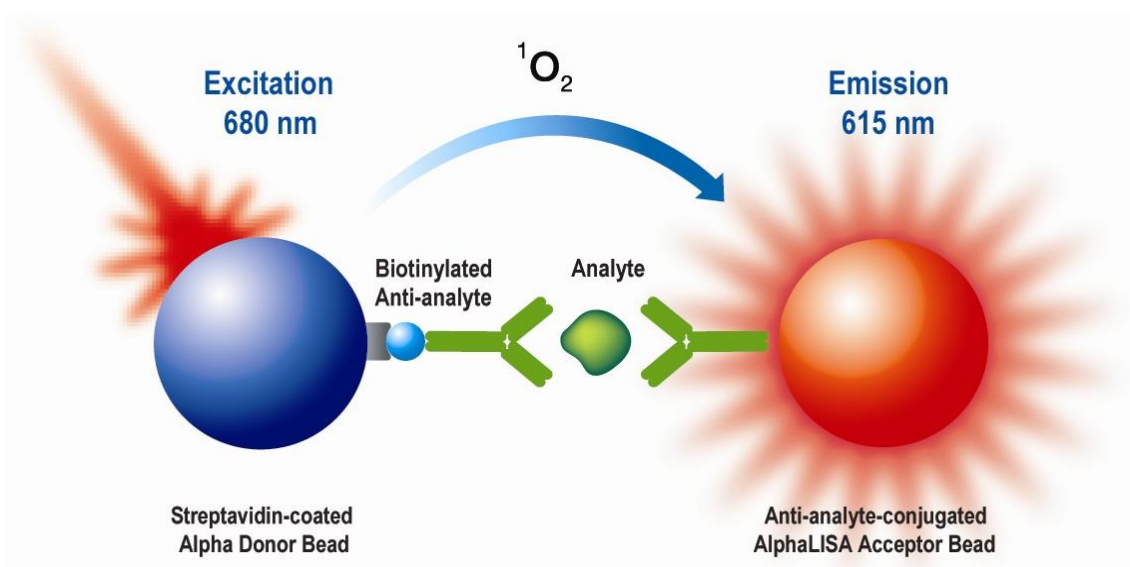
* Volumes based on the Quick manual.

Analyte of Interest

Tumor necrosis factor alpha (TNF α) is a multifunctional proinflammatory cytokine synthesized mainly by nucleated blood cells as a 233 aa type II transmembrane protein which is cleaved by ADAM17 between aa 76-77 to form a soluble homotrimeric complex. TNF α plays a role in lipid metabolism, coagulation, and endothelial function and has been associated with cancer, infection and inflammation (including inflammatory bowel disease), ischemia/reperfusion injury and heart failure, and insulin resistance.

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 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 (see figure below).



Recommendations

General 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 prewet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2 000 g, 10-15 sec). Resuspend 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 in 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. The standard curve should be performed in a similar matrix as the samples (e.g. FBS for serum samples).

Specific recommendations:

- AlphaLISA assays can be performed in cell culture medium with or without phenol red, with the following recommendations: If possible, avoid biotin-containing medium (e.g. RPMI medium) as lower counts and lower sensitivity are expected. Add at least 1% FBS or 0.1% BSA to cell culture medium.
- When analyzing serum samples, perform the standard curve in FBS and dilute the samples at least 2-fold with FBS before testing. Serum should not exceed 10% of final assay volume (i.e. 5 µL serum sample in 50 µL final assay volume).

Manuals

The two manuals described below are 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 a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly. 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 is calculated. One background point in triplicate (3 wells) can be used when LDL is not calculated.

Manual 1: Quick manual (2 incubation steps) – Dilution of standards in 1X AlphaLISA Immunoassay Buffer or cell culture medium

Manual 2: High sensitivity manual (3 incubation steps) – Dilution of standards in FBS

IMPORTANT: PLEASE READ THE RECOMMENDATIONS ABOVE BEFORE USE

Common Steps for Preparing Reagents (Manuals 1 & 2)

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 2.5 mL of 10X AlphaLISA Immunoassay Buffer to 22.5 mL H₂O.
- 2) Preparation of human TNF α analyte standard dilutions:
Reconstitute lyophilized human TNF α (0.1 μ g) in 100 μ L H₂O.
Prepare standard dilutions as follows (change tip between each standard dilution):

Tube	Vol. of human TNF α (μ L)	Vol. of diluent (μ L) *	[human TNF α] in standard curve	
			(g/mL in 5 μ L)	(pg/mL in 5 μ L)
A	10 μ L of reconstituted human TNF α	90	1E-07	100 000
B	60 μ L of tube A	140	3E-08	30 000
C	60 μ L of tube B	120	1E-08	10 000
D	60 μ L of tube C	140	3E-09	3 000
E	60 μ L of tube D	120	1E-09	1 000
F	60 μ L of tube E	140	3E-10	300
G	60 μ L of tube F	120	1E-10	100
H	60 μ L of tube G	140	3E-11	30
I	60 μ L of tube H	120	1E-11	10
J	60 μ L of tube I	140	3E-12	3
K	60 μ L of tube J	120	1E-12	1
L	60 μ L of tube K	140	3E-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

* Manual 1: Dilute standards in 1X AlphaLISA Immunoassay Buffer or cell culture medium.
Manual 2: Dilute standards in FBS.

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.

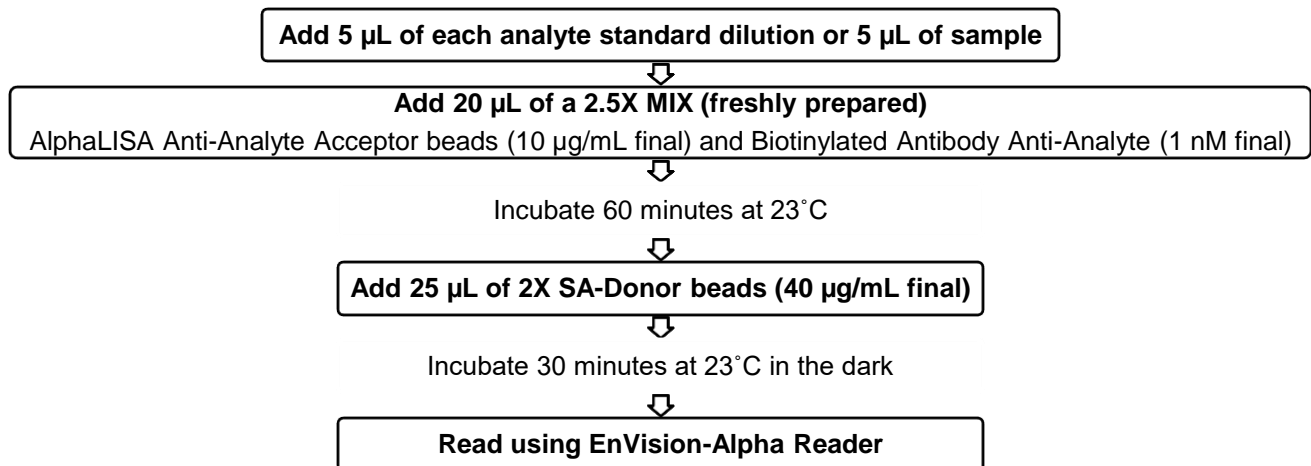
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

Manual 1: Quick Manual (2 Incubation Steps)

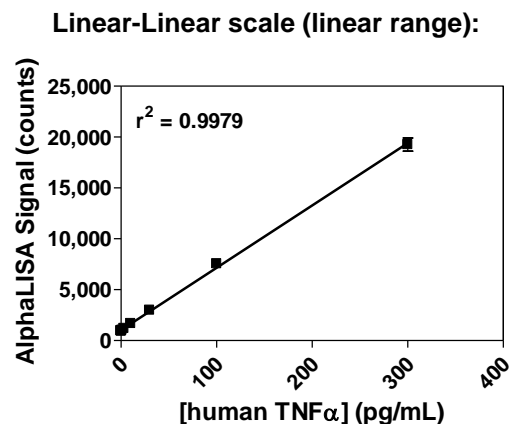
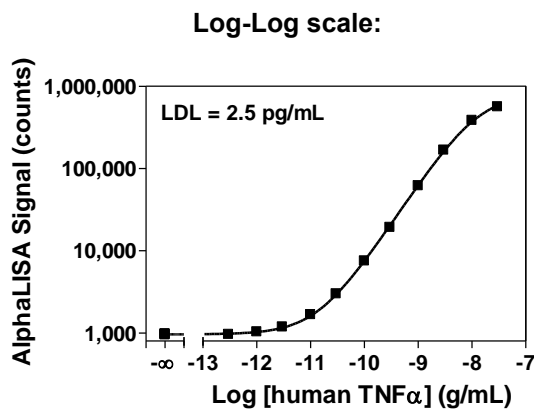
The manual described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards can be done in 1X AlphaLISA Immunoassay Buffer or cell culture medium.

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

- 3) Preparation of 2.5X AlphaLISA Anti-TNF α Acceptor beads + Biotinylated Antibody Anti-TNF α MIX (25 $\mu\text{g}/\text{mL}$ /2.5 nM):
Add 50 μL of 5 mg/mL AlphaLISA Anti-TNF α Acceptor beads and 50 μL of 500 nM Biotinylated Antibody Anti-TNF α to 9 900 μL of 1X AlphaLISA Immunoassay Buffer. Prepare just before use.
- 4) Preparation of 2X Streptavidin (SA) Donor beads (80 $\mu\text{g}/\text{mL}$): Keep the beads under subdued laboratory lighting.
Add 200 μL of 5 mg/mL SA-Donor beads to 12 300 μL of 1X AlphaLISA Immunoassay Buffer.
- 5) Samples: If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA Immunoassay Buffer or cell culture medium).
- 6) In a 96- or 384-well microplate:



Manual 1 - Typical results in 1X AlphaLISA Immunoassay Buffer



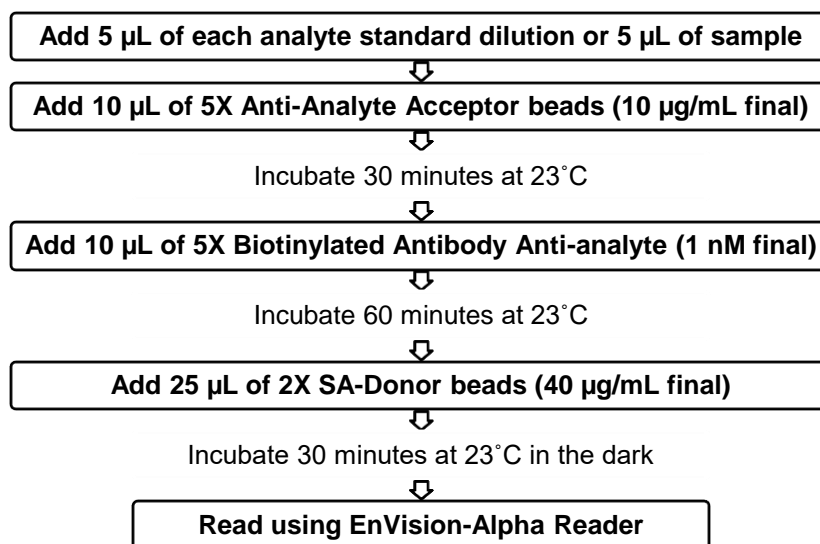
The data was generated using a white Optiplat-384 microplate and an EnVision-Alpha Reader 2102.

Manual 2: High Sensitivity Manual (3 Incubation Steps)

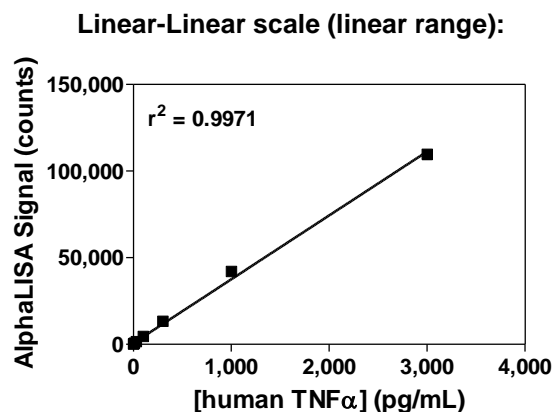
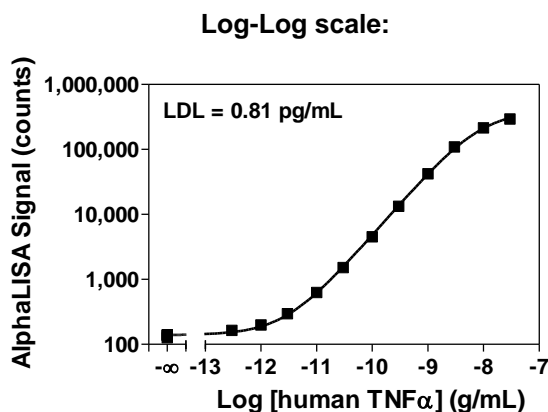
The manual described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards in FBS.

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

- 3) Preparation of 5X AlphaLISA Anti-TNF α Acceptor beads (50 $\mu\text{g}/\text{mL}$):
Add 50 μL of 5 mg/mL AlphaLISA Anti-TNF α Acceptor beads to 4 950 μL of 1X AlphaLISA Immunoassay Buffer.
- 4) Preparation of 5X Biotinylated Antibody Anti-TNF α (5 nM):
Add 50 μL of 500 nM Biotinylated Antibody Anti-TNF α to 4 950 μL of 1X AlphaLISA Immunoassay Buffer.
- 5) Preparation of 2X Streptavidin (SA) Donor beads (80 $\mu\text{g}/\text{mL}$): Keep the beads under subdued laboratory lighting.
Add 200 μL of 5 mg/mL SA-Donor beads to 12 300 μL of 1X AlphaLISA Immunoassay Buffer.
- 6) Samples: If applicable, dilute samples to be tested in diluent (e.g. FBS).
- 7) In a 96- or 384-well microplate:



Manual 2 - Typical results in FBS



The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2102.

Manuals 1 & 2 - Interpreting the Data

- 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.
- 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 manual 1 (dilution of standards in 1X AlphaLISA Immunoassay Buffer) or manual 2 (dilution of standards in FBS).

Sensitivity:

The LDL was calculated as described above. This value corresponds to the lowest concentration of analyte that can be detected in a volume of 5 μ L using the recommended assay conditions.

- Average LDL is 2.2 pg/mL * (using 5 μ L of analyte in AlphaLISA Immunoassay Buffer) (mean of 27 independent experiments).
- Average LDL is 0.78 pg/mL (using 5 μ L of analyte in FBS) (mean of 4 independent experiments).

* Note that LDL can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 μ L of analyte dilution in a final assay volume of 50 μ L).

Dynamic range: 2.2 – 30 000 pg/mL (in AlphaLISA Immunoassay Buffer)

Assay precision:

The following assay precision data were calculated from a total of 27 assays. Three operators performed three independent assays using three different kit lots. Each assay consisted of one standard curve and three control samples of high (A), medium (B) and low (C) concentration, assayed in triplicate. The assays were performed in 384-well format using AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 9 independent determinations in 9 replicates for each control sample.

Sample	Mean (pg/mL)	SD (pg/mL)	% CV (n = 9)
A	3 371	163	4.8
B	340	30.7	9.0
C	36	1.8	5.0

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 27 measurements for each control sample.

Sample	Mean (pg/mL)	SD (pg/mL)	% CV (n = 3)
A	3 440	225	6.5
B	336	22.9	6.8
C	37	2.6	7.1

- Recovery:

Three known concentrations of analyte were spiked in AlphaLISA Immunoassay Buffer. The % of measured versus theoretical amount was calculated for each concentration in 9 independent experiments.

Spike (ng/mL)	% Recovery
3	111
0.3	102
0.03	113

Human serum experiments:

In the following experiments, FBS was used as diluent in both the standard curve and dilution of samples. Additionally, all human serum samples tested were pre-diluted 2-fold with the diluent before being processed.

- Dilutional linearity:

The dilutional linearity was determined by serial dilutions of a pool of human sera spiked with 3 ng/mL of human TNF α . The recovery was calculated using the 2-fold diluted sample as the 100% value. The average recovery from two independent measurements is reported.

Dilution Factor	% Recovery
1	100
2	99
4	108
8	89
16	96

- **Recovery:**

Three known concentrations of analyte were spiked in a pool of human sera. All samples, including non-spiked serum, were measured in the assay. Values calculated for spiked samples reflect subtraction of the endogenous (no-spike) value. The % in serum versus expected (control spike value) was calculated for each concentration. The average recovery from two independent measurements is reported.

Spike (ng/mL)	% Recovery
3	72
0.3	77
0.03	96

Calibration:

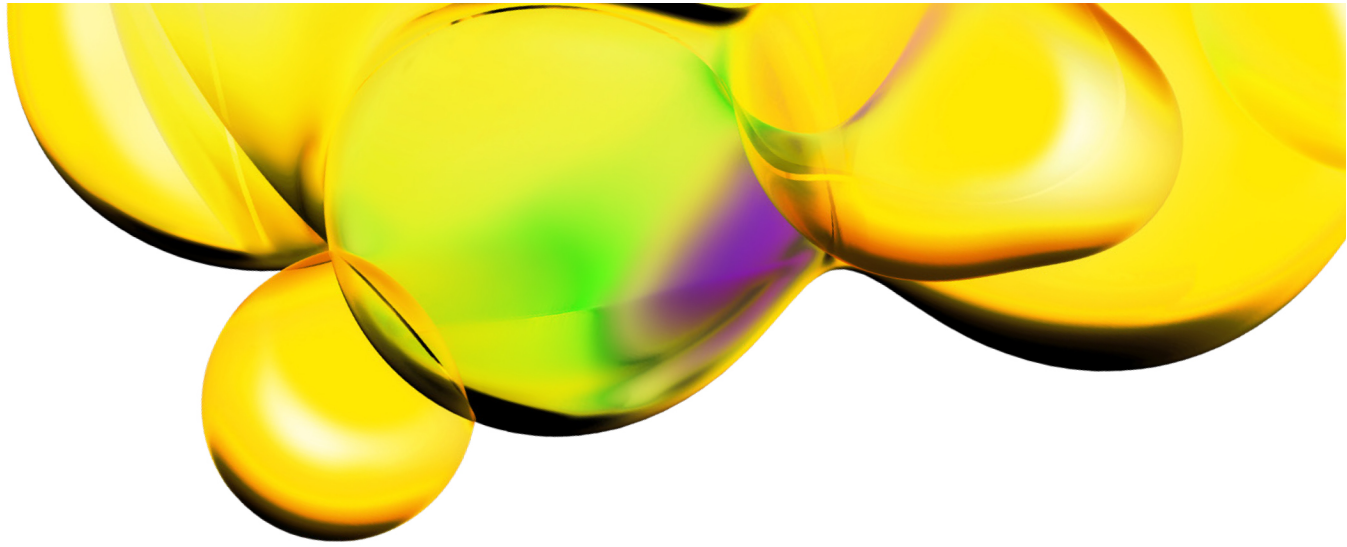
Human Tumor Necrosis Factor Alpha (NIBSC/WHO Second International Standard (code 88/786)) was tested using this kit: 1 International Unit (IU) Standard corresponds to 8.6 pg of AlphaLISA TNF α .

Specificity:

Cross-reactivity of the AlphaLISA TNF α Kit was tested using the following proteins at 0.1 μ g/mL in AlphaLISA Immunoassay Buffer.

Protein	% Cross reactivity
Human TNF β	0
Mouse TNF α	0
Rat TNF α	0
Bovine TNF α	0

The possible interference from human TNF Receptor I and II (TNF RI and RII) was investigated. The human TNF α was kept at a constant concentration (1 ng/mL). The binding proteins were titrated into the assay. No interference was observed up to 1 μ g/mL, which is the maximum concentration tested.



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www.revvity.com

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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

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