TECHNICAL DATA SHEET

AlphaPlex™ 645 Research Reagents

Human Tumor Necrosis Factor Alpha (hTNFα) AlphaPlex™ 645 Immunoassay Kit

Product number: **AP208SM-HV/C/F**

Research Use Only. Not for use in diagnostic procedures.

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Product Information

Application: This kit is designed for the quantitative determination of human TNF-alpha (hTNFα) in serum, plasma, and cell culture supernatants using a homogeneous AlphaPlex 645 assay (no wash steps). The assay shows negligible cross-reactivity with other cyotkines.

Sensitivity: Lower Detection Limit (LDL): 13.3 pg/mL

Lower Limit of Quantification (LLOQ): 43.7 pg/mL

EC_{50}: 25.7 ± 3 ng/mL

Dynamic range: Kit designed to detect [hTNFα] between: 0.3 - 100000 pg/mL (Figure 1).

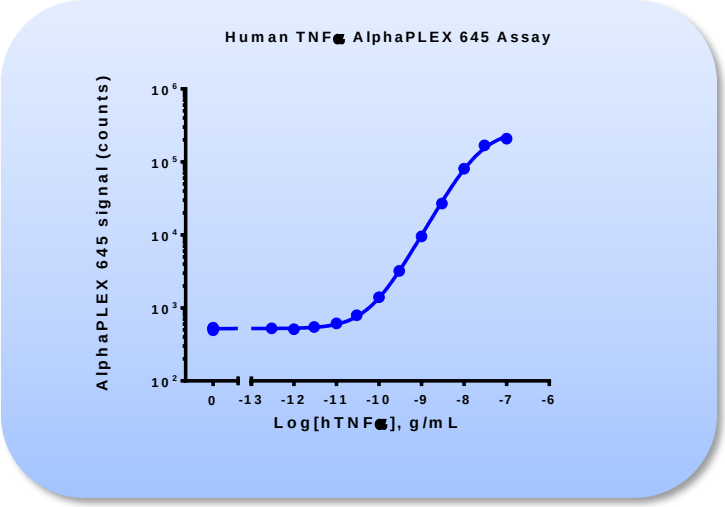


Figure 1. Typical sensitivity curves in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2104.

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

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. Note: Once reconstituted, the hTNF-alpha analyte is stable for at least 18 months when stored at -20°C.

Quality Control

Lot to lot consistency is confirmed in an AlphaPlex 645 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

Tumor Necrosis Factor alpha (TNFα) is a multifunctional proinflammatory cytokine. TNFα is released via proteolytic cleavage of the 233 Amino-Acid long TNF type II transmembrane proteins by ADAM17. Released TNFα self-assembles to form a soluble homotrimeric complex. TNFα has been observed to play a role in inflammation, apoptotic cell death, and as an important regulator of tumorigenesis and infection. TNFα dysregulation has also been observed in several major diseases such as Inflammatory Bowel Disease, Depression, Cancer, and Neurological Diseases. This kit has been designed for the detection of Human TNFα in serum, plasma, and cell culture supernatants.

Description of the AlphaPlex Assay

AlphaPlex 645 technology allows for 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 AlphaPlex 645 assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaPlex 645 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 transfers in the Acceptor beads, resulting in a sharp peak of light emission at 645 nm (Figure 2). Combining this assay with an AlphaLISA 615- or AlphaPlex 545 - based kit will allow the quantification of 2 (or more) analytes in the same well.

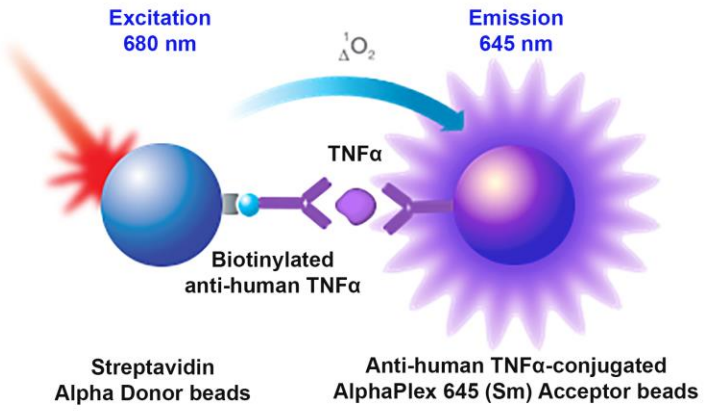


Figure 2. AlphaPlex 645 assay principle.

Precautions

- 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. The analyte included in this kit is from a 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.

Kit Content: Reagents and Materials

Kit components	AP208Sm-HV (100 assay points***)	AP208Sm-C (500 assay points***)	AP208Sm-F (5000 assay points***)
AlphaPlex 645 Anti-hTNFα Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	30 µL @ 5 mg/mL (1 brown tube, purple cap)	60 µL @ 5 mg/mL (1 brown tube, purple cap)	550 µL @ 5 mg/mL (1 brown tube, purple cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	105 µL @ 5 mg/mL (1 brown tube, black cap)	210 µL @ 5 mg/mL (1 brown tube, black cap)	2.1 mL @ 5 mg/mL (1 brown tubes, black caps)
Biotinylated Antibody Anti-hTNFα stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	30 µL @ 500 nM (1 tube, black cap)	60 µL @ 500 nM (1 tube, black cap)	550 µL @ 500 nM (1 tube, black cap)
AlphaPlex 645 hTNFα (0.1 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap	10 tubes, clear cap
AlphaLISA Immunoassay Buffer (10X) **	2.5 mL, 1 small bottle	10.5 mL, 1 small bottle	105 mL, 1 large bottle

* Reconstitute hTNFα 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 at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted hTNFα is stable for at least 18 months at -20°C. One vial contains an amount of hTNFα sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AP208Sm S).

** 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 (AL522HV) 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 AlphaPlex 645 signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaPlex 645 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	PerkinElmer Inc.	6050195
EnVision®-Alpha Reader	PerkinElmer Inc.	-

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 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 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 AlphaPlex 645 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: D670as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaPlex 645 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 AlphaLISA Immunoassay buffer or performed in FBS for serum and/or plasma samples.

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) and 452 samples. The protocols also include testing samples in 384 well plates. If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly, as shown in the table below. ****These calculations do not include excess reagents 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.

		Volume				
Format	**# of data points**	**Final**	**Sample**	**AlphaPlex 645 beads / Biotin Antibody MIX**	**SA-Donor beads**	**Plate recommendation**
AP208SmHV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AP208SmC	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	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)
AP208SmF	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)

Protocol (2-steps): Dilute standards and samples in 1X AlphaLISA Immunoassay Buffer or FBS. All other components (acceptor beads, biotinylated antibody, and donor beads) are diluted in Immunoassay Buffer.

***** The protocol described below is designed for 500 assay points including one standard curve (48 wells) and samples (452 wells).***

******Sample preparation – Dilute serum samples 2-fold in FBS and compare to an FBS analyte standard***

Standard Preparation:

1) Preparation of 1X AlphaLISA Immunoassay Buffer:

   - Add 10 mL of 10X AlphaLISA Immunoassay Buffer to 90 mL H_2O.

2) Preparation of hTNFα analyte standard dilutions:

   - hTNFα analyte is provided at 0.1 µg in lyophilized form. Reconstitute with 100 µL H_2O to create a 1 µg/mL solution. Prepare standard dilutions as follows (change tip between each standard dilution):

Tube	Vol. of hTNFα (µL)	Vol. of diluent (µL) *	[hTNFα] in standard curve		Final [hTNFα] in well
			(g/mL in 5 µL)	(pg/mL in 5 µL)	(g/mL in 50 µL)
A	10 µL of provided hTNFα	90	1.00E-07	100000	1.00E-08
B	60 µL of tube A	120	3.00E-08	30000	3.00E-09
C	60 µL of tube B	140	1.00E-08	10000	1.00E-09
D	60 µL of tube C	120	3.00E-09	3000	3.00E-10
E	60 µL of tube D	140	1.00E-09	1000	1.00E-10
F	60 µL of tube E	120	3.00E-10	300	3.00E-11
G	60 µL of tube F	140	1.00E-10	100	1.00E-11
H	60 µL of tube G	120	3.00E-11	30	3.00E-12
I	60 µL of tube H	140	1.00E-11	10	1.00E-12
J	60 µL of tube I	120	3.00E-12	3	3.00E-13
K	60 µL of tube J	140	1.00E-12	1	1.00E-13
L	60 µL of tube K	120	3.00E-13	0.3	3.00E-14
M ** (background)	0	100	0	0	0
N ** (background)	0	100	0	0	0
O ** (background)	0	100	0	0	0
P ** (background)	0	100	0	0	0

* 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).

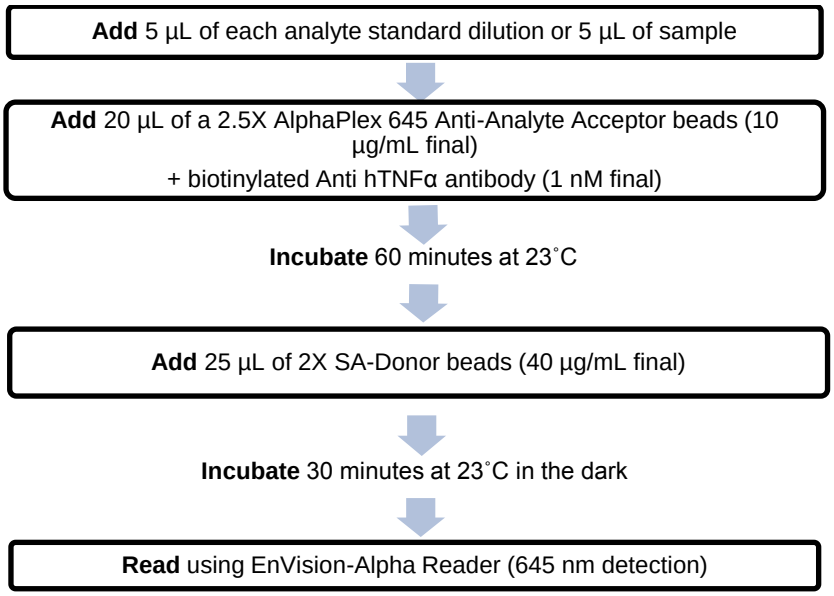
FBS can be used as the standard diluent with minimal effect on maximum and minimum counts and without altering the LDL, LLOQ, EC_{50} and S/B ratios.

Protocol (2 Steps):

1) Preparation of 2.5X AlphaPlex 645 Anti-hTNFα Acceptor beads (25 µg/mL) + biotinylated Anti hTNFα Antibody (2.5nM):
    a. Add 50 µL of 5 mg/mL **AlphaPlex 645 Anti hTNFα** Acceptor beads and 50 µL of 500nM **Anti hTNFα Antibody** to 9900 µL of 1X AlphaLISA Immunoassay Buffer.
    b. Prepare just before use.

2) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
    a. Keep the beads under subdued laboratory lighting.
    b. Add 200 µL of 5 mg/mL SA-Donor beads to 12300 µL of 1X AlphaLISA Immunoassay Buffer.
    c. Prepare just before use.

3) In a white Optiplate (384 wells):



Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaPlex 645 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

AlphaPlex assay performance described below was determined using the 2 step protocol.

Assay Sensitivity:

The LDL and LLOQ were calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL using the recommended assay conditions.

LDL (pg/mL)	LLOQ (pg/mL)	Buffer/Serum	# of experiments
13.4	43.7	AlphaLISA Immunoassay Buffer	15
14.1	46.9	FBS	8

* Note that LDL/ LLOQ 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).
** Only the analytes were prepared in serum (i.e. FBS). All of other components were prepared in Immunoassay Buffer.

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 AlphaLISA Immunoassay Buffer (IAB) or Fetal Bovine Serum (FBS). Each assay consisted of one standard curve comprising 12 data points in triplicate and 12 background wells containing no analyte. The assays were performed in a 384-well format using AlphaLISA Immunoassay Buffer.

Intra-assay precision:

The intra-assay precision was determined using 3 independent experiments for a total of 16 independent determinations in triplicate. CV% were calculated for each individual experiment then averaged. Shown is the average intra experimental CV%.

hTNFα	IAB	FBS
CV%	4.5%	6.2%

Inter-assay precision:

The inter-assay precision was determined using the data across 3 independent experiments with 16 measurements in triplicate. CV% was calculated by comparing the same measurement in each experiment. The CV% for all 16 measurements were then averaged. Shown is the inter experimental CV%.

hTNF-alpha	IAB	FBS
CV%	10.3%	11.7%

Spike Recovery:

Three known concentrations of hTNFα were spiked into IAB. All samples, including non-spiked Immunoassay Buffers were measured in the assay. The average recovery was reported from 4 independent experiments each with 3 measurements in triplicate.

Spiked hTNFα (ng/mL)	% Recovery
	IAB
3	101.3
0.3	84.1
0.03	90.0

Specificity for hTNFα:

Cross-reactivity of the AlphaPlex 645 hTNFα Kit was tested using the following proteins at 100 ng/mL in AlphaLISA Immunoassay Buffer.

Protein	% Cross-reactivity
Human TNFβ	0
Mouse TNFα	0
Rat TNFα	0
Bovine TNFα	0

The possible interference form human TNF Receptors I and II (TNF RI and RII) were investigated. The human TNFα was kept at a constant concentration (1 ng/mL). The binding proteins were titrated into the assay. No interference was observed up to 1 µg/mL, which was the maximum concentration tested.

Human Serum Experiments

Human serum was purchased and Fetal Bovine Serum (FBS) was used as the diluent**.** Human TNFα was not detected in the Human serum (data not shown). TNFα is not expected to be present at detectable levels in serum from normal healthy subjects.

Spike recovery in human serum:

Three known concentrations of hTNFα were spiked into Human serum diluted 2-fold with FBS. All samples, including non-spiked sera were measured in the assay. The average recovery was reported from 4 independent experiments each with 3 measurements in triplicate.

Spike (ng/mL)	% Recovery
3	74.1
0.3	91.8
0.03	99.8

Dilutional Linearity:

Dilutional linearity was determined by serial dilutions of Human serum diluted 2-fold in FBS then supplemented with 1.5 ng/mL of hTNFα. The undiluted (dilution factor 1) was considered the 100%. The sequential dilutions were multiplied by their respective dilution factors and referenced back to the 100% value.

Dilution Factor	% Recovery
1	100.0
2	105.3
4	104.3
8	105.2
16	111.8

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaPlex 645 Assay kit at:

http://www.perkinelmer.com/in/resources/technicalresources/applicationsupportknowledgebase/alphalisa-alphascreen-no-washassays/alpha_troubleshoot.xhtml

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