TECHNICAL DATA SHEET

AlphaPlex™-545 Research Reagents

IL-6 AlphaPlex™-545 Immunoassay Kit

Product number: AP223TB-HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Contents

Product Information

Application: This kit is designed for the quantitative determination of interleukin-6 in cell culture media, sera and plasma, using a homogeneous AlphaPlex assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 10 pg/mL

Lower Limit of Quantification (LLOQ): 20 pg/mL

EC_{50}: 9,5 ng/mL

Min/Max counts: 170/ 150000 counts

Dynamic range: 10 - 100 000 pg/mL (Figure 1).

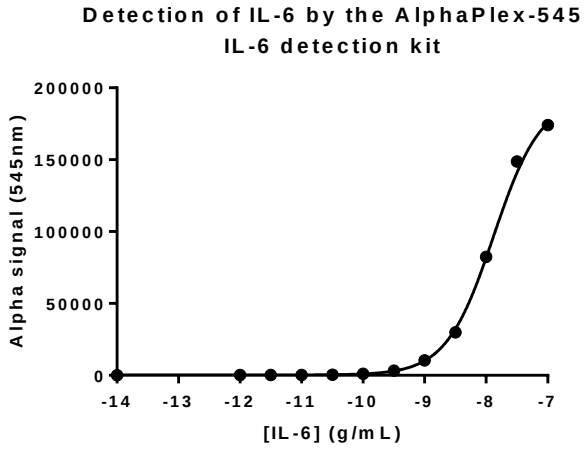


Figure. 1. Typical sensitivity curve in HiBlock buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2102.

Storage: The kit components must be stored dark at +4˚C.

Stability: This kit is stable for at least 4 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 AlphaPlex 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

Interleukin-6 is a 212 amino acids cytokine generated by macrophages and T cells. It binds to a IL-6 receptor complex to mediate its effects. IL-6 is known as a pro-inflammatory cytokine generated in the first response to pathogens. It is also involved in cancer generation and growth. Its secretion often parallels other cytokines involved in immune response (such as IL-1a and IL-8) or inflammation (such as TNFa). The present kit permits detection of glucagon (i.e. analyte) in different sample matrices.

Description of the AlphaPlex Assay

AlphaPlex 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 AlphaPlex assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaPlex 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 (Figure 2).

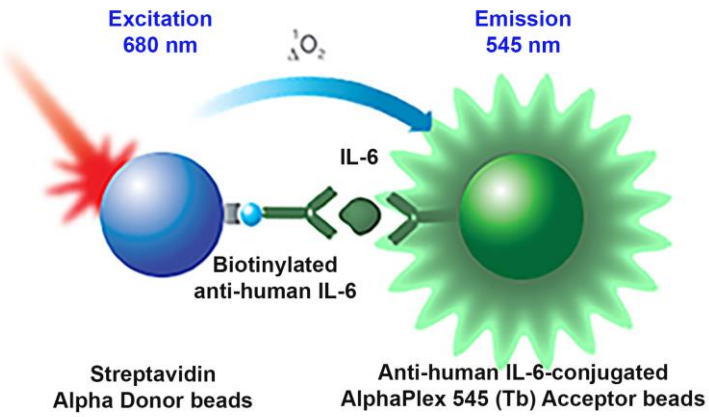


Figure 2. AlphaLISA Assay principle.

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.
- 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	AP223Tb-HV (100 assay points***)	AP223Tb-C (500 assay points***)	AP223Tb-F (5 000 assay points***)
AlphaPLEX 545 Anti-IL-6 Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, green cap)	50 µL @ 5 mg/mL (1 brown tube, green cap)	500 µL @ 5 mg/mL (1 brown tube, green cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	100 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2000 µL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Antibody Anti-IL-6 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)
AlphaPLEX IL-6 lyophilyzed standard	1 tube, clear cap	1 tube, clear cap	1 tube, clear cap
AlphaLISA Immunoassay Buffer (10X)	5 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* The analyte should be resuspended in 100µL of distilled water and 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 the IL-6 analyte solution is stable for at least 6 months at -20°C. One vial contains an amount of IL-6 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL223S).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AP223Tb-HV) 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 Adhesive Sealing Film	PerkinElmer Inc.	6050195
EnVision®-Alpha Reader	PerkinElmer Inc.	-

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 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.
- The AlphaPlex signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaPlex 545 settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: 535/40, Center Wavelength 535 nm, Bandwidth 40 nm, Transmittance 75%) for sequential readings (545 then 615 reads).
- AlphaPlex 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).
- AlphaPlex assays should be performed in cell culture medium without phenol red, as phenol red will lower counts and sensitivity. The following recommendations should also be followed: 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.

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). The protocols 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, 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.

		Volume				
Format	**# of data points**	**Final**	**Sample**	**AlphaPlex beads / Biotin Antibody MIX**	**SA-Donor beads**	**Plate recommendation**
AP223Tb-HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AP223Tb-C	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)
AP223Tb-F	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)

High sensitivity protocol (3 incubation steps) – Dilution of standards in 1X AlphaLISA Immunoassay Buffer

The protocol described below is recommended when generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations with manual pipetting). *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 1 mL of 10X AlphaLISA Immunoassay Buffer to 9 mL H_2O.

2) Preparation of IL-6 analyte standard dilutions:

   - The provided IL-6 standard is provided at as 0.1µg lyophilized stock. The analyte must be resuspended in 100µL of MilliQ grade water before use.

- Prepare standard dilutions as follows (change tip between each standard dilution) in 1X AlphaLISA Immunoassay Buffer or the matrix used for the analysis.

Tube	**Vol. of Glucagon (µL)**	**Vol. of diluent (µL) ***	**[Glucagon] in standard curve (pg/mL in 5 µL)**
A	10 µL of resuspended IL-6	90	100000
B	30 µL of tube A	70	30000
C	30 µL of tube B	60	10000
D	30 µL of tube C	70	3000
E	30 µL of tube D	60	1000
F	30 µL of tube E	70	300
G	30 µL of tube F	60	100
H	30 µL of tube G	70	30
I	30 µL of tube H	60	10
J	30 µL of tube I	70	3
K	30 µL of tube J	60	1
L	30 µL of tube K	70	0,3
M ** (background)	0	100	0
N ** (background)	0	100	0
O ** (background)	0	100	0
P ** (background)	0	100	0

* Dilute standards in diluent (buffer or corresponding matrix). FBS can be used, since bovine IL-6 is not recognixed by this kit.
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/LLOQ is calculated. If LDL/LLOQ does not need to be calculated, one background point in triplicate can be used (3 wells).

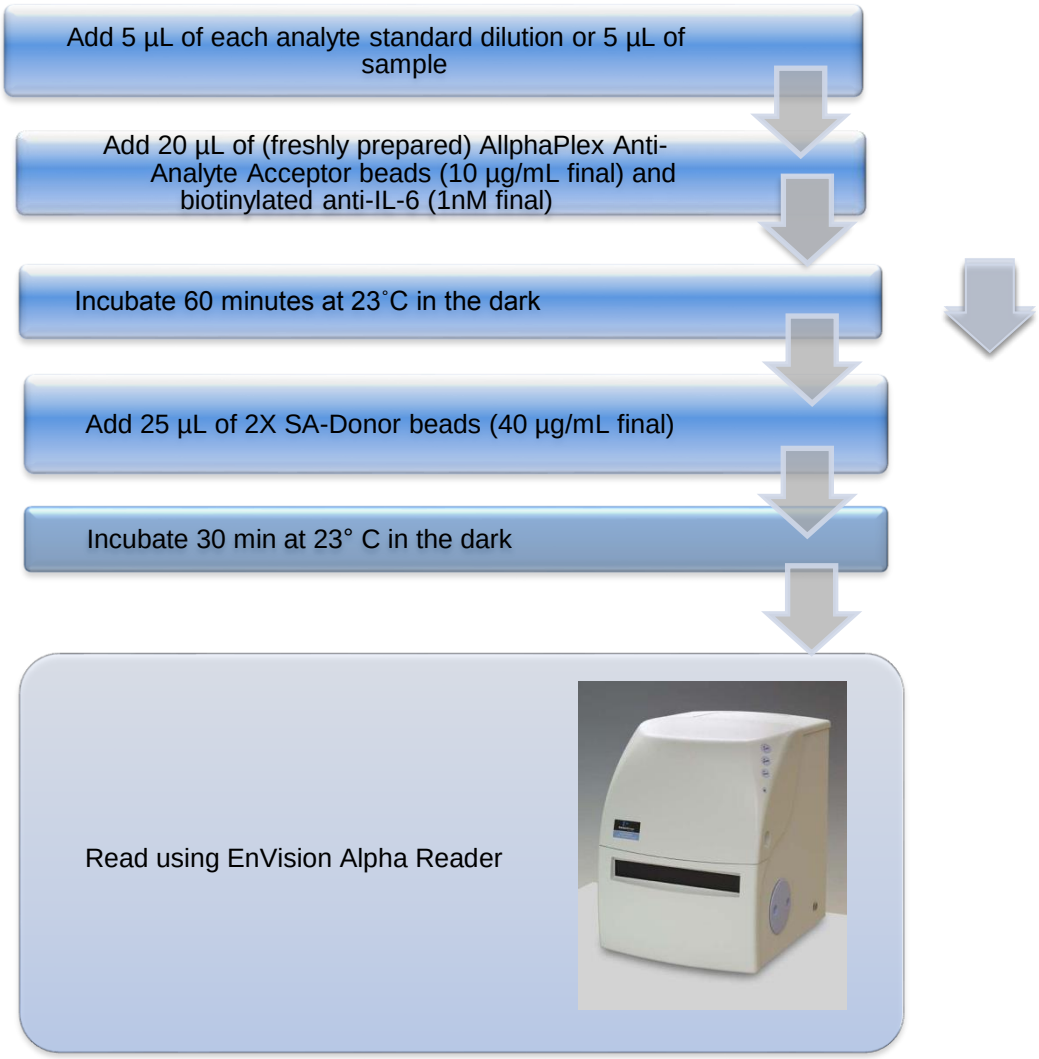
3) Preparation of 2.5X AlphaPlex-545 Anti-IL-6 Acceptor beads and biotinylated anti-IL-6 antibody (25 µg/mL beads and 2.5nM antibody):
Add 6 µL of 5 mg/mL AlphaPlex 545 Anti-IL-6 Acceptor beadsand 6µL of of 500 nM Biotinylated Antibody Anti-IL-6 to 1088 µL of 1X AlphaLISA immunoassay Buffer. Prepare just before use.

4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting. Add 32 µL of 5 mg/mL SA-Donor beads to 1968 µL of 1X AlphaLISA Immunoassay bufferk Buffer.

5) Samples:

- If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA Immunoassay Buffer).

6) In a 96- or 384-well microplate:



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

AlphaPlex assay performance described below was determined using the quick protocol.

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/Media used	# of experiments
10	20	AlphaLISA Immunoassay Buffer	5

* 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).

Assay precision:

The following assay precision data were calculated from the five independent assays using three different kit lots. In each lot, the analytes were prepared in AlphaLISA Immunoassay Buffer (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 using AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 6 independent determinations in triplicate. Shown CV%.

IL-6 (ng/ml)	HBB
10	13%

- Inter-assay precision:

The inter-assay precision was determined using a total of 6 independent determinations.

IL-6 (ng/ml)	HBB
10	11,5%

- Recovery:

Three known concentrations of analyte were spiked into DMEM cell culture media containing 10% FBS,RPMI cell culture media containing 10% FBS, HAT cell culture media containing 10% FBS and AlphaLISA Immunoassay Buffer (IAB). All samples were run alongside a standard curve diluted in AlphaLISA immnunoassay Buffer, this standard curve was used to interpolate the concentrations of the samples. The percent recovery is defined as assay measured concentration with respect to the spiked concentration. The average recovery from two independent measurements is reported.

Spike (Glucagon ng/mL)	% Recovery			
	AlphaLISA Immunoassay Buffer	DMEM with FBS	RPMI	HAT media
10	121	108	120	87
1	87	93	110	116
0.1	114	118	131	124

Troubleshooting Guide

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

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

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