Caution: For Laboratory Use. A research chemical for research purposes only.

Human Interleukin 1 beta (IL1β) AlphaPlex™ 645 Immunoassay Kit

Product number: AP220SM-HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Contents

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Product Information

Application: This kit is designed for the quantitative determination of human Interleukin 1 beta (IL-1β) in serum and cell culture supernatants using a homogeneous AlphaPlex 645 assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 1.7 pg/mL

Lower Limit of Quantification (LLOQ): 5.5 pg/mL

EC_{50}: 5.4 ± 0.6 ng/mL

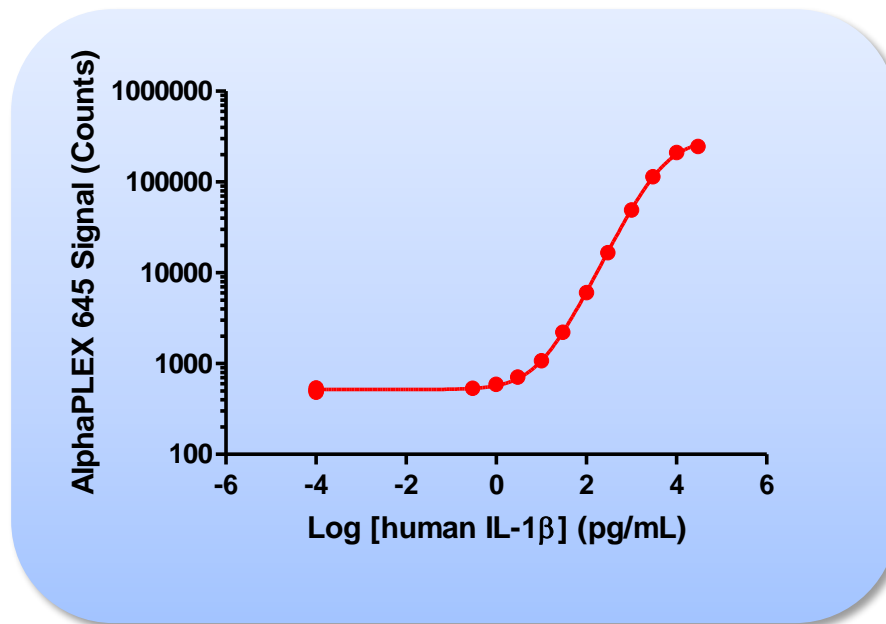**Dynamic range:** 2 - 300000 pg/mL (Figure 1).

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 2102.

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 human IL-1β analyte is stable for at least 18 months when stored at -20°C.

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.

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Analyte of Interest

IL1α and IL-1β are central players of the immune response, displaying roles in inflammation both at local and systemic levels. Despite they seem to display very similar functions, these proteins are encoded by two independent genes sharing only ~30% identity. IL-1β is synthesized as a 31 kDa precursor that is cleaved by Caspase-1 (ICE) into the active 17 kDa form, and eventually released into the extracellular space. Its production has been reported in many cell types including brain and, importantly, monocytic and periferal blood mononuclear cells. After binding to its receptor, IL-1RI, IL-1β triggers a cascade of kinase signaling pathways that lead to the activation of transcription factors like NFκB and AP-1, eventually activating the expression of genes such as MIP-2 and C-reactive protein.

Description of the AlphaPlex 645 Assay

AlphaPlex 645 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 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 transfer in the Acceptor beads, resulting in a sharp peak of light emission at 645 nm (Figure 2).

Combining this assay with an AlphaLISA or AlphaPlex 545- based kit will allow the quantification of 2 (or more) analytes in the same well. Indeed, the presence of two acceptor beads allow for the following assays:

- Two unrelated analyte measurements.
- Total versus modified analyte.
- Two different modifications on same analyte.
- Cascade effects.
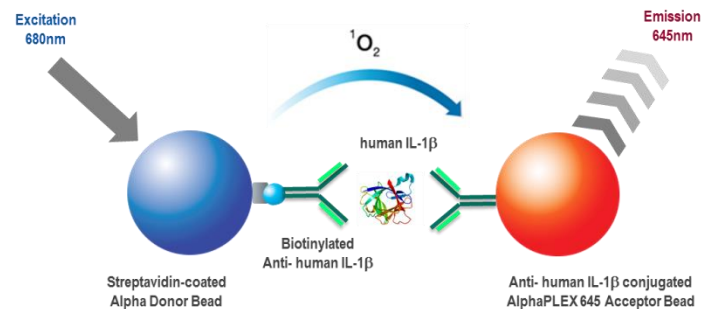- Protein-molecule interactions coupled to odification

Figure 2. AlphaPLEX 645 assay principle

Precautions

- The AlphaLISA 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 bovine 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.

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Kit Content: Reagents and Materials

Kit components	AP220Sm-HV (100 assay points***)	AP220Sm-C (500 assay points***)	AP220Sm-F (5000 assay points***)
AlphaPlex 645 Anti-human IL-1β Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, <u>purple</u> cap)	50 µL @ 5 mg/mL (1 brown tube, <u>purple</u> cap)	500 µL @ 5 mg/mL (1 brown tube, <u>purple</u> 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, <u>black</u> cap)	200 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	2 mL @ 5 mg/mL (1 brown tubes, <u>black</u> caps)
Biotinylated Antibody Anti-human IL-1β stored in PBS, 0.1% Tween-20, 0.05% NaN$_3$, pH 7.4	20 µL @ 500 nM (1 tube, <u>black</u> cap)	50 µL @ 500 nM (1 tube, <u>black</u> cap)	500 µL @ 500 nM (1 tube, <u>black</u> cap)
AlphaPlex human IL-1β (0.1 µg), lyophilized analyte *	1 tube, <u>clear</u> cap	1 tube, <u>clear</u> cap	1 tube, <u>clear</u> cap
AlphaLISA Immunoassay Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute human IL-1β in 100 µL Milli-Q® grade H$_2$O. 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 human IL-1β is stable for at least 18 months at -20°C. One vial contains an amount of human IL-1β sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AP220S).

** 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.	-

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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, <u>change tips</u> between each standard or sample dilution. When loading reagents in the assay microplate, <u>change tips</u> 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.

- AlphaPLEX 645 signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 1000 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D670as (Barcode# 605), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 224).

- 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 the bovine Immunoassay buffer 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). The protocols also include testing samples in 354 wells. If a different amount of samples are tested, <u>the volumes of all reagents have to be adjusted accordingly, as shown in the table below</u>. 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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Format	# of data points	Volume				Plate recommendation
		Final	Sample	AlphaPlex 645 beads / Biotin Antibody MIX	SA-Donor beads	
AP220Sm -HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AP220Sm -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)
AP220Sm -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)

The 2-step high sensitivity protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells).

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
   - Add 2.5 mL of 10X AlphaLISA Immunoassay Buffer to 22.5 mL H_2O.

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2) Preparation of human IL-1β analyte standard dilutions:

- Human IL-1β analyte is provided at 0.1 µg in lyophilized form. Reconstitute with 100 µL MiliQ H_2O to create a 1µg/mL solution. The first point of the curve is 0.1 µg/mL so a 10 fold dilution is required. Prepare standard dilutions as follows (change tip between each standard dilution):

Tube	Vol. of Human IL-1β (µL)	Vol. of diluent (µL) *	[human Il-1β] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of provided IL-1β	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 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 2.5X AlphaPLEX 645 anti-human IL-1β   Acceptor beads + 2.5X biotinylated anti- human IL-1β antibody
(25 µg /mL / 2.5nM)
Add 50 µL of 5 mg/mL AlphaPLEX 645 anti- human IL-1β acceptor beads and 50 µL of 500nM biotinylated anti-human IL-1β antibody to 9,900 µL of AlphaLISA Immunoassay Buffer.
Prepare just before use.

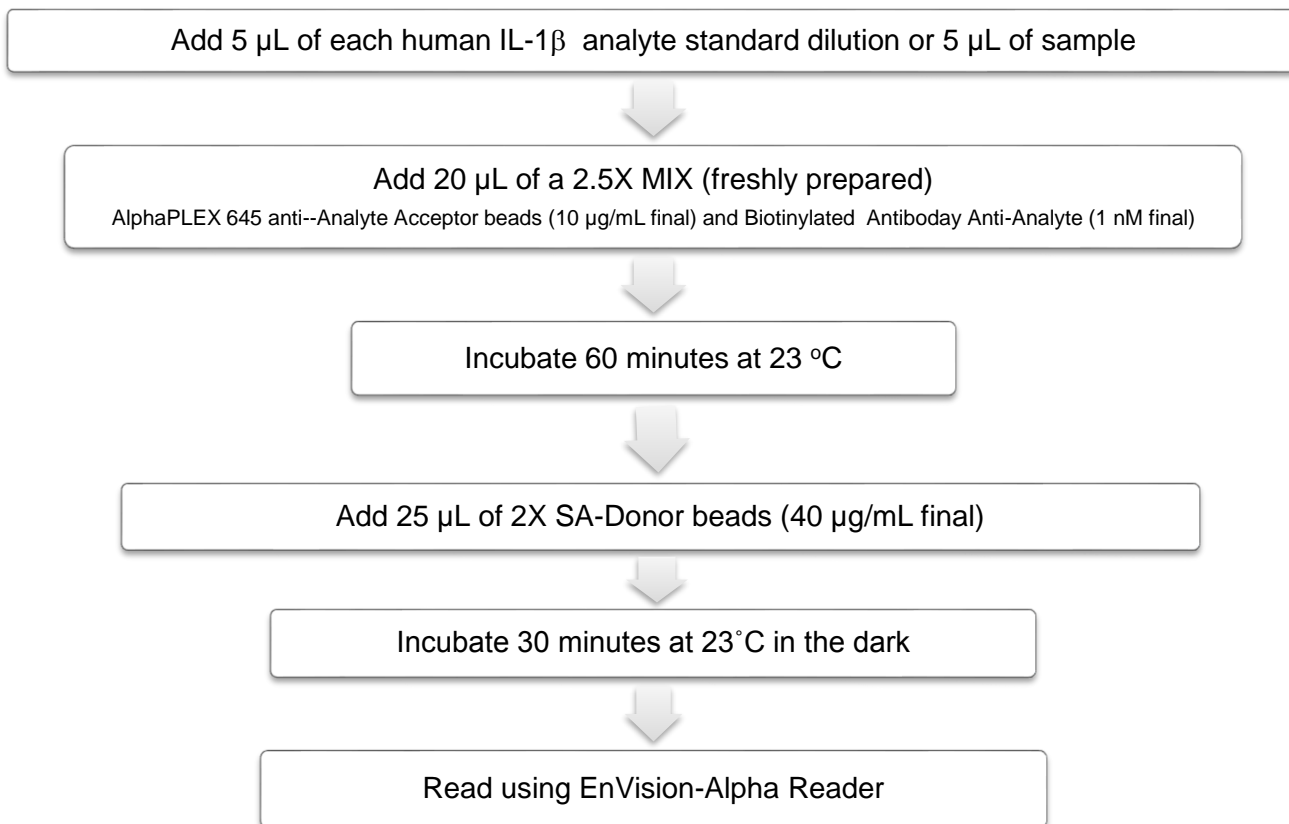4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
Keep the beads under subdued laboratory lighting.
Add 200 µL of 5 mg/mL SA-Donor beads to 12,300 µL of 1X AlphaLISA IImmunoassay Buffer.
Prepare just before use.

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

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Add 5 µL of each human IL-1β analyte standard dilution or 5 µL of sample

Add 20 µL of a 2.5X MIX (freshly prepared)
AlphaPLEX 645 anti--Analyte Acceptor beads (10 µg/mL final) and Biotinylated Antiboday Anti-Analyte (1 nM final)

Incubate 60 minutes at 23 °C

Add 25 µL of 2X SA-Donor beads (40 µg/mL final)

Incubate 30 minutes at 23˚C in the dark

Read using EnVision-Alpha Reader

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

- <u>Assay Sensitivity:</u>

…
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)	Buffer/Media	# of experiments
1.8	AlphaLISA Immunoassay Buffer (IAB)	10
2.3	FBS	3

* 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).

- <u>Assay Precision:</u>

The following assay precision data were calculated from the ten independent assays using two 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.

- <u>Intra-assay precision:</u>

The intra-assay precision was determined using a total of 10 independent measurements in 12 concentrations with each in triplicate. Shown is average CV(%).

Measurement	% CV
10	5.7

- <u>Inter-assay precision:</u>

The inter-assay precision was determined using a total of 7 independent measurements for following concentrations of human IL-1β.

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Sample	Mean	SD	% CV
1ng/mL	41966	4051	9.7
0.1ng/mL	5386	495	9.2
0.01ng/mL	1034	95	9.2

- Spike Recovery:

Three known concentrations of analyte were spiked in AlphaLISA Immunoassay Buffer (IAB), Fetal Bovine Serum (FBS) and Human Serum. The % recovery of measured versus theoretical amount was calculated for each concentration.

Spiked IL-1β (ng/mL)	% Recovery		
	IAB	FBS	Human Serum
1	90	105	98
0.1	97	107	89
0.01	101	84	84

- Specificity:

Cross-reactivity of the AlphaPLEX 645 human IL-1β Kit was tested using the following proteins at 30 ng/mL in AlphaLISA Immunoassay Buffer. Reactivity to human IL-1β is 100%.

Protein	% Cross-reactivity
Mouse IL-1β	33
Rat IL-1β	6
Human IL-1α	0

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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