TECHNICAL DATA SHEET

AlphaPlex™ 545 Research Reagents

Human Interferon-gamma (IFN-γ) AlphaPlex™ 545 Immunoassay Kit

Product number: AP217TB-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 human IFN-γ in serum, buffered solution or cell culture medium using a homogenous AlphaPlex 545 assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 12 pg/mL

Lower Limit of Quantification (LLOQ): 48 pg/mL

EC_{50}: 50 ± 30 ng/mL

Dynamic range: 10 - 300000 pg/mL (Figure 1).

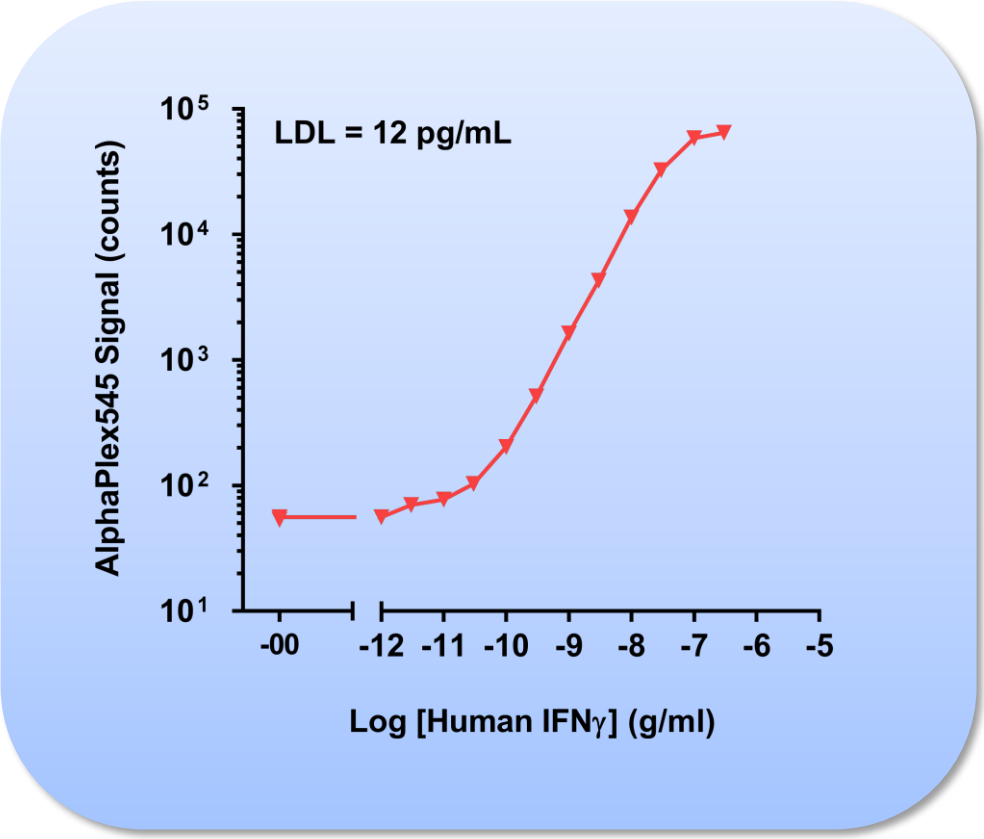


Figure 1. Typical sensitivity curve in 1X AlphaLISA HiBlock Buffer (Log-Log scale). The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2102.

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

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the human Interferon-gamma (IFN-γ) analyte is stable for at least 3 months when stored at -20°C.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA 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

Interferons (IFNs) activity has been discovered due to their antiviral effects. In humans, there are three families of IFNs: IFN type I (IFN-α, β, ω, ε, and κ), IFN type II (one single representative, IFN-γ), and IFN type III (IFN-λ1-3). Antigens and mitogens stimulate in Natural Killer (NK) and activated helper T lymphocytes (Th1) the production of IFN-γ. Human IFN-γ is a 140 amino acids polypeptide that shows multiple effects; it induces the production of cytokines, upregulates the expression of class I and II MHC antigens, and leukocyte adhesion molecules. It also activates macrophages, enhances the secretion of immunoglobulins by B cells, and potentiates Th1 cell expansion. Response to IFN-γ is mediated by the hetetodimeric IFN-γ Receptor, triggering a signalling cascade involving JAK1, JAK2, and STAT1. Importantly, IFNs have been proved to be effective in the treatment of several viral infections and cancers.

Description of the AlphaPlex 545 Assay

AlphaPlex 545 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 545 assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaPlex 545 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 545 nm (Figure 2).

Combining this assay with an AlphaLISA or AlphaPLEX 645- 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.

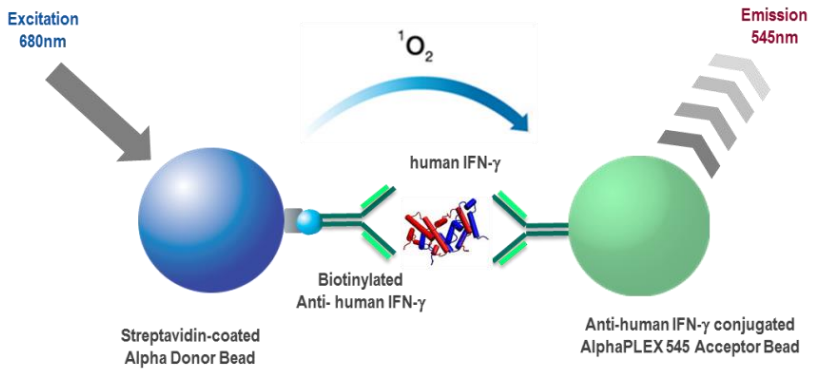


Figure 2. AlphaPlex 545 Assay principle.

Precautions

- The AlphaPLEX 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	AP217Tb-HV (100 assay points***)	AP217Tb-C (500 assay points***)	AP217Tb-F (5000 assay points***)
AlphaPlex 545 Anti-Human IFN-γ Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	30 µL @ 5 mg/mL (1 brown tube, green cap)	60 µL @ 5 mg/mL (1 brown tube, green cap)	600 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)	2 mL @ 5 mg/mL (1 brown tubes, black caps)
Biotinylated Antibody Anti-Human IFN-γ 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)	600 µL @ 500 nM (1 tube, black cap)
Human IFN-γ (0.3 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap	1 tube, clear cap
10X AlphaLISA HiBlock Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute Human IFN-γ 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 Human IFN-γ is stable for at least 60 days at -20°C. One vial contains an amount of Human IFN-γ sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL217S).

** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100 ,5% gelatin, 5% BSA and 0.5% Proclin-300. Extra buffer can be ordered separately (cat # AL004C: 10 mL, cat # AL004F: 100 mL).
Note: 10X buffer is slightly brown. However, this does not affect the assay results.
Once diluted, 1X AlphaLISA HiBlock Buffer contains 25 mM HEPES, pH 7.4, 0.1% Casein, 1 mg/mL Dextran-500, 0.5% Triton X-100, 0.5% gelatin, 0.5% BSA and 0.05% Proclin-300.

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AP 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 AlphaPlex545 signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaPlex 545 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 Bovine 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 545 signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaPLEX 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%).
- AlphaPlex 545 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.
- AlphaPlex 545 assays can be performed in cell culture medium but will have reduced performance in the presense of phenol red: if possible, avoid both phonel red and 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 two protocols described below are 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 384 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 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 545 beads / Biotin Antibody MIX**	**SA-Donor beads**	**Plate recommendation**
AP217Tb-HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AP217Tb-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)
AP217Tb-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)

Protocol 1: Quick protocol (2 incubation steps) – Dilution of standards in 1X AlphaLISA HiBlock Buffer or cell culture medium

Protocol 2: High sensitivity protocol (3 incubation steps) – Dilution of standards in human serum.

Common Steps for Preparing Reagents (Protocols 1 & 2)

1) Preparation of 1X AlphaLISA HiBlock Buffer Buffer:

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

2) Preparation of Human IFN-γ analyte standard dilutions:

- Human IFN-γ analyte is provided at 0.3 µg in lyophilized form. Reconstitute with 100 µL MiliQ H_2O to create a 3 µg/mL solution. The first point of the curve is 0.3 µg/mL so a 10 fold dilution is required. Prepare standard dilutions as follows (change tip between each standard dilution):

Tube	Vol. of Human IFN-γ (µL)	Vol. of diluent (µL) *	[Human IFN-γ] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted human IFN-γ	90	3.00E-07	300 000
B	60 µL of tube A	120	1.00E-07	100 000
C	60 µL of tube B	140	3.00E-08	30 000
D	60 µL of tube C	120	1.00E-08	10 000
E	60 µL of tube D	140	3.00E-09	3 000
F	60 µL of tube E	120	1.00E-09	1 000
G	60 µL of tube F	140	3.00E-10	300
H	60 µL of tube G	120	1.00E-10	100
I	60 µL of tube H	140	3.00E-11	30
J	60 µL of tube I	120	1.00E-11	10
K	60 µL of tube J	140	3.00E-12	3
L	60 µL of tube K	140	1.00E-12	1
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Protocol 1: Dilute standards in 1X AlphaLISA HiBlock Buffer or cell culture medium.
Protocol 2: Dilute standards in human serum.
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).

Protocol 1: Quick Protocol (2 Incubation Steps)

The protocol described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards can be done in 1X AlphaLISA HiBlock 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 AlphaPlex545 Anti-IFN-γ Acceptor beads + Biotinylated Antibody Anti-IFN-γ MIX

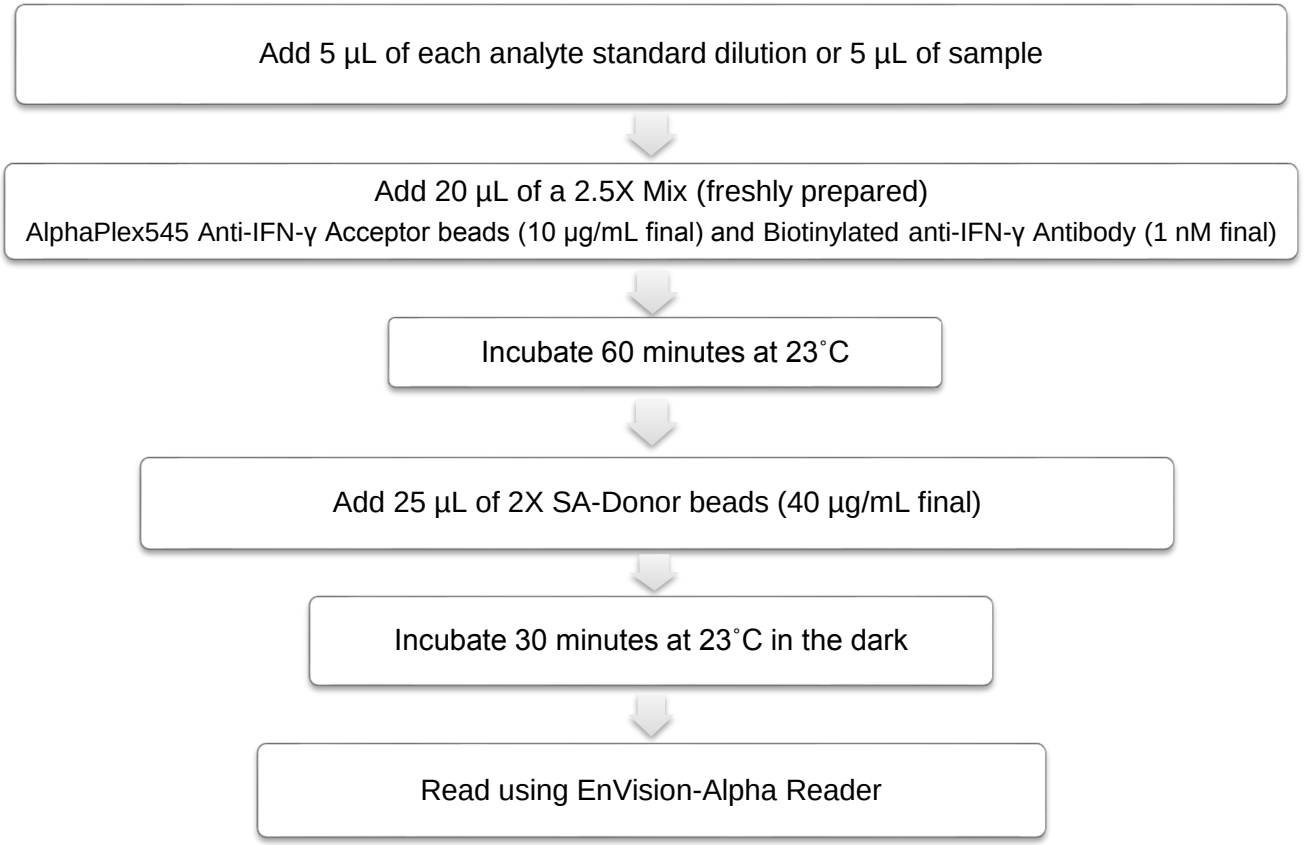
(25 µg/mL / 2.5 nM):

Add 50 µL of 5 mg/mL AlphaPlex545 Anti-IFN-γ Acceptor beads and 50 µL of 500 nM Biotinylated Antibody Anti-IFN-γ to 9 900 µL of 1X AlphaLISA HiBlock Buffer. Prepare just before use.

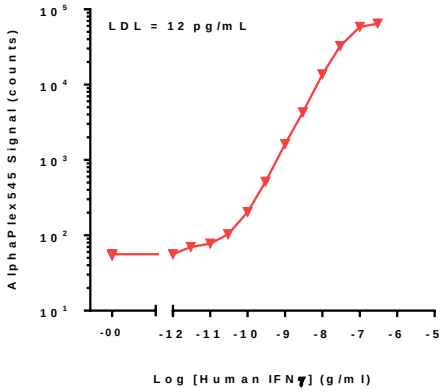
4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting. Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA HiBlock Buffer.

5) Samples: If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA HiBlock Buffer or cell culture medium).

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



Protocol 1 - Typical results in 1X AlphaLISA HiBlock Buffer (Log-Log scale)



Protocol 2: High Sensitivity Protocol (3 Incubation Steps)

The protocol described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards in human serum.
If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

3) Preparation of 5X AlphaLISA Anti-IFN-γ Acceptor beads (50 µg/mL):
Add 50 µL of 5 mg/mL AlphaLISA Anti-IFN-γ Acceptor beads to 4 950 µL of 1X AlphaLISA HiBlock Buffer.

4) Preparation of 5X Biotinylated Antibody Anti-IFN-γ (5 nM):
Add 50 µL of 500 nM Biotinylated Antibody Anti-IFN-γ to 4 950 µL of 1X AlphaLISA HiBlock Buffer.

5) 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 HiBlock Buffer.

6) Samples: If applicable, dilute samples to be tested in diluent (e.g. human serum).

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

Add 5 µL of each analyte standard dilution or 5 µL of sample

Add 10 µL of a 5X AlphaLISA anti-IFN-γ Acceptor beads (10 µg/mL final)

Incubate 30 minutes at 23 ºC

Add 10 µL of a 5X Biotinylated anti-IFN-γ Antibody (1 nM final)

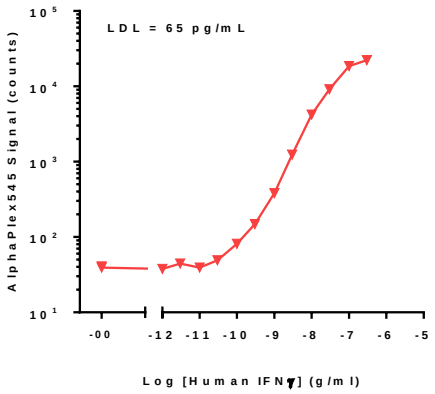
Incubate 60 minutes at 23˚C

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

Protocol 2 - Typical results in 1X human serum (Log-Log scale)



Protocols 1 & 2 - Interpreting the Data

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaPlex 545 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 545 assay performance described below was determined using the 2 step protocol.

- 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 using the recommended assay conditions.

LDL (pg/mL)	Buffer/Serum	# of experiments
13	AlphaLISA HiBlock Buffer	16
58	Human serum	4

* 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 three independent assays using two different kit lots. In each lot, the analytes were prepared in AlphaLISA Bovine Immunoassay Buffer (BIAB), AlphaLISA Immunoassay Buffer (IAB), DMEM, or RPMI. 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 16 independent determinations in triplicate. Shown are CV%.

Human IFN-γ	AlphaLISA HiBlock Buffer	Human Serum
CV%	5.2%	7.5%

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 9 measurements for 3 ng/mL sample. Shown are CV%.

Human IFN-γ (3 ng/ml)	AlphaLISA HiBlock Buffer	Human serum
CV%	9.6%	8.6%

- Spike Recovery:

Three known concentrations of analyte were spiked in BIAB, IAB, or in cell culture media containing 10% FBS. All samples, including non-spiked Immunoassay Buffers and culture media were measured in the assay. The average recovery from three independent measurements is reported.

Spiked Human IFN-γ (ng/ml)	% Recovery
30	99 %
3	92 %
0.3	102 %

- Specificity:

Cross-reactivity of the AlphaPlex 545 Human IFN-γ Kit was tested using the following proteins at 300 ng/mL in AlphaLISA HiBlock Buffer. Reactivity to Human IFN-γ is 100%.

Protein	% Cross-reactivity
Mouse IFN-γ	0
Rat IFN-γ	0
Canine IFN-γ	0
Rhesus Macaque IFN-γ	0

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaPlex 545 Assay kit at:

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

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