MANUAL

AlphaLISA® Human Interferon-gamma (IFN-γ) Kit

Product number: AL217 C/F

Research Use Only. Not for use in diagnostic procedures.

Material provided:

Format: AL217C: 500 assay points AL217F: 5000 assay points

The number of assay points is based on an assay volume of 50 µL 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 HiBlock 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 IFN-γ analyte is stable for at least 60 days at -20°C (see page 2: Reagents and Materials).

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

Sensitivity: Lower Detection Limit (LDL): 10.6 pg/mL (see page 9: Assay Performance Characteristics).

Dynamic range: 10.6 – 100 000 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	**AL217C (500 assay points)**	**AL217F (5 000 assay points)**
AlphaLISA Anti-IFN-γ Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Antibody Anti-IFN-γ stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
AlphaLISA human IFN-γ (0.3 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap
AlphaLISA HiBlock Buffer (10X) **	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, 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 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% Kathon. 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% 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
AL217C	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)
AL217F	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)

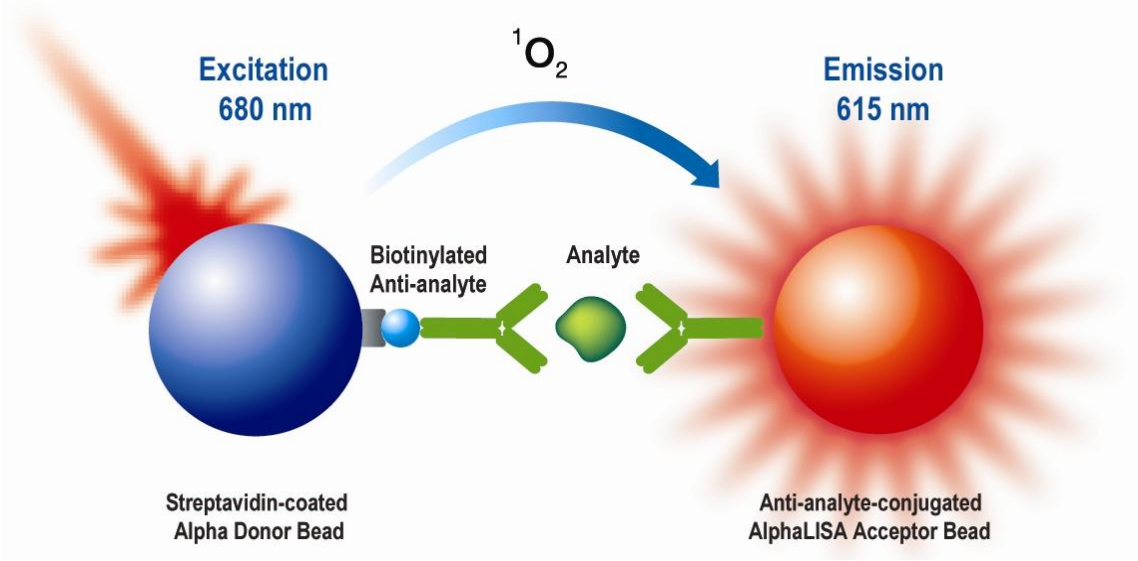
* Volumes based on the Quick manual.

Analyte of Interest

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-λ 1-3). 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 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 H_2O (18 MΩ•cm) to dilute 10X AlphaLISA HiBlock 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 analyte-depleted serum. 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 HiBlock Buffer or cell culture medium

Manual 2: High sensitivity manual (3 incubation steps) – Dilution of standards in analyte-depleted serum *

* See the analyte-depleted serum preparation manual in the "AlphaLISA Assay Development Guide" (page 20) at **www.revvity.com**

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 HiBlock Buffer:
Add 2.5 mL of 10X AlphaLISA HiBlock Buffer to 22.5 mL H_2O.

2) Preparation of human IFN-γ analyte standard dilutions:
Reconstitute lyophilized human IFN-γ (0.3 µg) in 100 µL H_2O.
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	3E-07	300 000
B	60 µL of tube A	120	1E-07	100 000
C	60 µL of tube B	140	3E-08	30 000
D	60 µL of tube C	120	1E-08	10 000
E	60 µL of tube D	140	3E-09	3 000
F	60 µL of tube E	120	1E-09	1 000
G	60 µL of tube F	140	3E-10	300
H	60 µL of tube G	120	1E-10	100
I	60 µL of tube H	140	3E-11	30
J	60 µL of tube I	120	1E-11	10
K	60 µL of tube J	140	3E-12	3
L	60 µL of tube K	120	1E-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

* Manual 1: Dilute standards in 1X AlphaLISA HiBlock Buffer or cell culture medium.
Manual 2: Dilute standards in analyte-depleted 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).

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 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 AlphaLISA Anti-IFN-γ Acceptor beads + Biotinylated Antibody Anti-IFN-γ MIX (25 µg/mL / 2.5 nM):
Add 50 µL of 5 mg/mL AlphaLISA 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:

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

⇩

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

⇩

Incubate 60 minutes at 23˚C

⇩

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

⇩

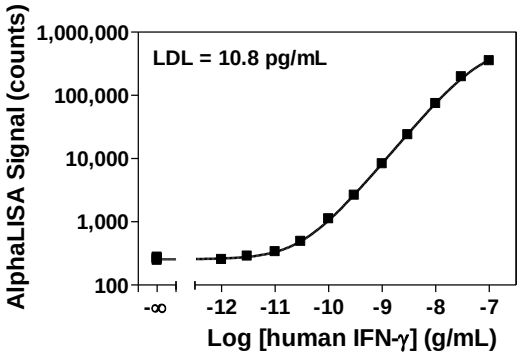
Incubate 30 minutes at 23˚C in the dark

⇩

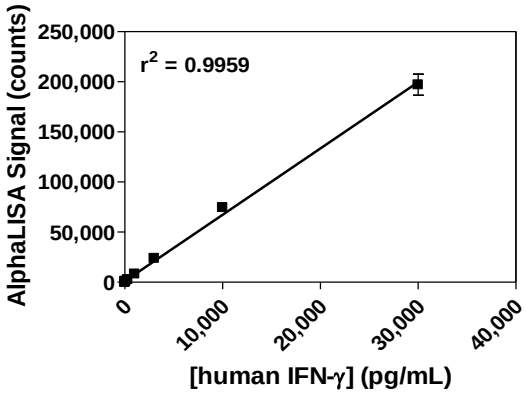
Read using EnVision-Alpha Reader

Manual 1 - Typical results in 1X AlphaLISA HiBlock Buffer

Log-Log scale:



Linear-Linear scale (linear range):



The data was generated using a white Optiplate-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 analyte-depleted 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. analyte-depleted 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 5X Anti-Analyte Acceptor beads (10 µg/mL final)

⇩

Incubate 30 minutes at 23˚C

⇩

Add 10 µL of 5X Biotinylated Antibody Anti-analyte (1 nM final)

⇩

Incubate 60 minutes at 23˚C

⇩

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

⇩

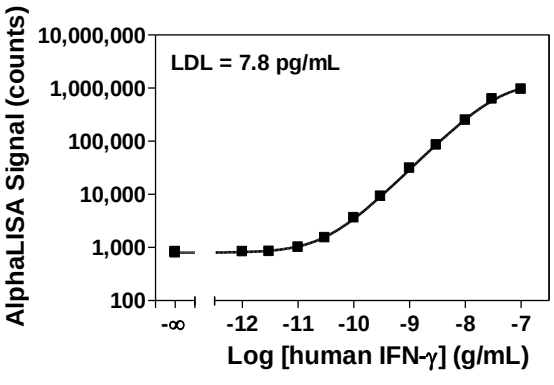
Incubate 30 minutes at 23˚C in the dark

⇩

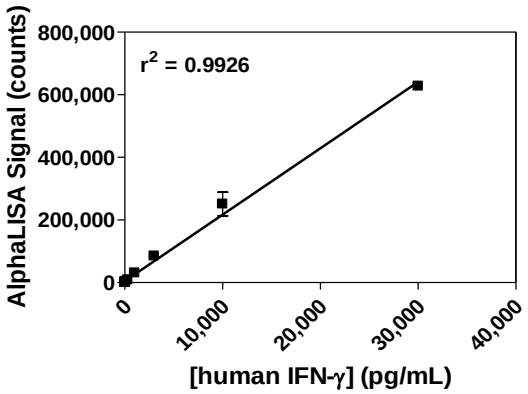
Read using EnVision-Alpha Reader

Manual 2 - Typical results in 1X AlphaLISA analyte-depleted serum

Log-Log scale:



Linear-Linear scale (linear range):



The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2101.

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 HiBlock Buffer) or manual 2 (dilution of standards in analyte-depleted serum).

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 µL using the recommended assay conditions.

- Average LDL is 10.6 pg/mL * (using 5 µL of analyte in AlphaLISA HiBlock Buffer) (mean of 27 independent experiments).
- Average LDL is 7.9 pg/mL (using 5 µL of analyte in analyte-depleted serum) (mean of 6 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 µL of analyte in a final assay volume of 50 µL).

Dynamic range: 10.6 – 100 000 pg/mL (in AlphaLISA HiBlock Buffer)

Assay precision:

The following assay precision data were calculated from a total of 18 assays. Two 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 HiBlock Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 18 independent determinations in triplicate for each control sample.

Sample	Mean (pg/mL)	SD (pg/mL)	% CV (n = 18)
A	39 443	1 780	4.5
B	3 714	134	3.6
C	393	22.0	5.6

- Inter-assay precision:

The inter-assay precision was determined using a total of 6 independent determinations with 9 measurements for each control sample.

Sample	Mean (pg/mL)	SD (pg/mL)	% CV (n = 6)
A	39 443	2 081	5.3
B	3 714	162	4.4
C	393	23.6	6.0

- Recovery:

Three known concentrations of analyte were spiked in AlphaLISA HiBlock Buffer or analyte-depleted serum. The % of measured versus theoretical amount was calculated for each concentration in 6 independent experiments (recovery in AlphaLISA HiBlock Buffer) or 6 independent experiments (recovery in serum).

Spike (ng/mL)	% Recovery (in Buffer)	% Recovery (in Serum)
30	112	88
3	106	88
0.3	110	93

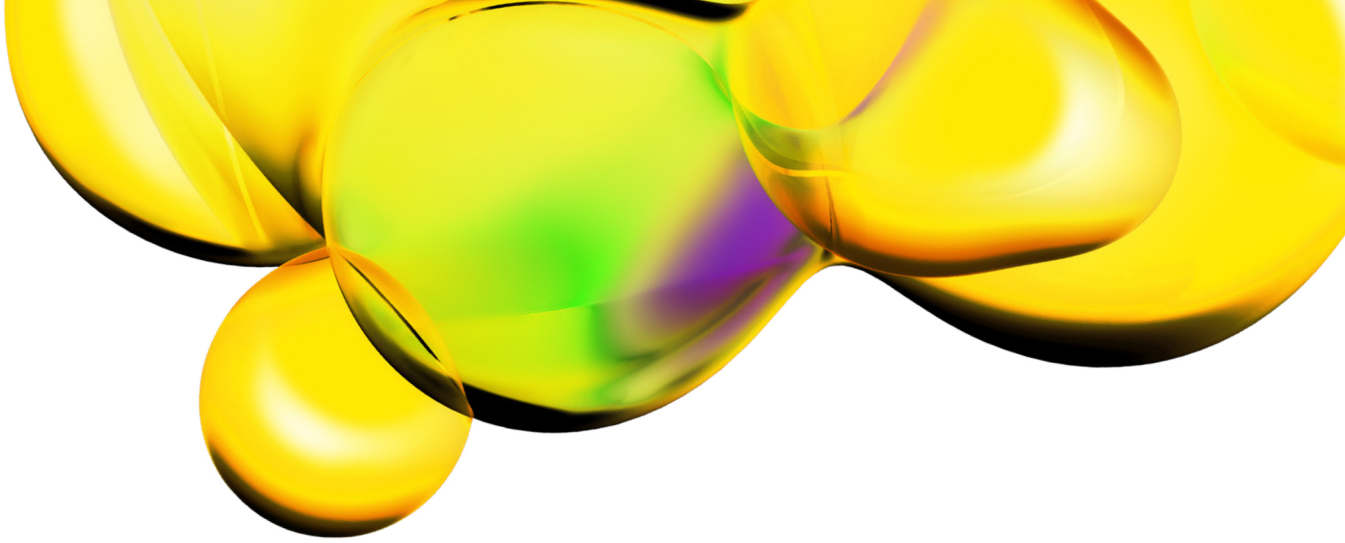
Calibration:

Human IFN-γ (NIBSC/Non WHO Reference Material (code 87/586)) was tested using this kit: 1 unit of Standard corresponds to 74.5 pg of AlphaLISA IFN-γ.

Specificity:

Cross-reactivity of the AlphaLISA IFN-γ Kit was tested using the following proteins at 0.3 µg/mL in AlphaLISA HiBlock Buffer.

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



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