TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Bovine Interferon Gamma (bIFNγ) AlphaLISA Immunoassay Kit

Product number: AL535

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 bovine IFN-gamma (bIFNγ) in buffer and cell culture media using a homogeneous AlphaLISA assay (no wash steps). The assay shows negligible cross-reactivity with other bovine cytokines.

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

Lower Limit of Quantification (LLOQ): 37 pg/mL

EC_{50}: 9.43 ± 6.5 ng/mL

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

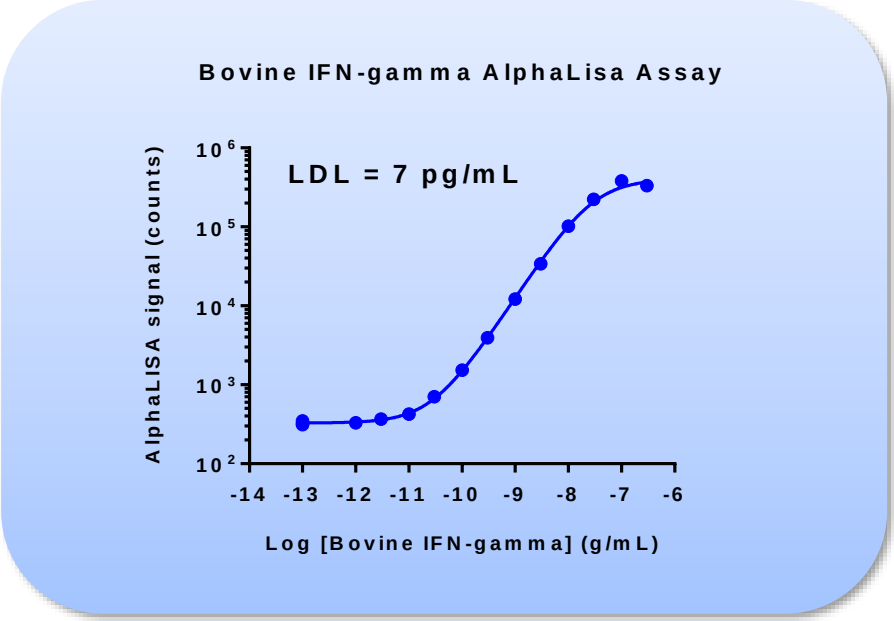


Figure 1. Typical sensitivity curves in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2102.

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

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the bovine IFNγ analyte is stable for at least 18 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

Cytokines are soluble mediators that impact a multitude of biologies including cell proliferation, survival, death, motility, cell-cell and cell-matrix interactions as well as immune response and leukocyte infiltration. Cytokines are intimately associated with acute and chronic diseases and response to vaccination. In particular, levels of cytokines such as IFNγ can be used to measure cellular immune responses to infection and/or vaccination.

IFNγ is mainly produced by activated T lymphocytes. Although by itself it has limited direct inflammatory effects, IFNγ is a major macrophage-activating factor, increasing TNFα and IL-1α production and thus contributing to activate acquired immunity to disease and passive immunity to vaccination. In particular, IFNγ has been associated to immune responses against bovine tuberculosis caused by *Mycoplasma bovis*.

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 Alpha 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 (Figure 2).

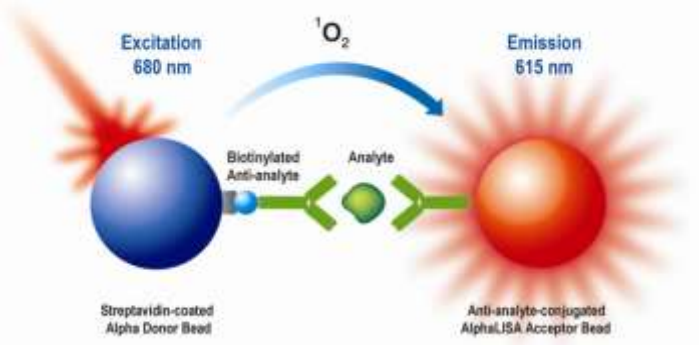


Figure 2. AlphaLISA Assay principle.

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. 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.

Kit Content: Reagents and Materials

Kit components	AL535HV (100 assay points***)	AL535C (500 assay points***)	AL535F (5000 assay points***)
AlphaLISA Anti-bIFNγ Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	30 µL @ 5 mg/mL (1 brown tube, white cap)	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% 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 (2 brown tubes, black caps)
Biotinylated Antibody Anti- bIFNγ stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	30 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
AlphaLISA bIFNγ (0.3 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap	1 tube, clear cap
AlphaLISA Bovine Immunoassay Buffer (10X)	2.5 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute bIFNγ 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 bIFNγ is stable for at least 18 months at -20°C. One vial contains an amount of bIFNγ sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL535S).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL535HV) 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

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 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 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.

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**	**AlphaLISAbeads / Biotin Antibody MIX**	**SA-Donor beads**	**Plate recommendation**
AL535HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL535C	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)
AL535F	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 3-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 Bovine Immunoassay Buffer:

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

2) Preparation of bIFNγ analyte standard dilutions:

- Bovine IFNγ 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 bIFNγ (µL)	Vol. of diluent (µL) *	[bIFNγ] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of provided IFN-gamma	90	3.00E-07	300000
B	60 µL of tube A	120	1.00E-07	100000
C	60 µL of tube B	140	3.00E-08	30000
D	60 µL of tube C	120	1.00E-08	10000
E	60 µL of tube D	140	3.00E-09	3000
F	60 µL of tube E	120	1.00E-09	1000
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

* Dilute standards in diluent (e.g. 1X AlphaLISA Bovine 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.

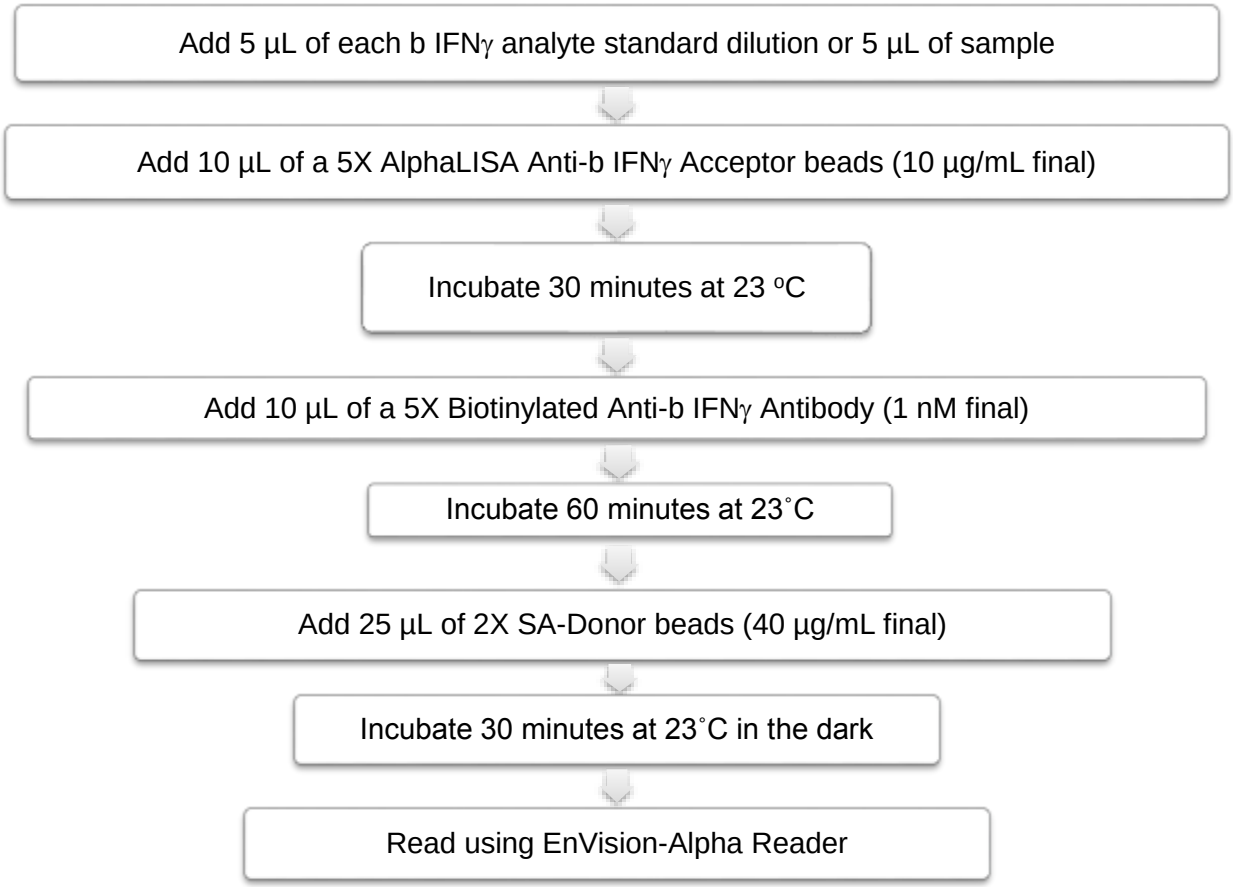
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X AlphaLISA Anti-bIFNγ Acceptor beads (50 µg /mL)
Add 60 µL of 5 mg/mL AlphaLISA Anti-bIFNγ Acceptor beads to 5940 µL of AlphaLISA Bovine Immunoassay Buffer. **Prepare just before use.**

4) Preparation of 5X AlphaLISA Anti-bIFNγ Antibody (5 nM):
Add 60 µL of 500nM biotinylated Anti-bIFNγ antibody to 5940 µL of 1X AlphaLISA Bovine Immunoassay Buffer**.** **Prepare just before use.**

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 Bovine Immunoassay Buffer.
Prepare just before use.

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



* Please note that levels of the target protein may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.

If cell culture supernatant samples are tested, preparing the standard curve in cell culture media containing 10%FBS is recommended.

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

AlphaLISA assay performance described below was determined using the 3 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)	Buffer/Media	# of experiments
12	AlphaLisa Bovine Immunoassay Buffer	25
10	AlphaLisa Immunoassay Buffer	6
7	DMEM+ 10% FBS	8
9	RPMI + 10% 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).

- 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%.

bIFNγ (3 ng/ml)	BIAB	IAB	DMEM	RPMI
CV%	5.2%	5.0%	4.9%	9.8%

- 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%.

bIFNγ (3 ng/ml)	BIAB	IAB	DMEM	RPMI
CV%	2.8%	8.4%	12.5%	9.8%

- 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 culture media and Immunoassay Buffers were measured in the assay. The average recovery from three independent measurements is reported.

Spiked bIFNγ (ng/mL)	% Recovery			
	BIAB	IAB	DMEM*	RPMI*
10	118	95	94 (121)	27 (110)
1	93	89	87 (91)	40 (94)
0.1	101	110	90 (104)	32 (96)

Note: * the numbers in parenthesis are the % recovery using the standards made in cell culture media.

- Specificity:

Cross-reactivity of the AlphaLISA bIFNγ Kit was tested using the following proteins at 10 ng/mL in AlphaLISA Bovine Immunoassay Buffer. Reactivity to bIFNγ is 100%.

Protein	% Cross-reactivity
BovineTNFα	0
Bovine IL-1α	0
Bovine IL-2	0
Bovine IL-6	0
Ovine IFNγ	111
Horse IFNγ	120
Swine IFNγ	33
Human IFNγ	3

Troubleshooting Guide

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

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

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