TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Human Immunoglobulin G subclass 4 (IgG4 isotyping) AlphaLISA Immunoassay Kit

Product number: AL310 HV/C/F

Lot specific kit information can be found at www.perkinelmer.com/COA

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

Contents

Product Information

Application: This kit is designed for the quantitative determination of human IgG4 in cell culture/non-human serum, using a homogeneous AlphaLISA assay (no wash steps). The assay shows negligible cross-reactivity with other human IgG isotypes and monkey IgG.

Sensitivity: Lower Detection Limit (LDL): 100 pg/mL

Lower Limit of Quantification (LLOQ): 300 pg/mL

EC_{50}: 220 ng/mL

Min/Max counts: 800/ 250 000 counts

Dynamic range: 300 - 500 000 pg/mL (Figure 1).

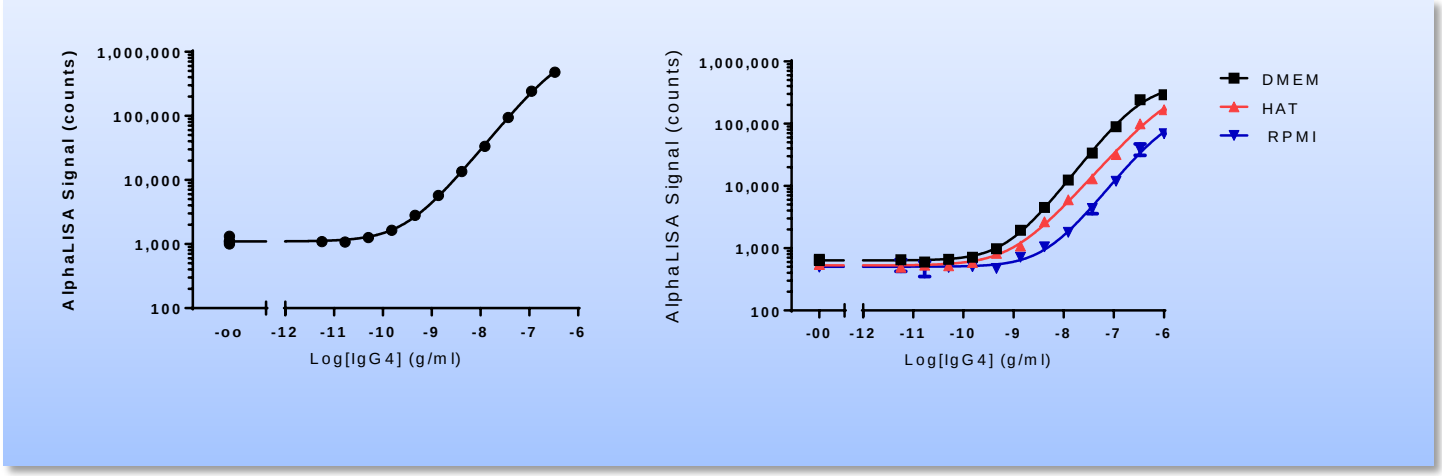


Figure. 1. Typical sensitivity curves in AlphaLISA HiBlock buffer (left) and culture media (right). The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

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 IgG4 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

Immunoglobulin G (IgG) is a major effector molecule of the humoral immune response and accounts for about 75% of the total immunoglobulins in plasma of healthy individuals. The remainder 25% comprises IgM, IgA, IgD and IgE, each of which has characteristic properties and functions. The basic IgG molecule has a four-chain structure, comprising two identical heavy (H) chains and two identical light (L) chains, linked together by inter-chain disulfide bonds. Four IgG subclasses have been identified: IgG1, IgG2, IgG3 and IgG4. Biotherapeutic antibody drugs, usually IgG1 or IgG4 molecules, are becoming increasingly important for treating debilitating diseases such as cancer and autoimmune disorders. Drug levels need to be accurately measured at various stages of drug development, including early antibody discovery, preclinical research *in vivo*, and commercial manufacturing. The present kit permits detection of human IgG4 (i.e. analyte) in different sample matrices, including different cell culture media and monkey serum.

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).

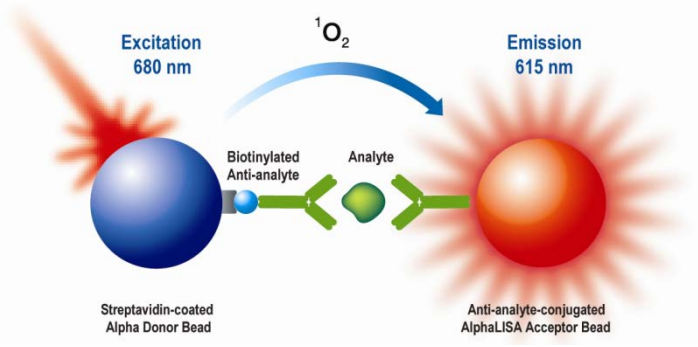


Figure 2. AlphaLISA Assay principle.

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 human source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated Anti-IgG4 Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL310HV (100 assay points***)	AL310C (500 assay points***)	AL310F (5 000 assay points***)
AlphaLISA Anti-IgG4 Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	25 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 x 1000 µL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Antibody Anti-IgG4 stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 250 nM (1 tube, black cap)	20 µL @ 250 nM (1 tube, black cap)	200 µL @ 250 nM (1 tube, black cap)
AlphaLISA human IgG4 (1 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap	1 tube, clear cap
AlphaLISA HiBlock Buffer (10X) **	2.5 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute human IgG4 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 IgG4 is stable for at least 6 months at -20°C. One vial contains an amount of human IgG4 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL310S).

** 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 tan. If not fully in suspension when diluted to the final 1X solution, it is recommended to centrifuge it for 5 min at 1000 rpm and use the supernatant. It should be noted however, that the appearance of the buffer does not affect its efficacy.

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL310HV) 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 HiBlock Buffer.
- 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. The standard curve should be performed in a similar matrix as the samples (e.g. FBS for serum samples).
- 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.

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**	**AlphaLISA beads / Biotin Antibody MIX**	**SA-Donor beads**	**Plate recommendation**
AL310HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL310C	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)
AL310F	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 (2 incubation steps) – Dilution of standards in 1X AlphaLISA HiBlock Buffer or cell culture medium

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) and 452 samples. *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 5 mL of 10X AlphaLISA HiBlock Buffer to 45 mL H_2O.

2) Preparation of human IgG4 analyte standard dilutions:

- Reconstitute lyophilized human IgG4 (1 µg) in 100 µL H_2O (10 µg/mL). The first point of the curve is 1 µg/ml.
- Prepare standard dilutions as follows (change tip between each standard dilution) in 1X AlphaLISA HiBlock Buffer or cell culture medium:

Tube	**Vol. of human IgG4 (µL)**	**Vol. of diluent (µL) ***	**[human IgG4] in standard curve (pg/mL in 5 µL)**
A	10 µL of reconstituted human IgG4	90	1 000 000
B	60 µL of tube A	120	333 333
C	60 µL of tube B	120	111 111
D	60 µL of tube C	120	37 037
E	60 µL of tube D	120	12 345
F	60 µL of tube E	120	4 115
G	60 µL of tube F	120	1 371
H	60 µL of tube G	120	457
I	60 µL of tube H	120	152
J	60 µL of tube I	120	51
K	60 µL of tube J	120	17
L	60 µL of tube K	120	6
M ** (background)	0	100	0
N ** (background)	0	100	0
O ** (background)	0	100	0
P ** (background)	0	100	0

* Dilute standards in diluent (e.g. 1X AlphaLISA HiBlock Buffer, or cell culture medium).
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).

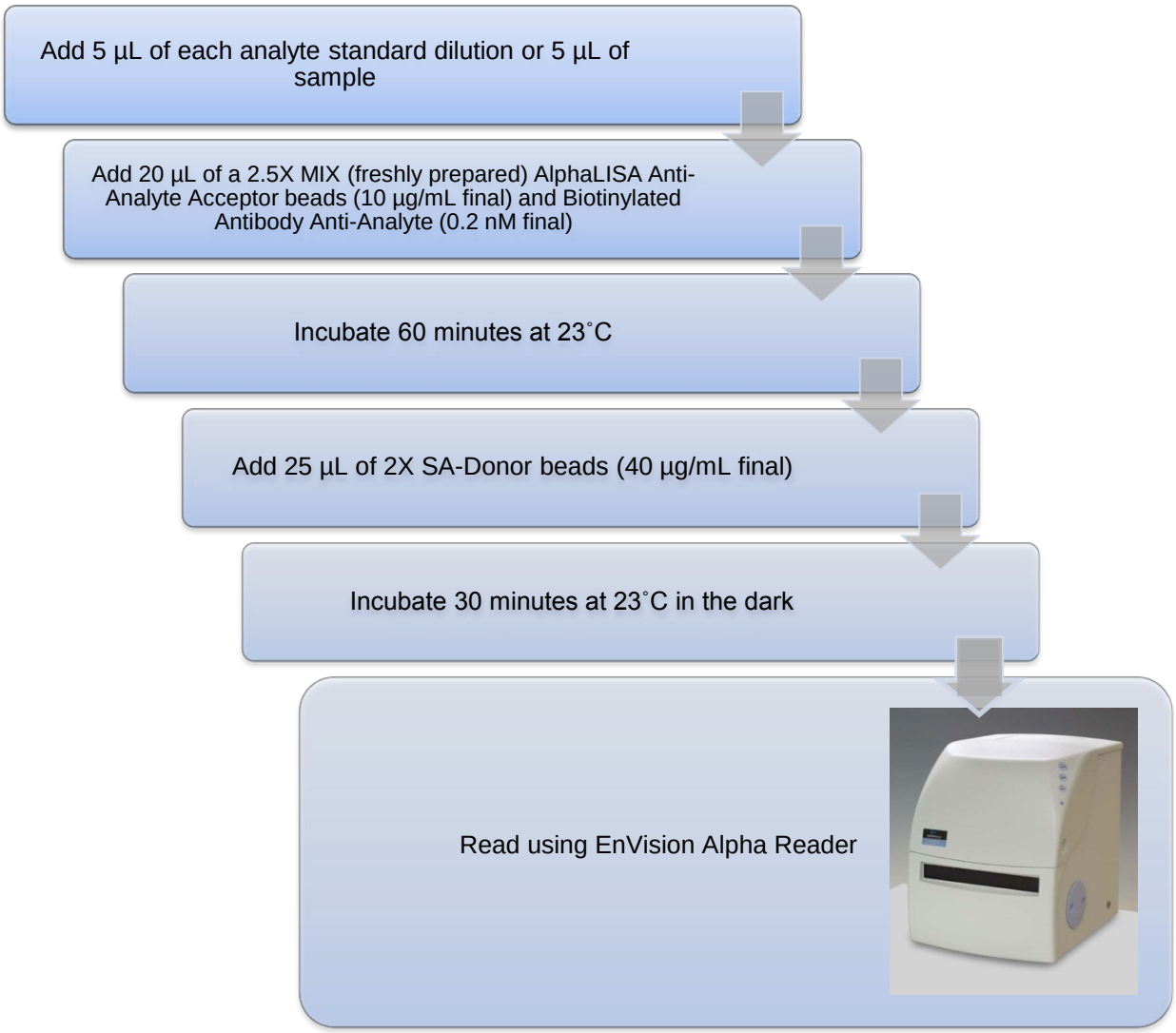
3) Preparation of 2.5X MIX AlphaLISA Anti-IgG4 Acceptor beads (25 µg/mL) and Biotinylated Anti-IgG4 Antibody (0.5 nM):
   a. Prepare just before use.
   b. Add 50 µL of 5 mg/mL AlphaLISA Anti-IgG4 Acceptor beads.
   c. Add 20 µL of 250 nM Biotinylated Antibody Anti-IgG4.
   d. Add 9930 µL of AlphaLISA HiBlock buffer.

4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. 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:



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 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)	Buffer/Media used	# of experiments
100	AlphaLISA HiBlock Buffer	6
170	DMEM+ 10% FBS	6
270	HAT + 10% FBS	6
2000	RPMI + 10% FBS	6
800	10X diluted monkey serum	1

* 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 HiBlock Buffer (HBB), DMEM, HAT, 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 HiBlock *Buffer.*

- Intra-assay precision:

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

Human IgG4	HBB	DMEM	HAT	RPMI
CV%	10%	8%	14%	10%

- Inter-assay precision:

The inter-assay precision was determined using a total of 6 independent determinations. Shown as CV%.

Human IgG4	Buffer	DMEM	HAT	RPMI
CV%	5%	12%	9%	17%

- Recovery:

Three known concentrations of analyte were spiked in a cell culture media containing 10% FBS and AlphaLISA HiBlock Buffer (HBB). All samples, including non-spiked culture media and AlphaLISA HiBlock Buffer were measured in the assay. Values calculated for control spiked samples in AlphaLISA HiBlock Buffer considered as 100% recovery. The % in cell culture media vs. expected (control spike value) was calculated for each concentration. The average recovery from two independent measurements is reported.

Spike (IgG4 ng/mL)	% Recovery			
	AlphaLISA HiBlock Buffer	DMEM	HAT	RPMI
10	96	102	90	100
4	81	116	112	80

- Specificity:

Cross-reactivity of the AlphaLISA IgG4 Kit was tested using the following proteins at 100 ng/mL in AlphaLISA HiBlock Buffer.

Protein	% Cross-reactivity
Human IgG1	0.4
Human IgG2	0.3
Human IgG3	0.7
Cynomolgus Monkey IgG	0.5
Bovine IgG	0.9

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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