MANUAL

AlphaLISA® Human Immunoglobulin G subclass 1 (IgG_1 isotyping) Immunoassay Kit

Product number: AL3171 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of human IgG_1 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): 3.82 ng/mL

Lower Limit of Quantification (LLOQ): 4.29 ng/mL

EC_{50}: 3.68 µg/mL

Min/Max counts: 700/200 000 counts

Dynamic range: 4.29 – 1 000 000 ng/mL (Figure 1).

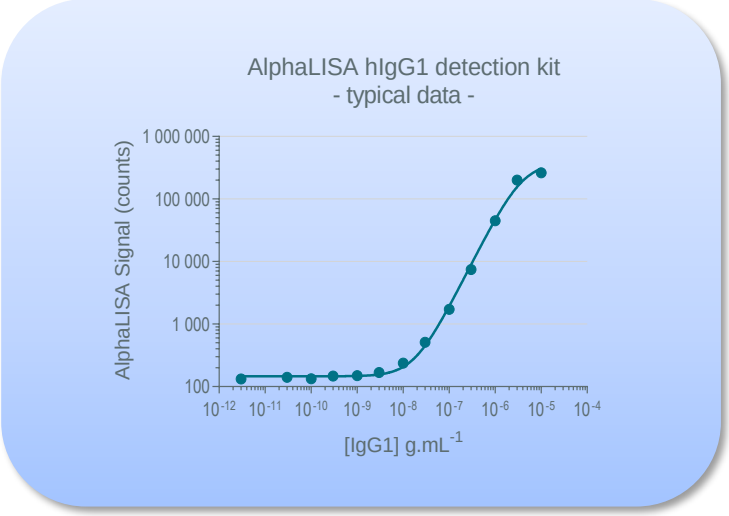


Figure. 1. Typical sensitivity curves in AlphaLISA HiBlock buffer. The data was generated using a grey Alphaplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2102.

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 IgG_1 analyte is stable for at least 1 months when stored at -20°C.

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 remaining 25% comprises IgM, IgA, IgD and IgE, each of which have 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: IgG_1, IgG_2, IgG_3 and IgG_4. Biotherapeutic antibody drugs, usually IgG_1 or IgG_4 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 IgG_1 (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).

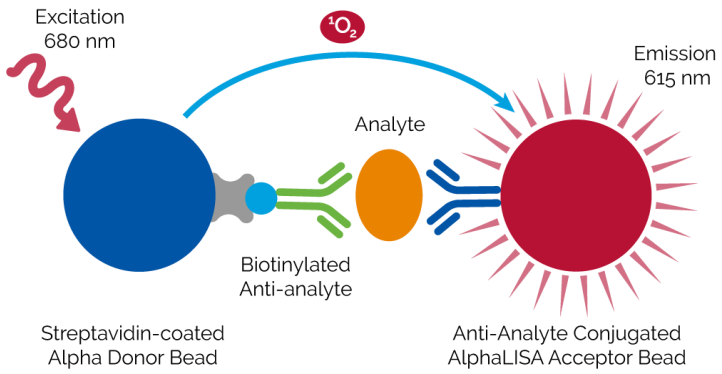


Figure 2. AlphaLISA Assay principle.

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-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3171HV (100 assay points***)	AL3171C (500 assay points***)	AL3171F (5 000 assay points***)
AlphaLISA Anti-IgG_1 Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	20 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% Kathon, pH 7.4	80 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 x 1,000 µL @ 5 mg/mL (2 brown tube, black caps)
Biotinylated Antibody Anti-IgG_1 stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	40 µL @ 500 nM (1 tube, black cap)	100 µL @ 500 nM (1 tube, black cap)	1,000 µL @ 500 nM (1 tube, black cap)
AlphaLISA human IgG_1 (10 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap	1 tube, clear cap
AlphaLISA HiBlock Buffer (10X) **	2 mL, 1 small bottle	10mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute human IgG_1 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 -20ºC for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted human IgG_1 is stable for at least 18 months at -20ºC. One vial contains an amount of human IgG_1 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3171S).

** Extra buffer can be ordered separately (cat # AL004C: 10 mL, cat # AL004F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL3171HV) 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	Revvity Inc.	6050195
EnVision®-Alpha Reader	Revvity 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 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. 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 manual described below is 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, 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 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/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**
AL3171HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3171C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	5 µL	5 µL	40 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3171F	5 000	50 µL	5 µL	5 µL	40 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)

The manual described below is for one standard curve (48 wells) and samples (452 wells). *If different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly*.

1. Preparation of 1X Alpha HiBlock Buffer (for 30 mL)

- Add 3 mL of 10X AlphaLISA HiBlock Buffer and 27 mL of MilliQ water.

2. Preparation of human IgG1 analyte standard dilutions:

- Reconstitute lyophilized human IgG1 (10 µg) in 100 µL H2O.
- Prepare standard dilutions as follows (change tip between each standard dilution) in 1X AlphaLISA HiBlock Buffer or cell culture medium:

Tube	Vol. of human IgG_1 (µL)	Vol. of diluent (µL) *	[human IgG_1] in standard curve (g/mL in 5 µL)
A	20 µL of reconstituted human IgG1	180	1.00E-05
B	60 µL of tube A	140	3.00E-06
C	60 µL of tube B	120	1.00E-06
D	60 µL of tube C	140	3.00E-07
E	60 µL of tube D	120	1.00E-07
F	60 µL of tube E	140	3.00E-08
G	60 µL of tube F	120	1.00E-08
H	60 µL of tube G	140	3.00E-09
I	60 µL of tube H	120	1.00E-09
J	60 µL of tube I	140	3.00E-10
K	60 µL of tube J	120	1.00E-10
L	60 µL of tube K	140	3.00E-11
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, 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.

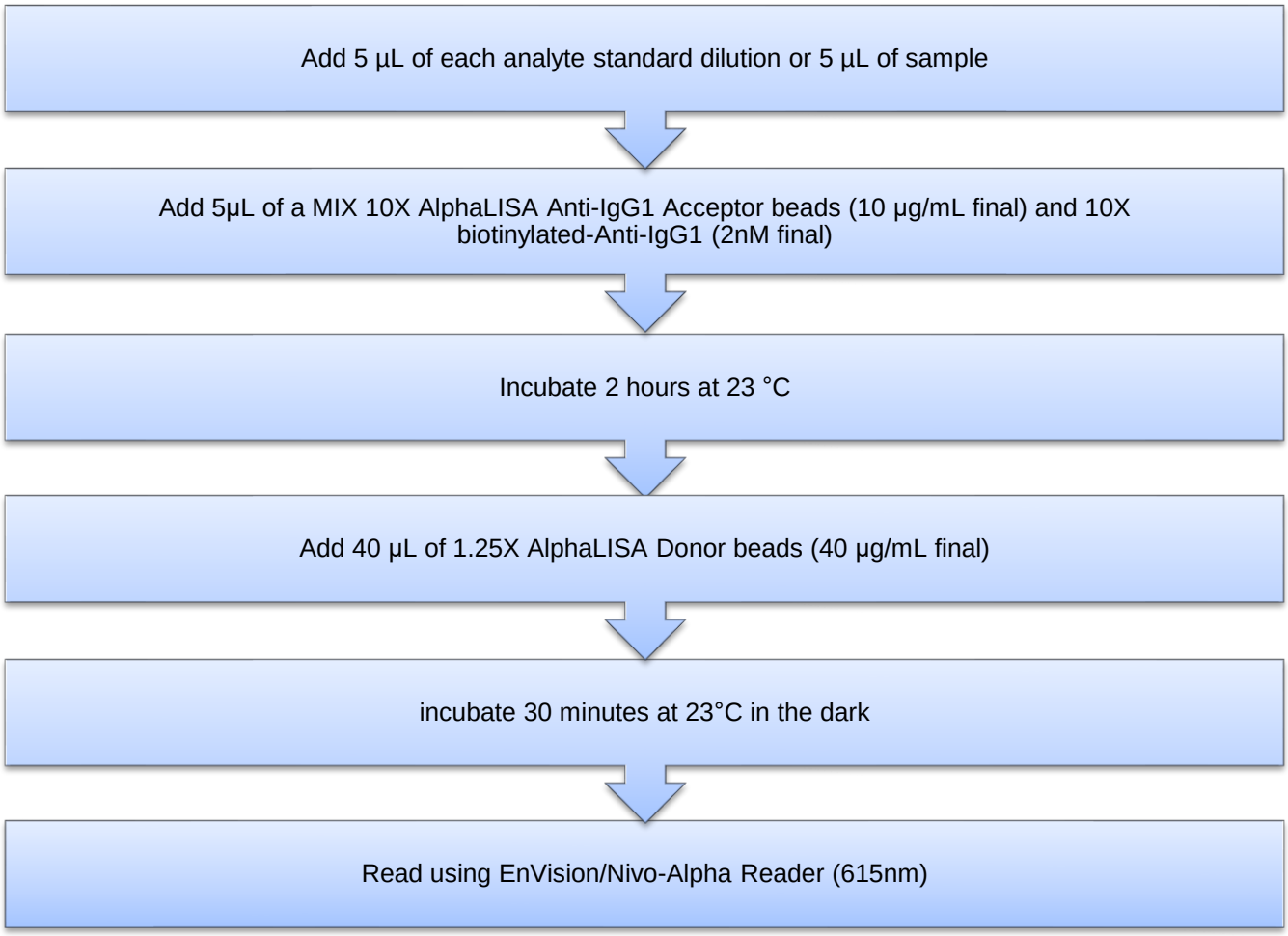
** Four background points in triplicate (12 wells) are used when LDL/LLOQ is calculated. If LDL/LLOQ does not need to be calculated, one background point in triplicate can be used (3 wells).

3. Preparation of MIX of 10X AlphaLISA Anti-hIgG1 Acceptor beads (100 µg/mL) and 10X Biotinylated Anti-hIgG1 (20 nM):
   Add 50 µL of 5 mg/mL AlphaLISA Anti-hIgG1 Acceptor beads and 100µL of 500nM of biotinylated anti-hIgG1 in 2,350 µL of 1X AlphaLISA HiBlock Buffer.

4. Preparation of 1.25 X Streptavidin (SA) Donor beads (50 µg/mL):
   Add 200 µL of 5 mg/mL SA-Donor beads to 19.8 mL of 1X AlphaLISA HiBlock Buffer.
   Keep the beads under subdued laboratory lighting.

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

6. In AlphaPlate (384 wells):



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

- **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 (ng/mL)	Buffer/Media used	# of experiments
3.82	HiBlock Buffer	14
2.77	DMEM+ 10% FBS	3
2.64	RPMI + 10% FBS	3
1.05	FCS	3

- **Specificity:**

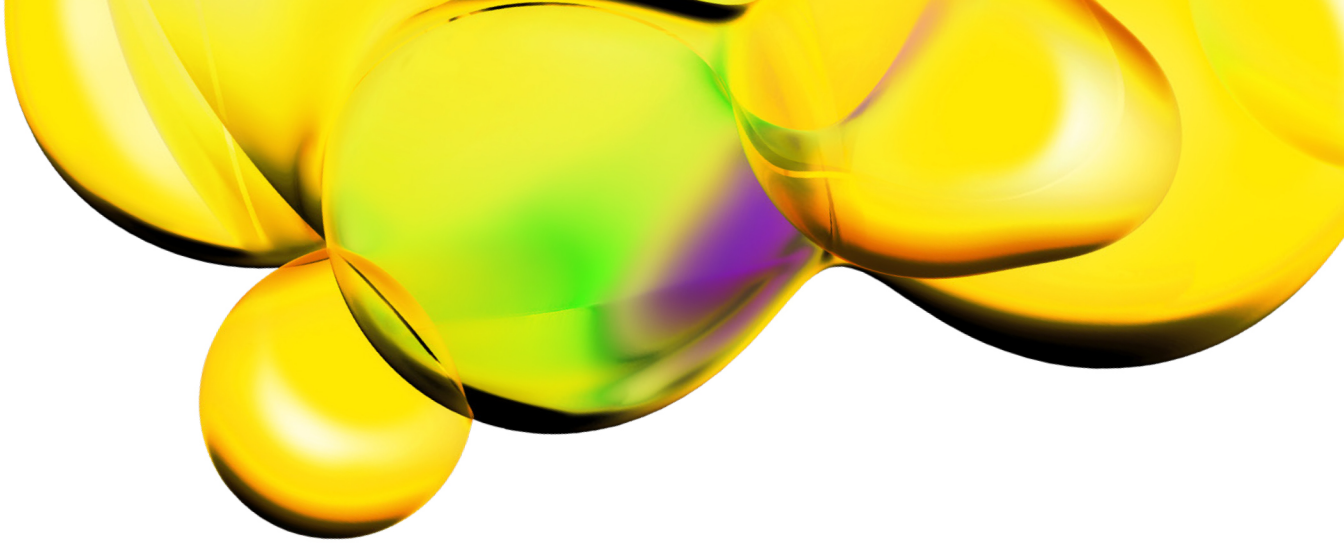
Cross-reactivity of the AlphaLISA IgG_1 Kit was tested using the following sample:
- purified proteins at 1µg/mL for IgGs in AlphaLISA HiBlock buffer
- purified proteins at 0.3µg/mL for IgG1 species in AlphaLISA Immunoassay Buffer

Protein	% Cross-reactivity
Human IgG_2	0.26
Human IgG_3	0.15
Human IgG_4	0.01
Mouse IgG_1	0.00
Bovine IgG_1	0.00
Monkey IgG_1	22

Troubleshooting Guide

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

RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.



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