MANUAL

AlphaLISA® Human FCGR3A/CD16a (176Val/V158) Detection Kit

Product number: AL348 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the detection of binding activity between FCGR3A (176Val) and human IgG Fc fragment using a homogeneous AlphaLISA assay (no wash steps). This assay can facilitate the design and development of antibody therapeutics by using competitive binding.

Sensitivity: EC_{50} 0.21 nM (average)

Signal to background ratio: 5082 (average)

Kit contents: The kit contains 4 components: Human IgG Fc fragment conjugated Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human FCGR3A (176Val), and AlphaLISA HiBlock buffer.

Storage: The kit components must be stored at 4 ˚C and in the dark. After reconstitution, store unused protein in -20 °C. Avoid multiple freeze/thaw cycles.

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.

Description of the AlphaLISA Assay

The Fc-Gamma Receptors (FCGRs) are members of immunoglobulin superfamily and play a critical role in the function of therapeutic antibodies. FCGRs are divided into three classes. FCGR3 (CD16) is expressed as two distinct forms (FCGR3A and FCGR3B) encoded by two different highly homologous genes in a cell type specific manner. FCGR3A is a low/intermediate affinity receptor for polyvalent immune-complexed IgG. It is involved in phagocytosis, secretion of enzymes and inflammatory mediators, antibody-dependent cellular cytotoxicity (ADCC), mast cell degranulation and clearance of immune complexes. In humans, a single nucleotide polymorphism creates two isoforms: high binding (176Val/V158) and low binding (176Phe/F158) forms that, when homozygous, may influence susceptibility to autoimmune diseases or response to therapeutic IgG antibodies. FCGR3A has been considered as an important therapeutic target. This AlphaLISA assay can be used to determine the binding activity of human IgG Fc fragment to human FCGR3A and also can be used to study how other antibodies bind to FCGR3A in a competition assay.

The AlphaLISA detection of FCGR3A and IgG Fc binding uses IgG Fc AlphaLISA® acceptor beads to capture the Biotinylated human FCGR3A and Streptavidin-coated donor beads to capture the biotinylated human FCGR3A. Donor beads and acceptor beads come into proximity through IgG Fc fragment binding to FCGR3A. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 1).

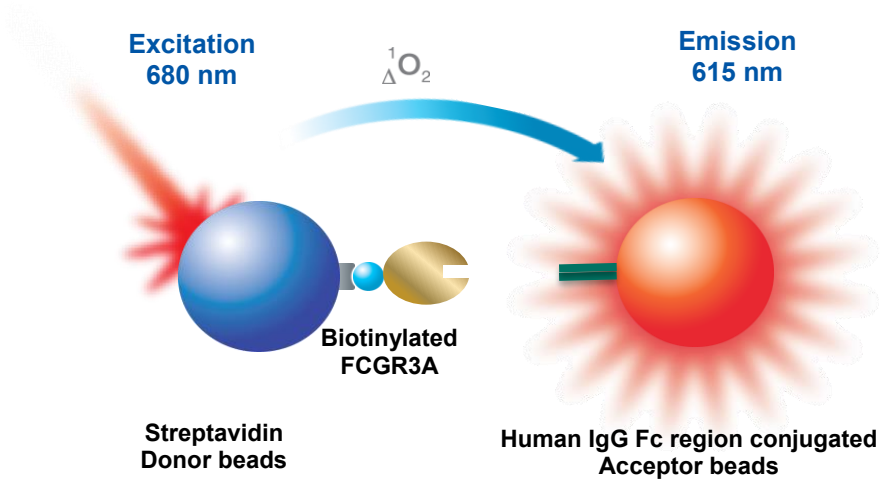


Figure 1. AlphaLISA assay principle.

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.

Kit Content: Reagents and Materials

Kit components	AL348HV (100 assay points)**	AL348C (500 assay points)**	AL348F (5000 assay points)**
AlphaLISA Human IgG Fc fragment Acceptor beads stored in PBS, 0.05% Kathon-300, pH 7.2	16 µL @ 5 mg/mL (1 brown tube, white cap)	80 µL @ 5 mg/mL (1 brown tube, white cap)	800 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-300, pH 7.4	16 µL @ 5 mg/mL (1 brown tube, black cap)	80 µL @ 5 mg/mL (1 brown tube, black cap)	800 µL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated human FCGR3A (176Val) (lyophilized) ***	0.5 µg (1 tube, clear cap)	0.5 µg (1 tube, clear cap)	5 x 0.5 µg (5 tubes, clear caps)
AlphaLISA HiBlock Buffer (10X)*	2 mL, 1 small bottle	10 mL, 1 large bottle	100 mL, 1 large bottle

* Extra buffer can be ordered separately (cat # AL004 C: 10 mL, cat # AL004F: 100 mL).

** The number of assay points is based on an assay volume of 40 µL in 96-well assay plates using the kit components at the recommended concentrations.

*** After reconstitution, aliquot and store unused protein at -20 °C for 3 months. Avoid multiple freeze/thaw cycles.

Additional Reagents and Materials

The following items are recommended for the assays:

Item	Supplier	Catalog number
½ AlphaPlate-96, white	Revvity	6005560 (50/box) 6005569 (200/box)
TopSeal™-A Adhesive Sealing Film	Revvity	6050195
EnSpire® or EnVision® Multilabel Alpha Reader	Revvity	Please consult our website

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
IgG1, Human Plasma	Athens Research Technology	16-16-090707-1
IgG2, Human Plasma	Athens Research Technology	16-16-090707-2
IgG3, Human Plasma	Athens Research Technology	16-16-090707-3
IgG4, Human Plasma	Athens Research Technology	16-16-090707-4
ChromPure Human IgG $F(ab')_2$ Fragment	JacksonImmunoResearch	009-000-006
ChromPure Human IgG Fc Fragment	JacksonImmunoResearch	009-000-008
ChromPure Human IgG,whole molecule	JacksonImmunoResearch	009-000-003
Anti-human CD16a antibody	AbD Serotec	MCA1193GA

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 quickly all tubes before use to improve recovery of content (2 000 ×*g*, 10-15 sec). Resuspend all reagents by gentle mixing before use.

- Use Milli-Q® grade H_2O to dilute 10X HiBlock Buffer 1.

- When reagents are added in the microplate, make sure the liquids are at the bottom of the well by tapping or swirling the plate gently on a smooth surface.

- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Sealing Film to reduce evaporation during incubation with the Alpha beads. 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 (barcode 444), Emission Filter: M570w (barcode 224), Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).

- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation time and temperature should be used for each plate.

Competition Assay Manual

- Assay specificity can be demonstrated by competing the binding of human FCGR3A with all subclasses of human IgG or comparing the binding with human IgG Fab fragments.

The competition assay described below is an example for determining IC_{50} of human IgG subclass competitive binding to human FCGR3A in 40 µL final assay volume (96 wells, duplicate determinations) by AlphaLISA technology. This manual can test full curves of 3 antibodies in 96 wells. If a different number of samples are tested, the volumes of all reagents have to be adjusted accordingly. The manual is provided for information only. As needed, the number of replicates or the range of concentrations tested can be modified.

1. Preparation of 1x HiBlock Buffer 1 (for 10 mL)

   Add 1 mL of 10X HiBlock Buffer and 9 mL of ultrapure water (18 MΩ.cm).

2. Preparation of serial dilution of human IgG subclasses

   Prepare serial dilutions of 4X IgG in 1x HiBlock buffer as follows:

Tube	Volume of IgG	Volume of 1X buffer	[IgG] (g/mL) (4X)	[IgG] (g/mL) (1X)
A	1.2 mg/mL stock	0	1.20E-03	3.00E-04
B	30 µL of tube A	60 µL	4.00E-04	1.00E-04
C	30 µL of tube B	70 µL	1.20E-04	3.00E-05
D	30 µL of tube C	60 µL	4.00E-05	1.00E-05
E	30 µL of tube D	70 µL	1.20E-05	3.00E-06
F	30 µL of tube E	60 µL	4.00E-06	1.00E-06
G	30 µL of tube F	70 µL	1.20E-06	3.00E-07
H	30 µL of tube G	60 µL	4.00E-07	1.00E-07
I	30 µL of tube H	70 µL	1.20E-07	3.00E-08
J	30 µL of tube I	60 µL	4.00E-08	1.00E-08
K	30 µL of tube J	70 µL	1.20E-08	3.00E-09
L	30 µL of tube K	60 µL	4.00E-09	1.00E-09
M	30 µL of tube L	70 µL	1.20E-09	3.00E-10
N	30 µL of tube M	60 µL	4.00E-10	1.00E-10
O	30 µL of tube N	70 µL	1.20E-10	3.00E-11
P	30 µL of tube O	60 µL	4.00E-11	1.00E-11

3. Preparation of 4X human FCGR3A (176Val) (1.2 nM, 1000 µL)

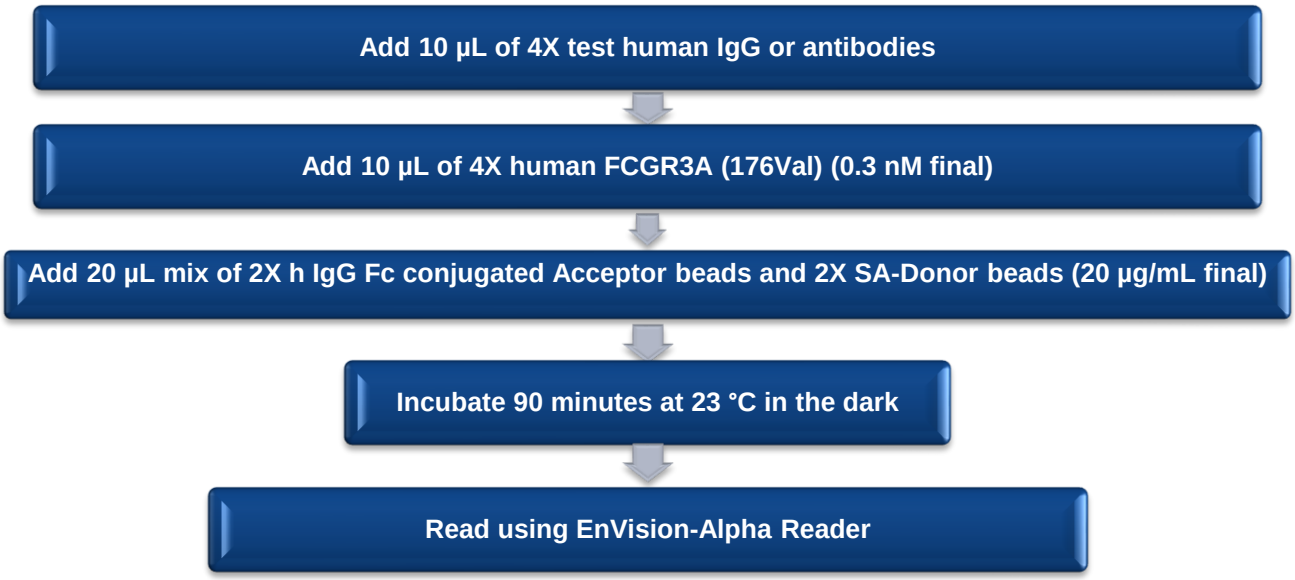
   Spin the vial containing 0.5 µg lyophilized protein briefly in microfuge and reconstitute it with 100 µL sterile distilled water to make 0.2 µM stock concentration of human FCGR3A (176Val).

   Add the 6 µL of 0.2 µM human FCGR3A (176Val) into a new tube containing 994 µL 1X HiBlock Buffer to make 1.2 nM human FCGR3A (Val).

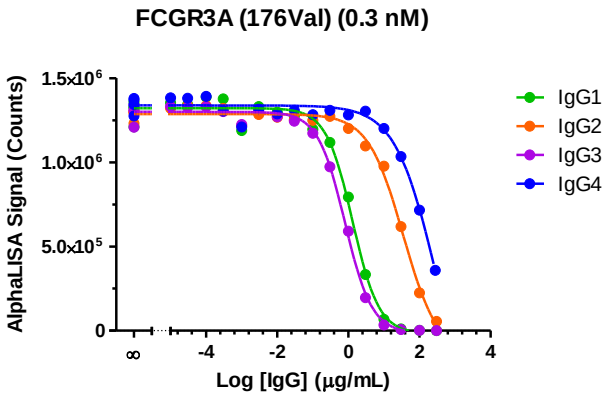
   Prepare just before use.

4. Preparation the mix of 2X human IgG Fc Conjugated Acceptor Beads and 2X Streptavidin (SA) Donor Beads (40 µg/mL, 40 µg/mL, 2000 µL).
   Add 16 µL of 5 mg/mL human IgG Fc conjugated Acceptor beads and 16 µL of 5 mg/mL SA-Donor beads into 1968 µL 1X HiBlock buffer
   Keep the beads under subdued laboratory lighting and prepare just before use.

5. In a ½ AreaPlate (96 wells):

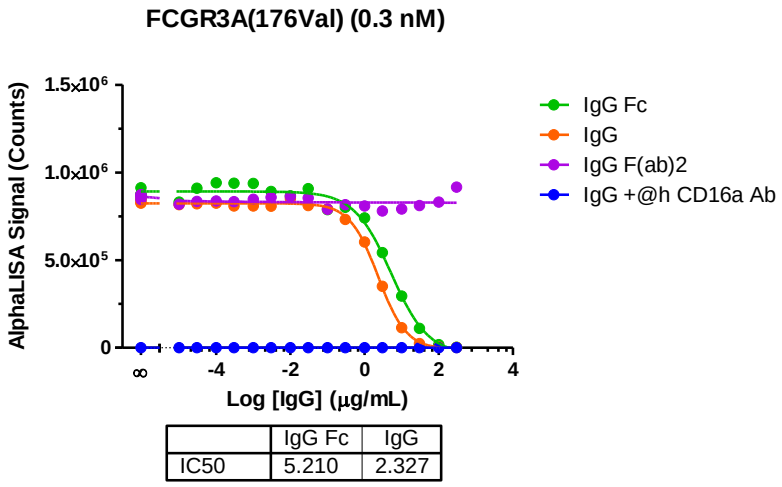


Typical competitive binding Data:



	IgG1	IgG2	IgG3	IgG4
IC50	1.317	35.04	0.8060	283.7

Figure 2. Human IgG subclasses competitive bind to FCGR3A (176Val). The IC50 values are 1.3, 35, 0.8 and 284 µg/mL for IgG1, IgG2, IgG3 and IgG4, respectively and were calculated by using nonlinear regression fitting with GraphPad Prism 5.



	IgG Fc	IgG
IC50	5.210	2.327

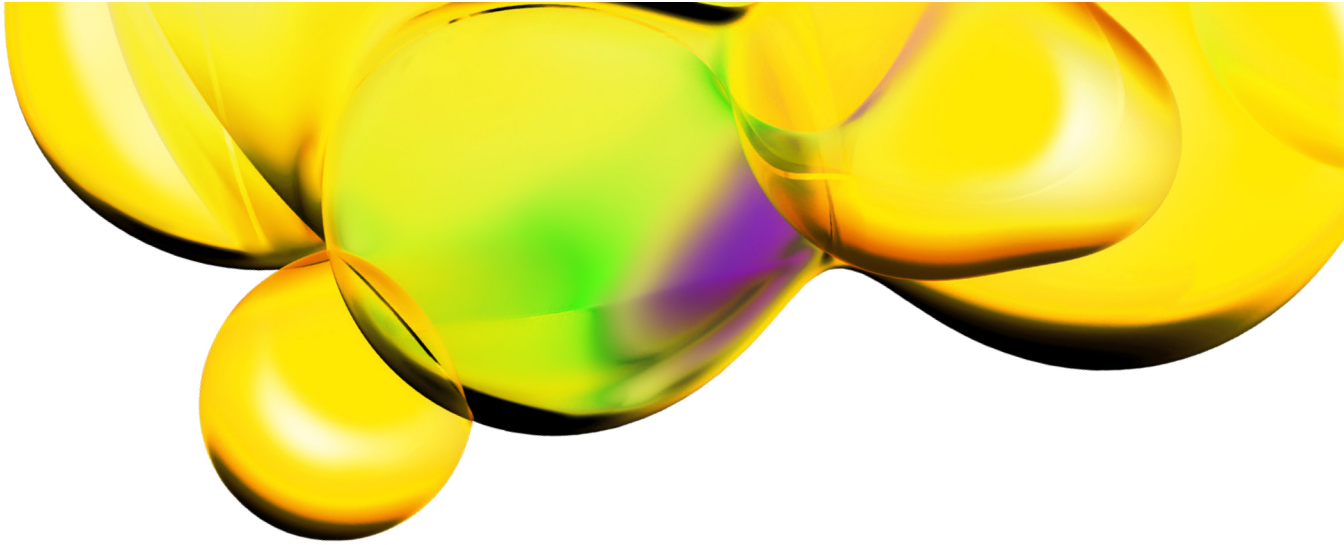
Figure 3. Human IgG fragments competitive bind to FCGR3A (176Val). Blue points showed human IgG whole molecule which was pre-incubated with anti-human CD16a antibody for 10 minutes at room temperature as a negative control. The IC_{50} values are 5.2 and 2.3 µg/mL for IgG Fc fragment and IgG whole molecule respectively and were calculated by using nonlinear regression fitting with GraphPad Prism 5.

Troubleshooting Guide

You will find below recommendations for common situations that you might encounter with your AlphaLISA detection assay. If further assistance is needed, do not hesitate to contact our technical support team for assistance.

Issue	Recommendations and Comments
High background signal	• Buffer is not freshly made. Make new. • Incubation time is longer than recommended range.
Low AlphaLISA signal	• Optimize EnVision with Plate format.
High variation between replicates or low Z' values	• Make sure that reagents are at the bottom of the well by tapping or swirling the plate gently on a smooth surface after each addition.

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



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