MANUAL

AlphaLISA® Human FCGR3B/CD16b Binding Kit

Product number: AL3094 C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the detection of binding between FCGR3B /CD16b 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: IC_{50} 1.87 µg/mL (average, with human IgG3)

Signal to background ratio: 2581 (average)

Kit contents: The kit contains 4 components: Human IgG Fc fragment conjugated Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human FCGR3B, and AlphaLISA HiBlock buffer.

Storage: Store kit in the dark at 4 ˚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. After reconstitution, store unused protein in -20 °C. Avoid multiple freeze/thaw cycles.

Product Information

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 Fc-Gamma Receptor 1 (CD64), FCGR1; Fc-Gamma Receptor 2 (CD32), FCGR2 and Fc-Gamma receptor 3 (CD16), FCGR3. FCGR3 is expressed as two distinct forms (FCGR3A and FCGR3B) encoded by two different highly homologous genes in a cell type specific manner.

FCGR3 is a low/intermediate affinity receptor for polyvalent immune-complexed IgG. FcGR3B binds complexed or aggregated IgG and also monomeric IgG. Contrary to FCGR3A, FCGR3B is not capable to mediate antibody-dependent cytotoxicity and phagocytosis. It may serve as a trap for immune complexes in the peripheral circulation which does not activate neutrophils.

Description of the AlphaLISA Assay

The AlphaLISA detection of FCGR3B and IgG Fc fragment binding uses IgG Fc AlphaLISA® acceptor beads to capture the human FCGR3B and Streptavidin-coated donor beads to capture the biotinylated human FCGR3B. Donor beads and acceptor beads come into proximity through IgG Fc fragment binding to FCGR3B. 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).

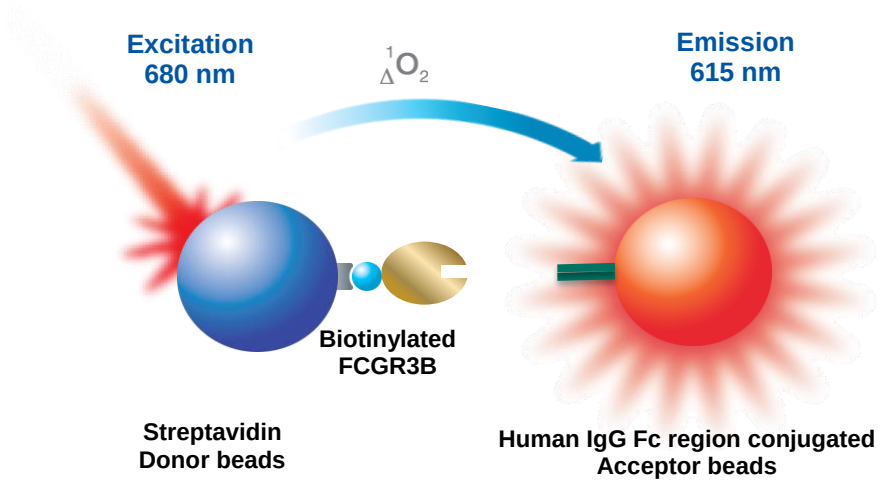


Figure 1. AlphaLISA assay principle.

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	AL3094C (500 assay points)**	AL3094F (5000 assay points)**
AlphaLISA Human IgG Fc fragment Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	40 µL @ 5 mg/mL (1 brown tube, white cap)	400 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	40 µL @ 5 mg/mL (1 brown tube, black cap)	400 µL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated human FCGR3B lyophilized solid***	0.6 µg (1 tube, clear cap)	10 x 0.6 µg (10 tubes, clear caps)
AlphaLISA HiBlock Buffer (10X)*	10 mL, 1 large bottle	100 mL, 1 large bottle

* Extra HiBlock 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 AreaPlate-96, white	Revvity Inc.	6005560 (50/box) 6005569 (200/box)
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.	6050185
EnSpire® or EnVision® Multilabel Alpha Reader	Revvity Inc.	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 CD16 antibody	Bio-Rad Laboratories	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® H2O 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. Alternatively, the assay plate may be centrifuged.

- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal™-A Plus Adhesive 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 FCGR3B with all human IgG subclasses or human IgG fragments.

The competition assay described below is an example for determining IC_{50} of human IgG subclasses competitive binding to human FCGR3B in 40 µL final assay volume (96 wells, duplicate determinations) by AlphaLISA technology. This manual can test 4 full curves of antibodies in 96 wells. If a different number of samples are tested, the total 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 covered can be modified.

1. Preparation of 1x HiBlock Buffer 1 (for 10 mL)
   Add 1 mL of 10X HiBlock Buffer and 9 mL of MilliQ water.

2. Preparation of serial dilution of human IgG subclasses
   Prepare serial dilutions of 4X IgG in 1x HiBlock buffer as follows. Change tips between each dilution:

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		60 µL	0	0

3. Preparation of 4X human FCGR3B (120 ng/mL)
   a. Spin the vial containing 0.6 µg lyophilized protein briefly in microfuge and reconstitute it with 100 µL Milli-Q water to make 6 µg/mL stock concentration of human FCGR3B. After reconstitution, aliquot and store unused protein at -20 °C for 3 months. Avoid multiple freeze/thaw cycles.

   b. Add the 20 µL of 6 µg/mL human FCGR3B into a new tube containing 980 µL 1X HiBlock Buffer to make 120 nM FCGR3B.

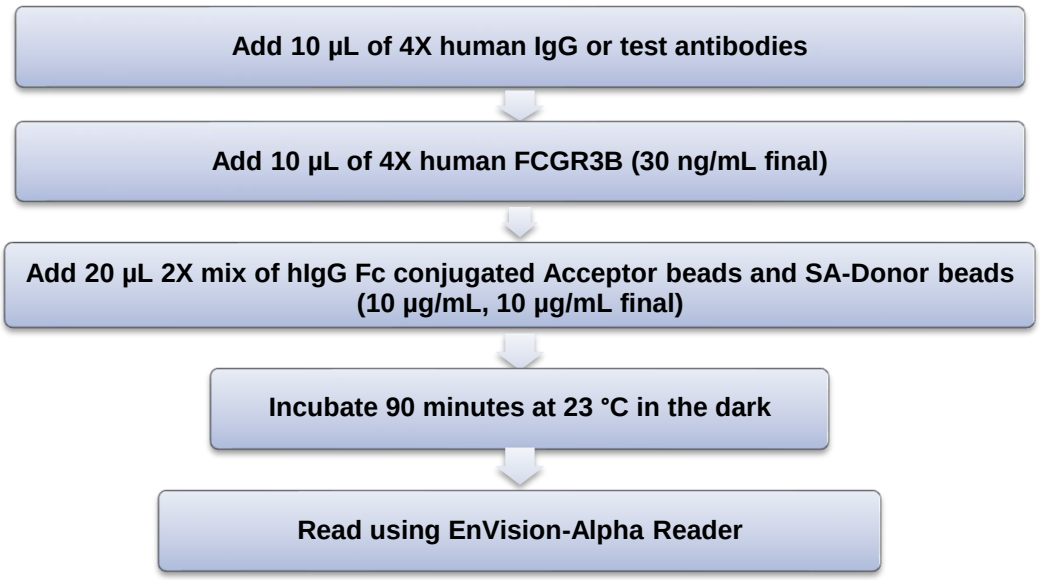
   c. Prepare just before use.

4. Preparation of 2X mix of human IgG Fc Conjugated Acceptor Beads (20 µg/mL) and Streptavidin (SA) Donor Beads (20 µg/mL).

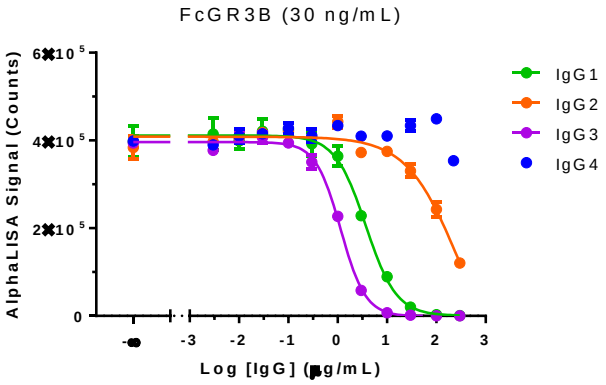
a. Add 8 µL of 5 mg/mL human IgG Fc conjugated Acceptor beads and 8 µL of 5 mg/mL SA-Donor beads into 1984 µL 1X HiBlock buffer.

b. Keep the beads under subdued laboratory lighting and prepare just before use.

5. In a ½ AreaPlate (96 wells):

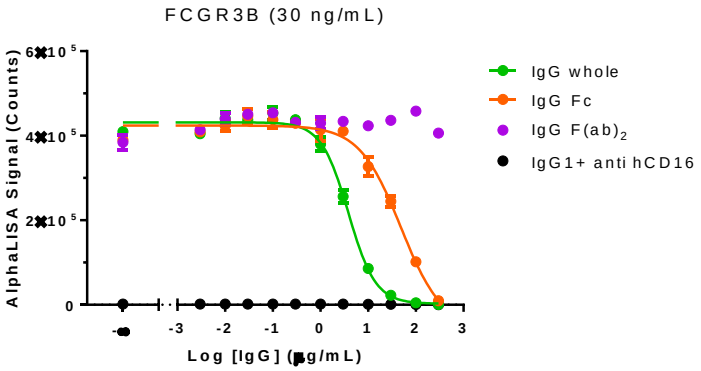


Typical competitive binding Data:



	IgG1	IgG2	IgG3
IC50	3.722	278.6	1.149

Figure 2. Human IgG subclasses competitive bind to FCGR3B. The IC_{50} values were 3.7, 278.6 and 1.15 µg/mL for IgG1, IgG2 and IgG3 respectively. The IC_{50} was not measurable for IgG4. All IC_{50} were calculated by using nonlinear regression fitting with GraphPad Prism 7. Each IgG subclass has gone through a zeba column (ThermoFisher, Cat. no. 89882) for a buffer exchange with PBS before testing to remove NaN_3. The concentrations of IgGs were measured with NanoDrop (E 1%=13.6).



	IgG whole	IgG Fc
IC50	3.862	48.44

Figure 3. Human IgG fragments competitive bind to FCGR3B. Black points showed human IgG whole molecule which was pre-incubated with anti-human CD16 antibody for 5 minutes at room temperature as a negative control. The IC_{50} values were 3.9 and 48.4 µg/mL for IgG whole molecule and IgG Fc fragment respectively and were calculated by using nonlinear regression fitting with GraphPad Prism 7. The IC_{50} was not measurable for IgG $F(ab)_2$.

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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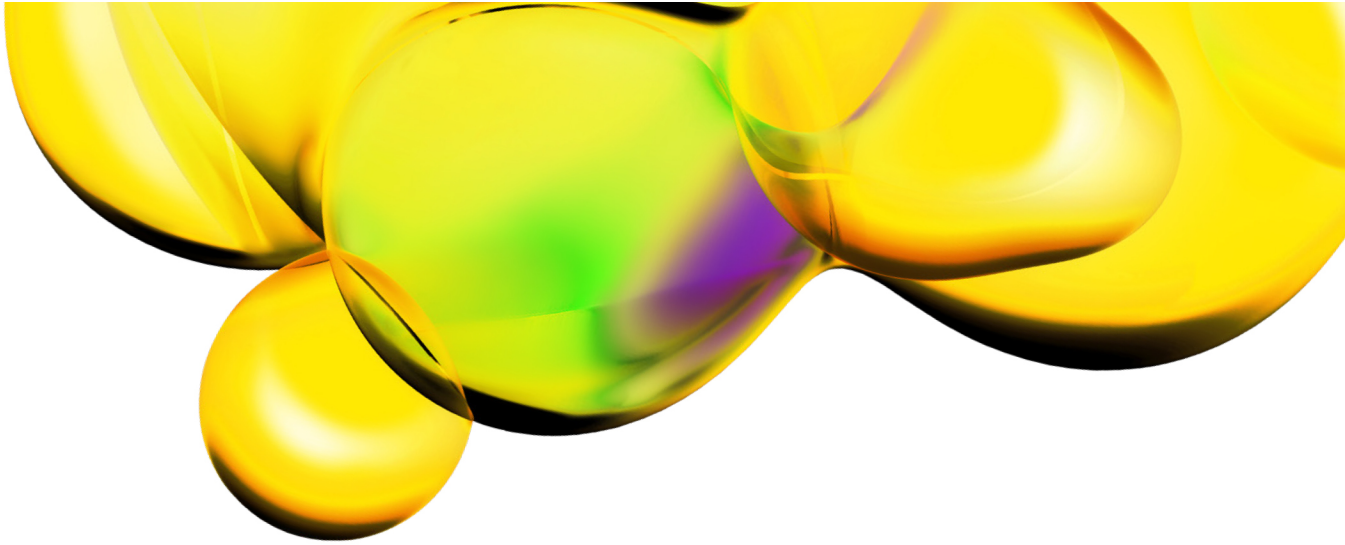

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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

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