MANUAL

AlphaLISA® Human FcRn Binding Kit

Product number: AL3095 C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for detection of the binding between FcRn and human IgG 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} 0.61 µg/mL (average, with IgG)

Signal to background ratio: 778 (average)

Kit contents: The kit contains 4 components: Human IgG conjugated Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human FcRn, and AlphaLISA MES 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.

Analyte of Interest

Human FcRn also known as Neonatal Fc receptor is a heterodimer of FCGRT (Fc Fragment of IgG Receptor and Transporter) and B2M (beta 2 microglobulin). Among several Fc receptors known to interact with IgG antibodies, FcRn plays a critical role in maintaining IgG homeostasis. After IgG binds to its target on the cell surface, it is pinocytozed to the endosome where FcRn binds to the antibodies at acidic pH (6.0) and recycles the antibodies back into circulation at physiological pH (7.4). The pH dependency of FcRn binding comes mostly from histidine residues His 310, His 435, and His 436 of IgG, which are protonated at pH 6 creating a strong interaction with the anionic pocket of FcRn; they are neutralized at pH 7.4 releasing IgG from FcRn. As a result, antibodies can be protected from lysosomal degradation, leading to enhanced in vivo stability and efficacy. Hence, profiling of FcRn binding is commonly required by regulatory agencies.

Description of the AlphaLISA Assay

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

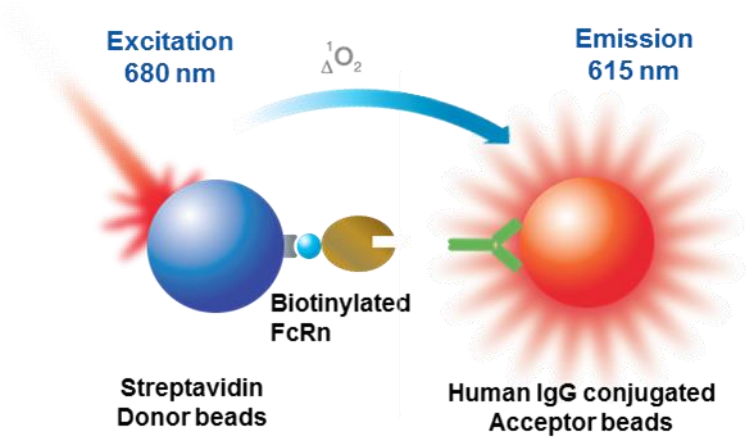


Figure 1. AlphaLISA assay principle.

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	AL3095C (500 assay points)**	AL3095F (5000 assay points)**
AlphaLISA Human IgG Acceptor beads stored in PBS, 0.05% ProClin-300, pH 7.4	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% ProClin-300, pH 7.4	80 µL @ 5 mg/mL (1 brown tube, black cap)	800 µL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated human FcRn lyophilized solid***	4 µg (1 tube, clear cap)	10 x 4 µg (10 tubes, clear caps)
AlphaLISA MES Buffer (5X)*	10 mL, 1 small bottle	100 mL, 1 large bottle

* Extra AlphaLISA MES Buffer (5X) can be ordered separately (cat # AL017 C: 10 mL, cat # AL017F: 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 Area OptiPlate-96, White	Revvity	6002290 (50/box) 6002299 (200/box)
TopSeal™-A Plus Adhesive Sealing Film	Revvity	6050185
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

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 H2O to dilute AlphaLISA MES Buffer (5X).

- 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 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 FcRn with 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 FcRn in 40 µL final assay volume (96 wells, duplicate determinations) by AlphaLISA technology. This manual can test 4 full curves of antibodies. 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 AlphaLISA MES Buffer (for 10 mL)
   Add 2 mL of AlphaLISA MES Buffer (5X) and 8 mL of Milli-Q water.

2. Preparation of serial dilution of human IgG subclasses
   Prepare serial dilutions of 4X IgG in 1x AlphaLISA MES 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

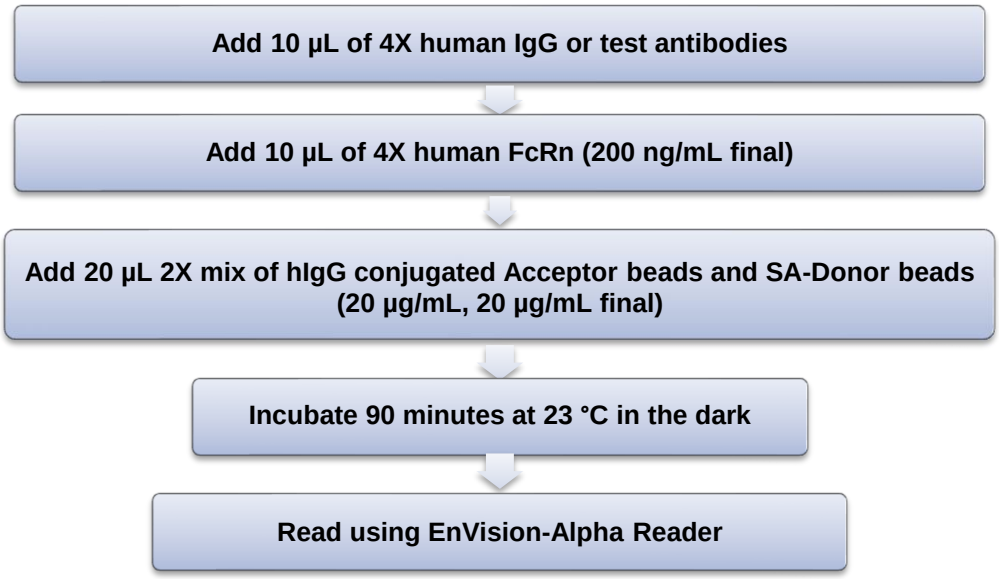
3. Preparation of 4X human FcRn (800 ng/mL)
   a. Spin the vial containing 4 µg lyophilized protein briefly in microfuge and reconstitute it with 100 µL Milli-Q water to make 40 µg/mL stock concentration of human FcRn.
   b. Add the 20 µL of 4 µg/mL human FcRn into a new tube containing 980 µL 1X AlphaLISA MES Buffer to make 800 nM FcRn. After reconstitution, aliquot and store unused protein at -20 °C for 3 months. Avoid multiple freeze/thaw cycles.
   c. Prepare just before use.

4. Preparation of 2X mix of human IgG Conjugated Acceptor Beads (40 µg/mL) and Streptavidin (SA) Donor Beads (40 µg/mL).

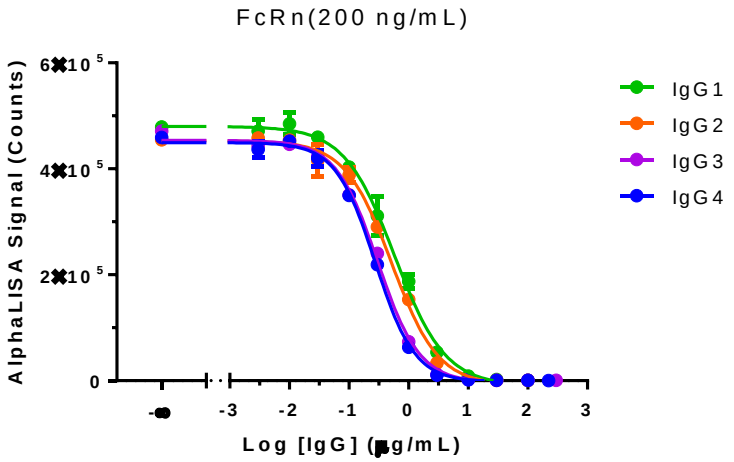
a. Add 16 µL of 5 mg/mL human IgG conjugated Acceptor beads and 16 µL of 5 mg/mL SA-Donor beads into 1978 µL 1X AlphaLISA MES buffer.

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

5. In a ½ AreaPlate (96 wells):

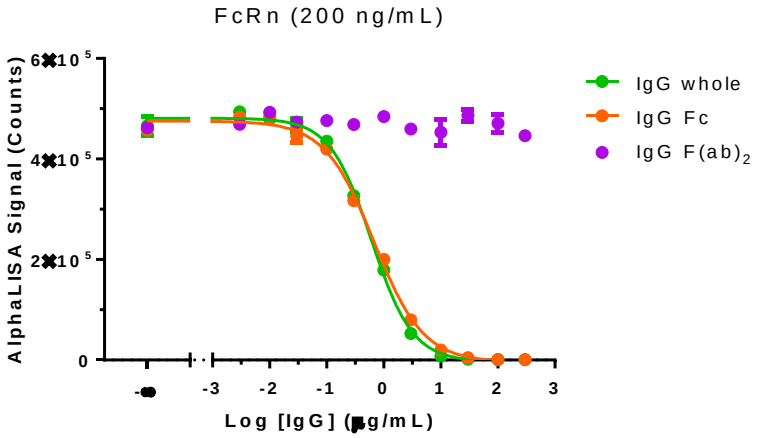


Typical competitive binding Data:



	IgG1	IgG2	IgG3	IgG4
IC50	0.5856	0.5192	0.3024	0.2758

Figure 2. Human IgG subclasses competitive bind to FcRn. The IC_{50} values were 0.6, 0.5, 0.3 and 0.3 µg/mL for IgG1, IgG2, IgG3 and IgG4 respectively. 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	0.6078	0.6758

Figure 3. Human IgG and IgG Fc fragment competitive bind to FcRn. The IC_{50} values were 0.6 and 0.7µg/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 $F(ab)_2$.

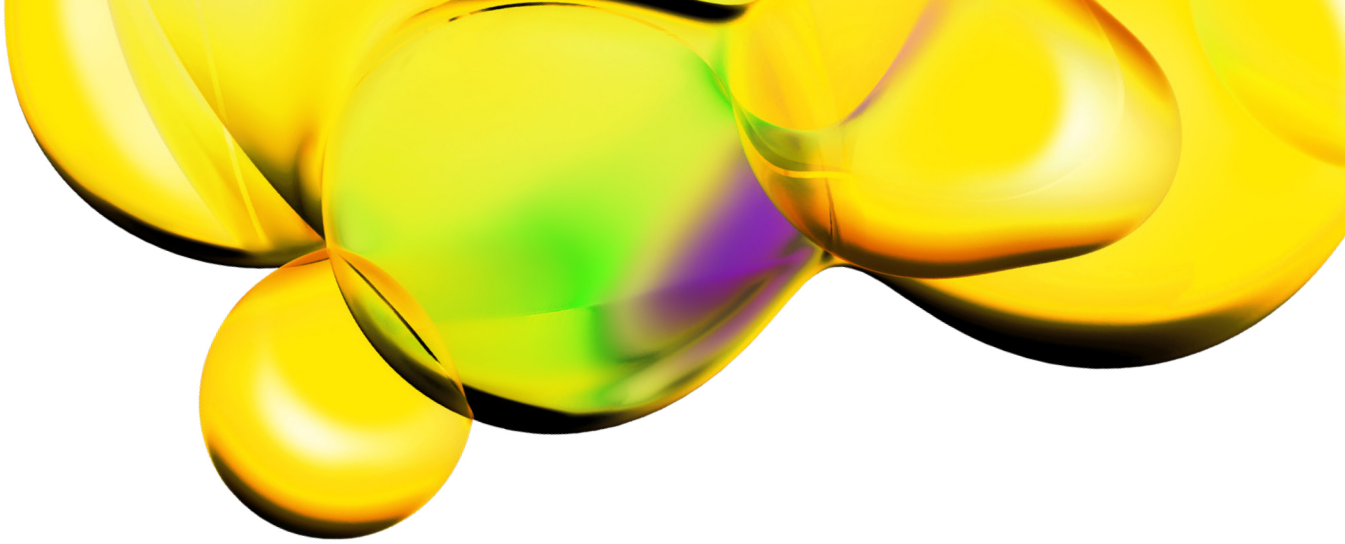
Troubleshooting Guide

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

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.



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