Fc-Receptor binding ADCC assays utilizing AlphaLISA technology: Characterization of hIgGs and FcγRIIIa.

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Introduction

Fc Receptors (FcRs) are cell-surface proteins found on a wide variety of cell types - including B lymphocytes, follicular dendritic cells, natural killer cells, macrophages, neutrophils, eosinophils, basophils, human platelets, and mast cells - and are involved in some of the actions of the adaptive immune system. These receptors take their name from the fact that they bind to the constant region, or tail, of an antibody (Figure 1), as opposed to the variable, or antigen binding, region. There are several different types of FcRs, which are classified based on the isotype of antibody that they recognize (e.g. IgE, IgG), and these classes may be further differentiated by the cell type(s) that express them and their downstream signaling mechanisms.
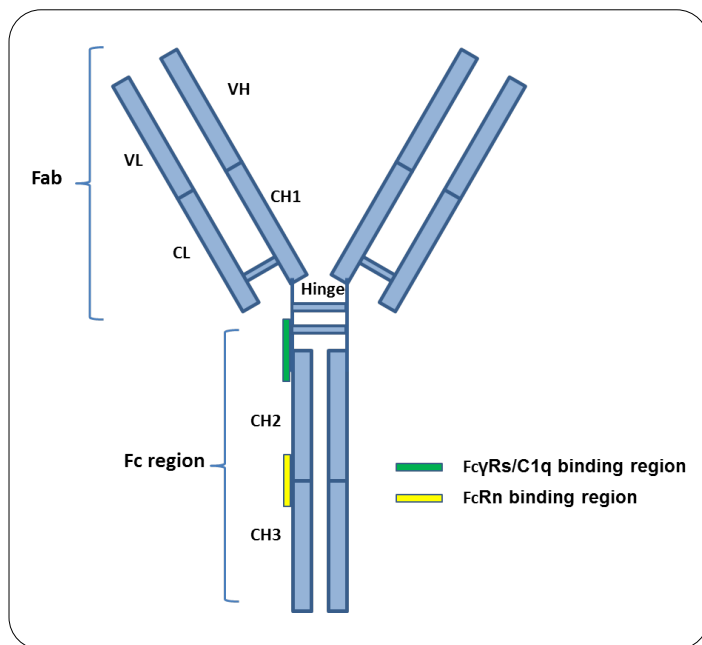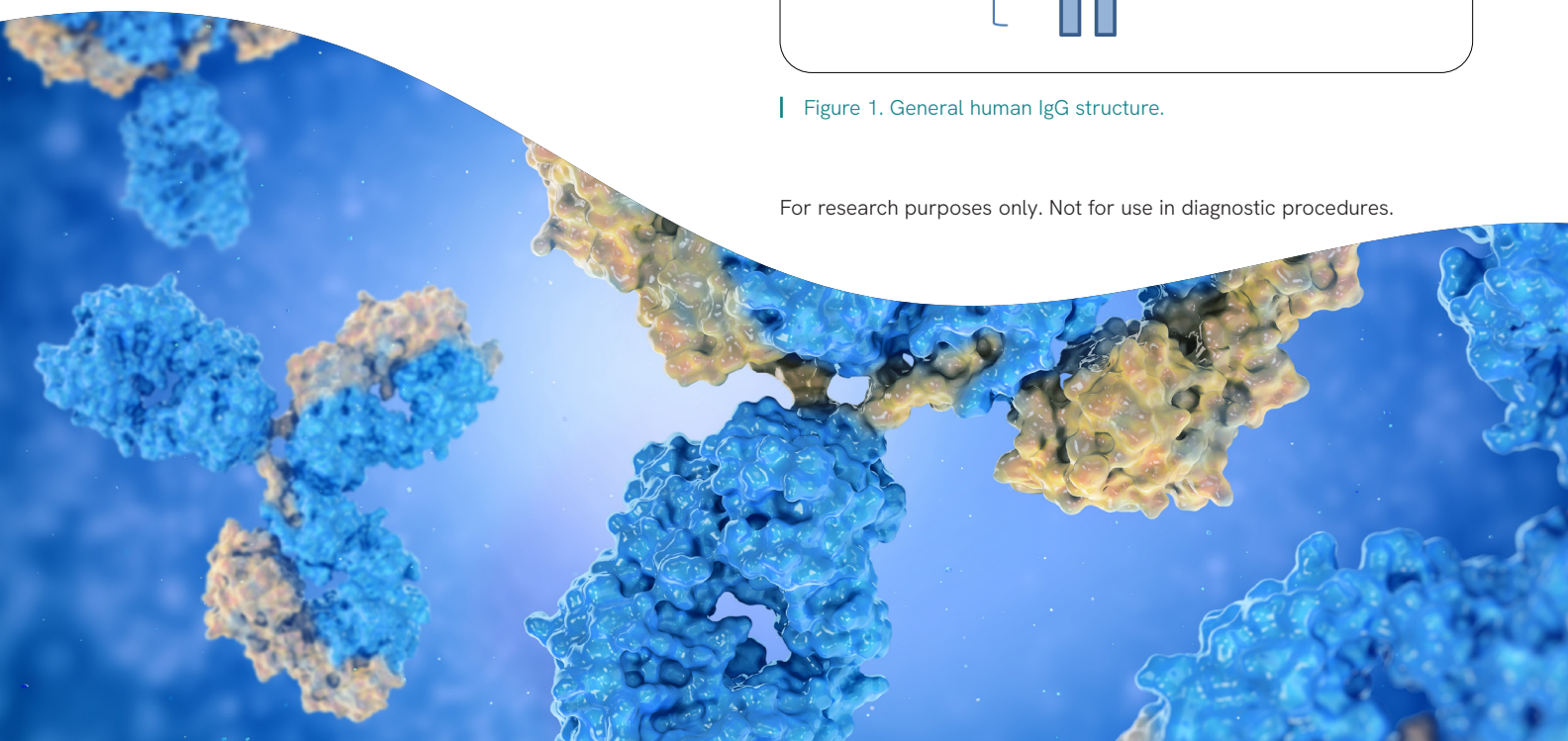

Figure 1. General human IgG structure.

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The Fc-Gamma Receptors (FcγRIIIa) are members of immunoglobulin superfamily and play a critical role in the function of therapeutic antibodies. FcγRIIIa are divided into three classes and FcγRIII (CD16) is expressed as two distinct forms (FcγRIIIa and FcγRIIIb) encoded by two different highly homologous genes in a cell type specific manner. FcγRIIIa 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. Therefore FcγRIIIa 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 FcγRIIIa and also can be used to study how other antibodies bind to FcγRIIIa by competition assay.

The AlphaLISA™ detection of FcγRIIIa and IgG Fc binding uses IgG Fc AlphaLISA Acceptor beads to capture the biotinylated human FcγRIIIa and Streptavidin-coated Donor beads to capture the biotinylated human FcγRIIIa. Donor beads and Acceptor beads come into proximity through IgG Fc fragment binding to FcγRIIIa. 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 2).
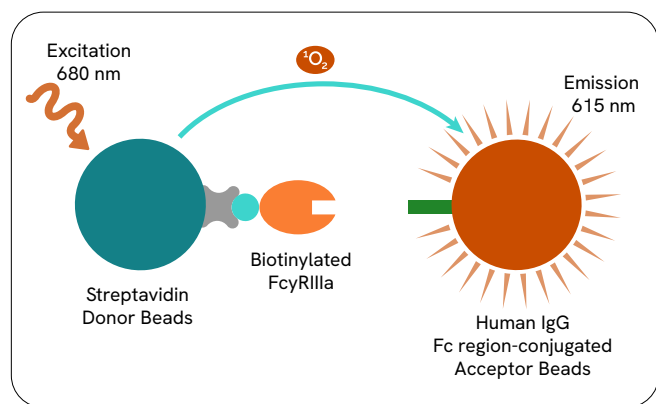

Figure 2. General Assay schematic used in both AlphaLISA FcγRIIIa assay kits.

Anti-CD20, also known as rituximab, was one of the first biotherapeutic antibodies approved. Its patent expired in 2015, and there are multiple biosimilars in development.

Some of these biosimilars have engineered Fc fragments to enhance the affinity of FcγRIIIa to bind to them in an effort to increase the therapeutics' ability to induce ADCC. Here we show how using these AlphaLISA kits allows for determining the affinity of Anti-CD20 with two different Fc regions to FcγRIIIa, and their utility in determining a binding profile for antibodies with enhanced or reduced binding to both isoforms of this receptor.

Materials and methods

AlphaLISA human FcγRIIIa/CD16a detection kits (AL347, 176Phe/F148 and AL348, 176Val/V158) were used according to the product's technical data sheet with the modification of using a 384-well white OptiPlate (Revvity 6007290), thereby reducing the assay volume to 20 μL (see Figure 3). The AlphaLISA human IgG4 Detection kit (Revvity AL310) was used according to the protocol on the technical data sheet.

The monoclonal antibodies (mAbs) tested were Anti-hCD20-hIgG1 (hcd20-mab1), Anti-hCD20-hIgG4 (S228P) (hcd20- mab14), Anti-β-Gal-hIgG1 (bgal-mab1), and Anti- β-Gal-hIgG4 (S228P) (bgal-mab14) (isotype controls) from InvivoGen.
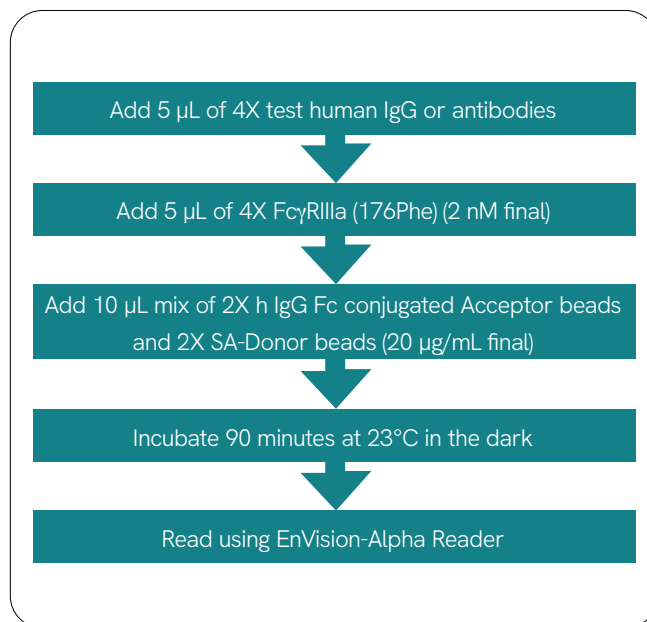

Figure 3. Assay protocol utilized in the AlphaLISA FcγRIIIa assay kits.

The antibodies were diluted in a 384-well V-bottom StorPlate (Revvity 6008590), according to the dilution protocol in Table 1.

Table 1. Dilution schema utilized for testing all four mAbs in the AlphaLISA FcγRIIIa assays.

	Volume of IgG	Volume of 1X buffer	[IgG] (g/mL) (4X)	[IgG] (g/mL) (1X)
A	1.0 mg/mL stock	0	1.00E-03	2.50E-04
B	30 µL of well A	60 µL	3.33E-04	8.33E-05
C	30 µL of well B	70 µL	1.00E-04	2.50E-05
D	30 µL of well C	60 µL	3.33E-05	8.33E-06
E	30 µL of well D	70 µL	1.00E-05	2.50E-06
F	30 µL of well E	60 µL	3.33E-06	8.33E-07
G	30 µL of well F	70 µL	1.00E-06	2.50E-07
H	30 µL of well G	60 µL	3.33E-07	8.33E-08
I	30 µL of well H	70 µL	1.00E-07	2.50E-08
J	30 µL of well I	60 µL	3.33E-08	8.33E-09
K	30 µL of well J	70 µL	1.00E-08	2.50E-09
L	30 µL of well K	60 µL	3.33E-09	8.33E-10
M	30 µL of well L	70 µL	1.00E-09	2.50E-10
N	30 µL of well M	60 µL	3.33E-10	8.33E-11
O	30 µL of well N	70 µL	1.00E-10	2.50E-11
P	30 µL of well O	60 µL	3.33E-11	8.33E-12

After the 90 minute incubation the plate was then read on an EnVision™ Multimode Plate Reader using standard Alpha settings (Figure 4). The EnVision also incorporates unique temperature control for sensitive Alpha and AlphaPlex assays, ensuring that results are both reproducible and accurate. In addition to fast, sensitive Alpha technology detection, the EnVision Multilabel microplate reader provides fluorescence intensity, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence detection technologies.

Figure 4. The EnVision multilabel plate reader.

Results

Four different monoclonal antibodies were characterized in the three different AlphaLISA assays utilized; every possible combination of Fc and Fab was represented among the chosen mAbs. As expected the antibodies of the same subtypes had similar affinities for the FcγRIIIa, regardless of their target. The data in Figures 5 and 6 show the binding affinity of the respective mAbs for each of the isoforms of FcγRIIIa. The IC50s determined were 4.9 and 5.2 µg/mL for the two IgG1 subtype antibodies, and 37 and 34 µg/mL for the two IgG4 subtype antibodies in the AL347 kit (low binding 176Phe/F158 isoform), while they were 3.7 and 2.2 µg/mL for the IgG1s and 136 and 126 µg/mL for the IgG4s in the AL348 kit (high binding 176Val/V158 isoform). Data shown are the average of two replicates, and were plotted using a four-parameter logistic fit with variable slope in GraphPad Prism.
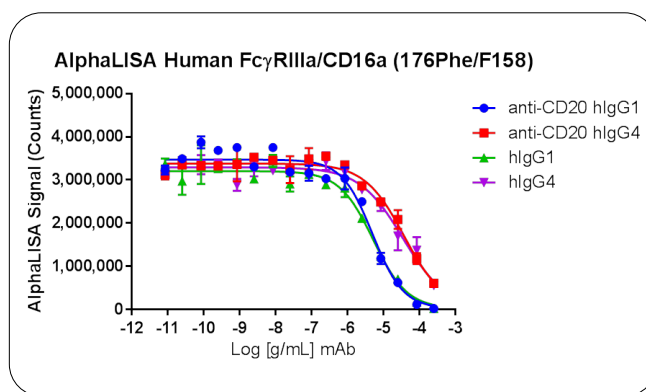

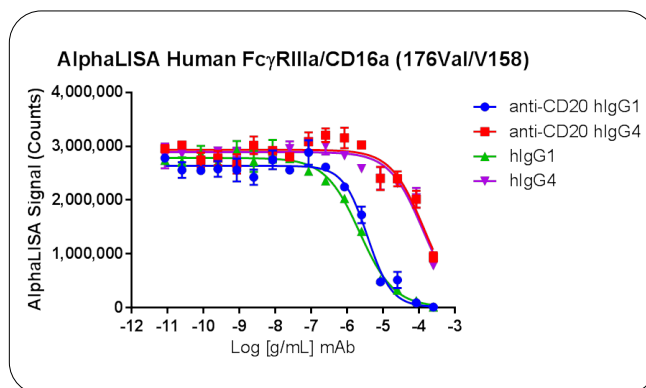Figure 5. Competition curves generated utilizing the AL347 assay kit.

Figure 6. Competition curves generated utilizing the AL348 assay kit.

To further confirm that the observed data are based solely on the identity of the constant portion of the antibody and not the variable region, one of the subtyping kits, the AlphaLISA IgG4 kit, was used. Here only the antibodies of subtype IgG4 should show any response, while those of IgG1 should not

show a response. Indeed the data shown in Figure 7 show that both IgG4 subtype antibodies showed a response that was roughly twenty times above the background signal of 3200 counts (a value corresponding to 2 ng/mL in the assay), the level of response that the IgG1 subtype antibodies showed in the assay, based on the standard curve shown in Figure 8.
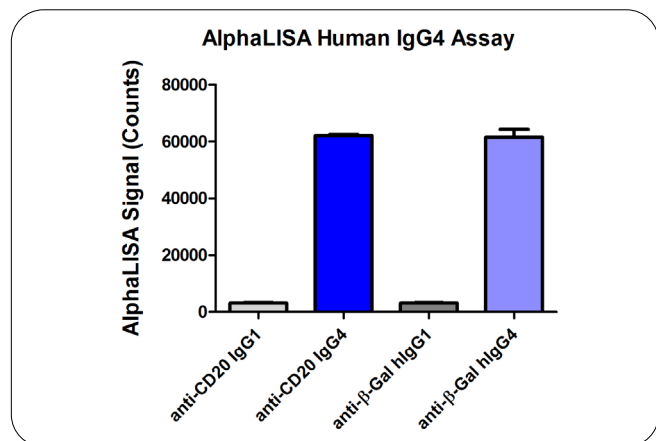

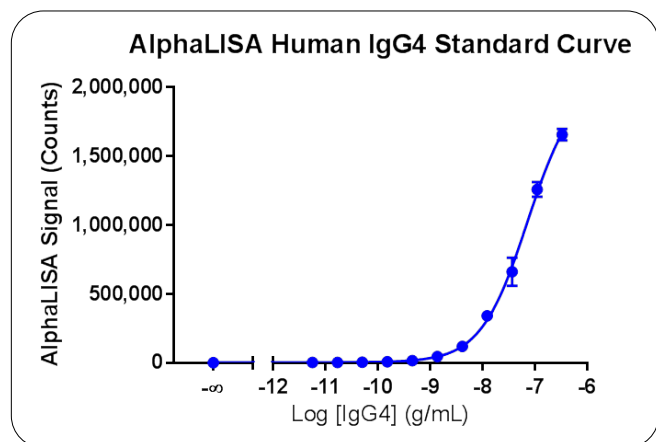Figure 7. Relative signal intensity for IgG4 detection.

Figure 8. Standard curve of Human IgG4 in the Human IgG4 subtyping kit.

Conclusions

Different FcγRIIIa have different affinities for the different subtypes of IgG molecules. The present study used antibodies of two different subtypes, both with variable regions that were targeted against two very different proteins, one a cell-surface receptor and one an intracellular enzyme. The affinities of the FcγRIIIa for both antibodies of the same subtype were comparable, and allowed for grouping of the mAbs by subtype, regardless of the target of the particular mAb. This was further confirmed using the subtyping assay, as only the IgG4 subtype antibodies and not the IgG1 subtype ones gave a signal in the IgG4 subtyping kit. These assays demonstrate the ease and utility of utilizing AlphaLISA, a homogeneous assay format, for determining binding profiles of therapeutic mAbs to FcγRIIIa. Due to its no-wash format, it allows for determination of low-affinity interactions, e.g. when engineering a mAb to have no ADCC activation one can pick up lower levels of affinity for an FcγR than with other assay technologies.