TECHNICAL NOTE

Fast, simple, homogeneous assays for characterization of Fc receptor binding using AlphaLISA.

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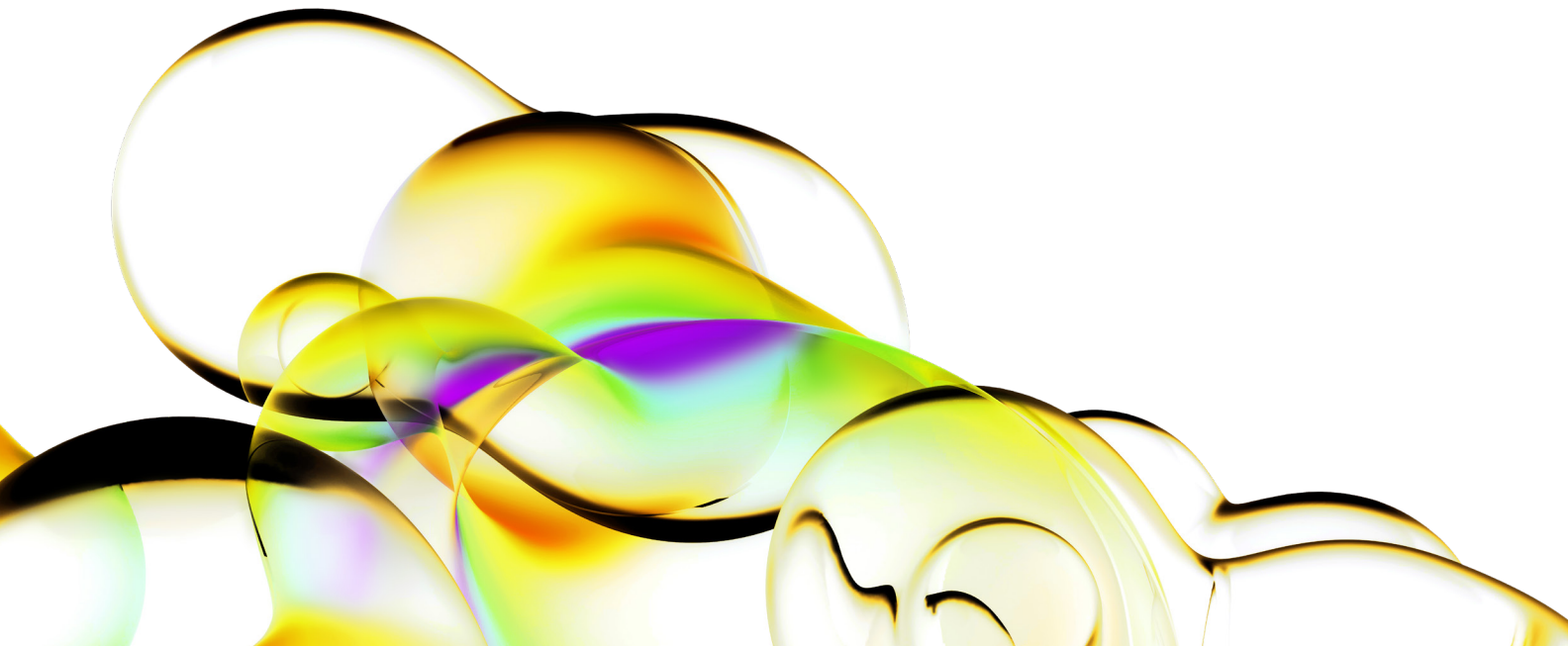
Introduction

In recent years, therapeutic antibodies have proven to be effective and safe treatments for various diseases. Understanding the binding of therapeutic antibodies to Fc receptors provides insights into their mechanism of action (MOA), efficacy, and safety. The interactions of therapeutic antibodies with fragment crystallizable gamma receptors (FcGRs) and neonatal Fc receptors (FcRn) can be measured *in vitro* as indicators of antibody functional performance. During early drug development, as potential therapeutic antibodies are selected and engineered, changes in Fc receptor affinity can be indicative of changes in effector function and serum half-life. When measured in scaled-up production, binding of therapeutic antibodies to Fc receptors can serve as a metric for quality control (QC).

FcGRs are members of the immunoglobulin superfamily and play a critical role in the function of therapeutic antibodies. FcGRs are divided into three classes: FcGR1, FcGR2, and FcGR3. Both FcGR2 and FcGR3 can be divided into two distinct forms (FcGR2A /FcGR2B and FcGR3A /FcGR3B) encoded by two different highly homologous genes in a cell type specific manner.



FcGRs have been categorized based upon their affinity for specific IgG subclasses and whether their binding initiates activating or inhibitory signals. Following binding of antibody to target, the subsequent binding of the FcGRs is a crucial step for the initiation and control of cell-mediated effector functions of the immune system. Each of the different subclasses of IgG (IgG1, IgG2, IgG3, and IgG4) presents different effector function profiles based upon their differential binding properties to each FcGR. While FcGR1 is capable of binding IgG with high affinity, both FcGR2 and FcGR3 have the capacity to bind IgG with a low/intermediate affinity. FcGR2A and FcGR3A deliver an activating signal upon ligand binding. In contrast, FcGR2B delivers an inhibitory signal and FcGR3B is not capable of mediating antibody-dependent cytotoxicity and phagocytosis.

Among FcGRs known to interact with IgG antibodies, FcRn plays a critical role in maintaining IgG homeostasis. FcRn binds to antibodies at acidic pH (6.0) and releases the antibodies back into circulation at physiological pH (7.4). 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.

AlphaLISA™ biochemical Fc receptor binding assays provide easy, robust assays to measure binding of potential therapeutic antibodies to human Fc receptor proteins. We offer a portfolio of AlphaLISA Fc receptor binding kits for therapeutic antibody development and production. These chemiluminescent, no-wash assays facilitate the design and development of antibody therapeutics. In the AlphaLISA binding assay, streptavidin-coated Donor beads are used to capture various biotinylated human Fc receptors. AlphaLISA Acceptor beads conjugated to human Fc-fragment or whole human IgG compete for binding with the therapeutic antibody to the biotinylated Fc receptor. In the absence of therapeutic antibody binding, Donor and Acceptor beads come into proximity. Excitation of the Donor beads at 680 nM provokes the release of singlet oxygen, which diffuses to the Acceptor bead and triggers a cascade of energy transfer reactions in the bead, resulting in a sharp peak of light emission at 615 nM. In the presence of therapeutic antibody binding to the biotinylated Fc receptor, the interaction between the Donor and Acceptor beads is disrupted. Binding of therapeutic antibody to Fc receptor is inversely proportional to the amount of light generated (Figure 1).

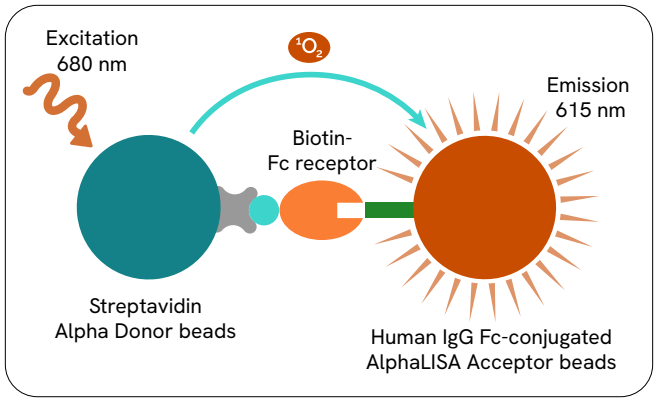


Figure 1: Assay principle for a biochemical AlphaLISA Fc receptor binding kit.

Materials and methods

AlphaLISA Fc receptor binding kits (FcGR1 (CD64), #AL3081; FcGR2A (167H) (CD32a), #AL3086; FcGR2A (167R) (CD32a), #AL3087; FcGR2B (CD32b), #AL3080; FcGR3A (176Phe/F158) (CD16a), #AL347; FcGR3A (176Val/V158) (CD16a), #AL348; FcGR3B (CD16b), #AL3094; FcRn, #AL3095) were supplied by Revvity. Antibodies and suppliers are indicated in Table 1. Buffer exchange with PBS (Gibco, #10010) was performed for each IgG subclass using Zeba™ spin columns (ThermoFisher, #89882) to remove NaN_3. All assays were performed as instructed in the protocol in each kit's technical data sheet, in white 96-well ½ AreaPlates™ (Revvity, #6005560). A general assay workflow is shown in Figure 2. All FcGR binding assays were run in AlphaLISA HiBlock Buffer while the FcRn binding assay was run in AlphaLISA MES Buffer (the buffer supplied in each kit). Plates were measured using the EnVision™ multimode plate reader from Revvity. IC_{50} values were calculated by using nonlinear regression fitting with GraphPad Prism® 7.

Table 1: Antibodies and suppliers.

Antibody	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	Jackson ImmunoResearch	009-000-006
ChromPure Human IgG Fc Fragment	Jackson ImmunoResearch	009-000-008
ChromPure Human IgG, whole molecule	Jackson ImmunoResearch	009-000-003
Anti-human CD16 antibody	Bio-Rad Laboratories	MCA1193GA

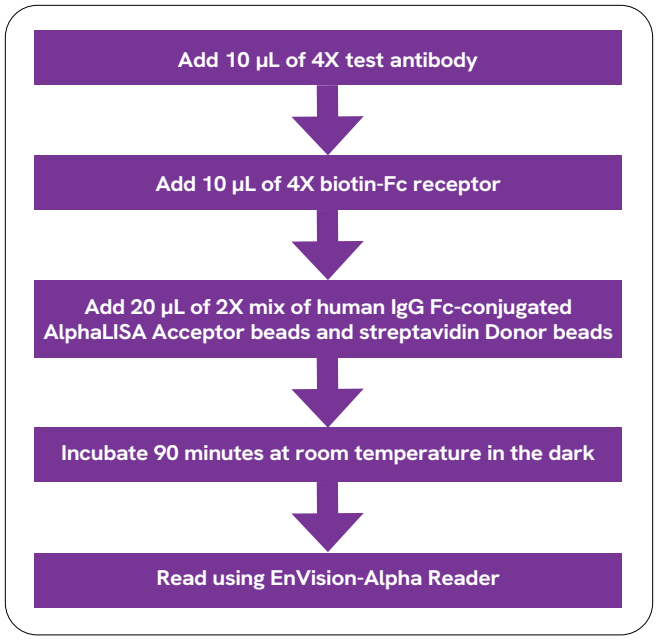


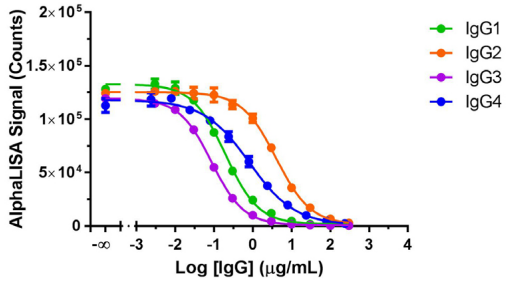
Figure 2: General AlphaLISA Fc receptor binding assay protocol.

Results

Competitive binding of IgG1, IgG2, IgG3, and IgG4 antibodies

For each AlphaLISA Fc receptor binding kit, competition curves were generated by titration of four human IgG antibodies. In these experiments, all antibodies were prepared in 1X AlphaLISA Buffer. The IC_{50} for each antibody was calculated for each Fc receptor. As indicated in the data, binding affinities were both antibody- and Fc receptor-dependent.

A



	IgG1	IgG2	IgG3	IgG4
IC_{50}	0.1963	4.036	0.08653	0.8150

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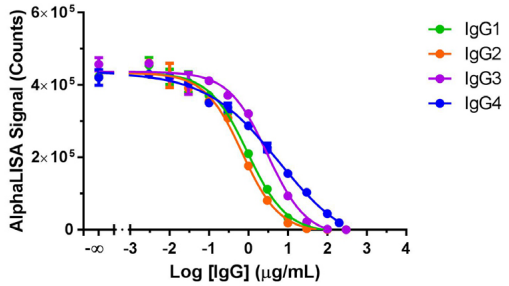

	IgG1	IgG2	IgG3	IgG4
IC_{50}	0.9582	0.6913	3.027	5.813

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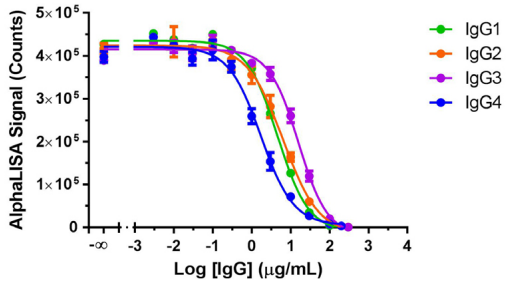

	IgG1	IgG2	IgG3	IgG4
IC_{50}	4.605	6.388	15.77	1.726

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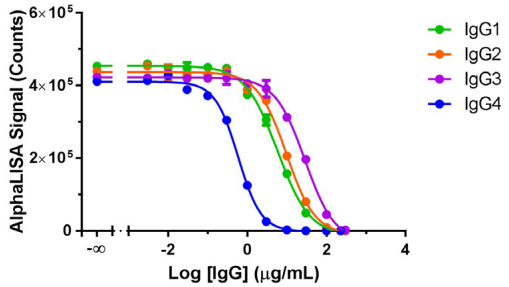

	IgG1	IgG2	IgG3	IgG4
IC_{50}	5.760	9.629	28.49	0.5814

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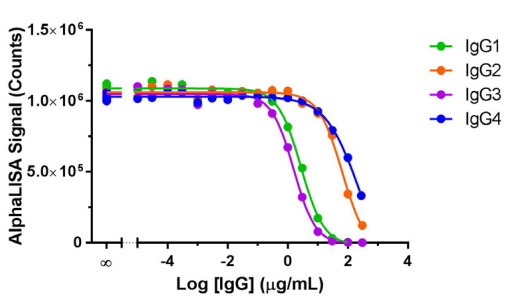

	IgG1	IgG2	IgG3	IgG4
IC_{50}	2.757	65.09	1.571	221.1

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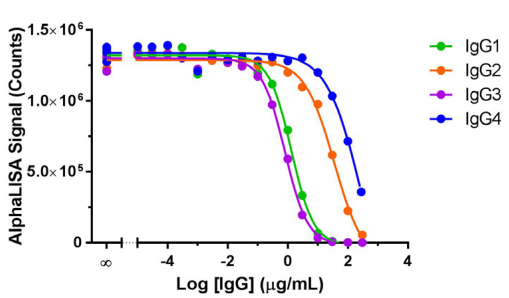

	IgG1	IgG2	IgG3	IgG4
IC_{50}	1.317	35.04	0.8060	283.7

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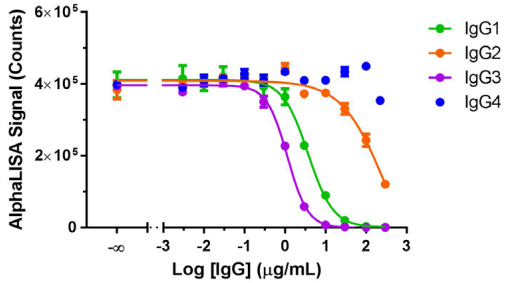

	IgG1	IgG2	IgG3
IC_{50}	3.722	278.6	1.149

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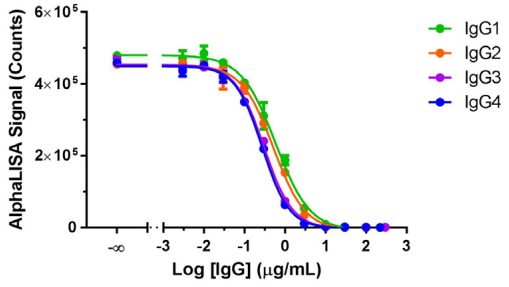
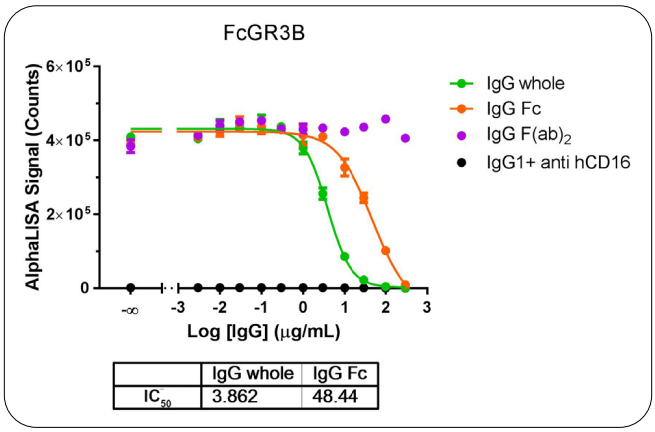


	IgG1	IgG2	IgG3	IgG4
IC_{50}	0.5856	0.5192	0.3024	0.2758

Figure 3: Competitive binding of antibodies to Fc receptors using each AlphaLISA Fc receptor binding kit. A) FcGR1, B) FcGR2A (167H), C) FcGR2A (167R), D) FcGR2B, E) FcGR3A (176Phe/F158), F) FcGR3A (176Val/V158), G) FcGR3B, H) FcRn. IC_{50}s are indicated in units of µg/mL.

Binding specificity

To verify specificity of the assay for Fc receptor binding proteins, titration curves were also performed with human IgG F(ab')2 fragment. Sample results are shown in Figure 4. As seen in the results, whole IgG and Fc-fragment could compete with binding to biotinylated Fc receptor.
As expected, F(ab')2 fragment could not compete for binding as it lacks an Fc region. This provides further confirmation of the specificity of the AlphaLISA Fc receptor binding assay.



	IgG whole	IgG Fc
IC_{50}	3.862	48.44

Figure 4: Human IgG antibody fragment competition assay using the AlphaLISA FcGR3B assay kit.

Conclusions

AlphaLISA Fc receptor binding kits enable fast, simple measurement of Fc receptor binding with no wash steps and no separation steps. These assays are specific, robust, and highly sensitive for reliable results. Revvity provides a full portfolio of Fc receptor binding kits for biotherapeutic antibody development and large scale manufacturing QC.

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