APPLICATION NOTE

# Biochemical binding ADCC assays utilizing AlphaLISA toolbox reagents for the characterization of hIgGs and FcγR1A.


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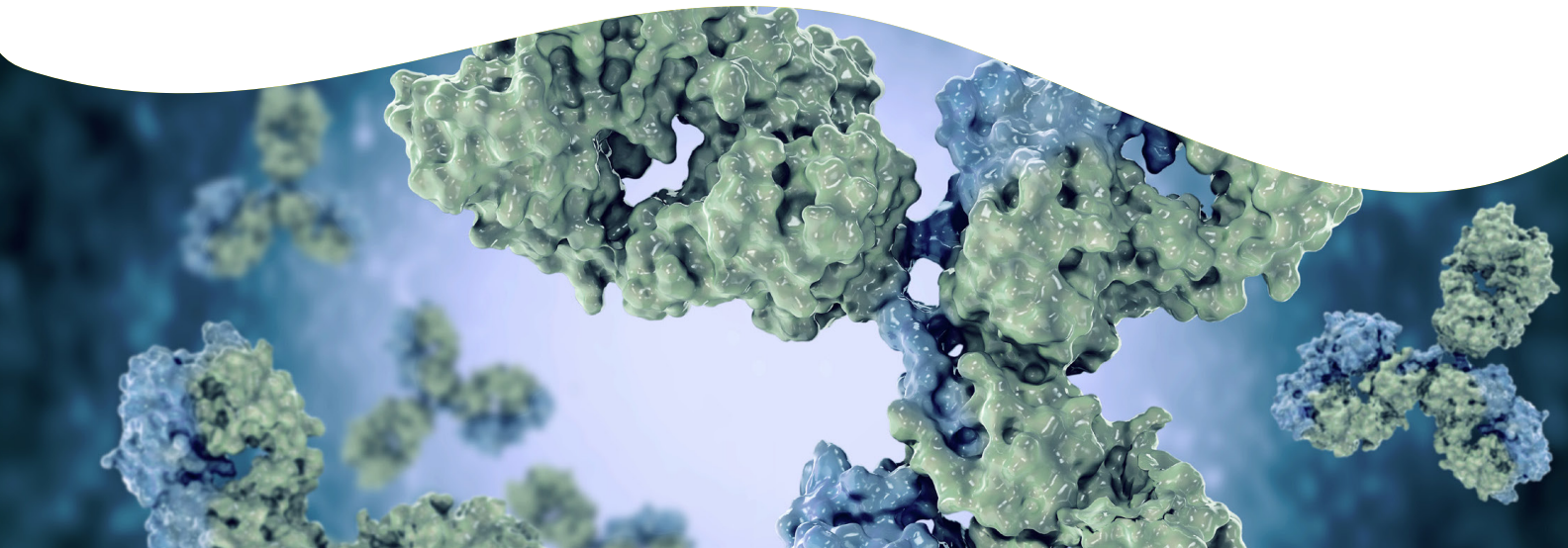
## Introduction

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.

Fc Gamma Receptors (FcγRs) are members of the immunoglobulin superfamily and play a critical role in the function of therapeutic antibodies. There are many effector functions that are FcγR-mediated, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), that will be the primary mechanism of action (MOA) of a given therapeutic. It is also important to know what other mechanism(s), or lack thereof, that a given therapeutic will activate (data which may be used to try to mitigate unwanted side effects of a given therapeutic). FcγRs are the largest family of Fc receptors in humans, comprising six members with differing affinities for the different IgG isotypes. FcRn (the neonatal Fc receptor), which also specifically binds IgGs, is from a different family, binds to a slightly different portion of the Fc region of IgGs, and is involved in pre- and post-natal antibody transfer and protection of IgGs from degradation (Figure 1).





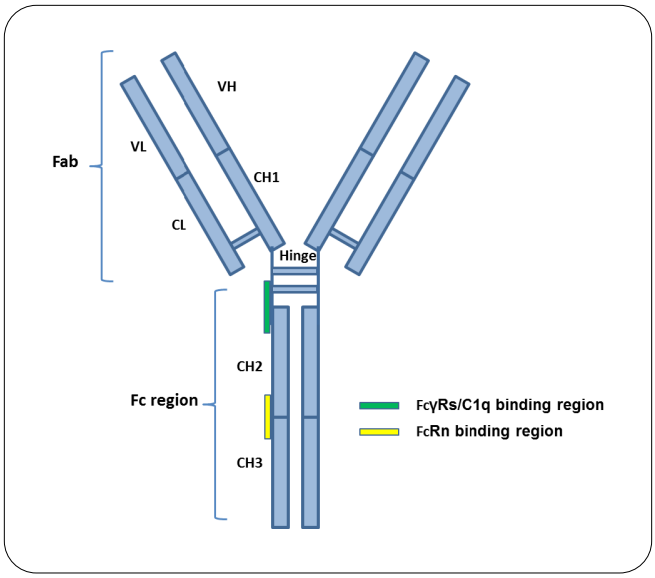



Figure 1: General structure of a Human IgG antibody. N.B. FcγRs bind closely to the hinge region whereas FcRn binds closer to the tail of the Fc region.

FcγRs have different affinities for the different subtypes of IgG molecules, due in part to the fact that they bind the antibody molecules in close proximity to the hinge region, unlike FcRn, and that the structure of the hinges is slightly different among all four subtypes of IgGs (Figure 2). As such, one goal for therapeutic antibody engineering programs may be to increase the affinity for certain FcγRs (e.g. IIIa, or CD16a), while concurrently decreasing the affinity for another (e.g. IIb, or CD32b) (Lazar et al.). There is a need to have robust, transferable assays to determine the binding affinity for a particular therapeutic antibody to all of the FcγRs, to generate a 'characterization binding profile' that can be used to help determine a therapeutic antibody's MOA and potential off-target effects. One example might involve the development of a therapeutic antibody that is solely desired to have as its MOA the ability to inhibit an interaction and not to activate ADCC or CDC.

For this application we chose to utilize AlphaLISA™ technology, an HTS-amenable assay format for interrogating protein: protein interactions. A competition assay format was chosen, wherein a characterization binding assay utilizing a poly-His tagged FcγR, captured by a Nickel-Chelate AlphaLISA Acceptor bead binds to biotinylated Human

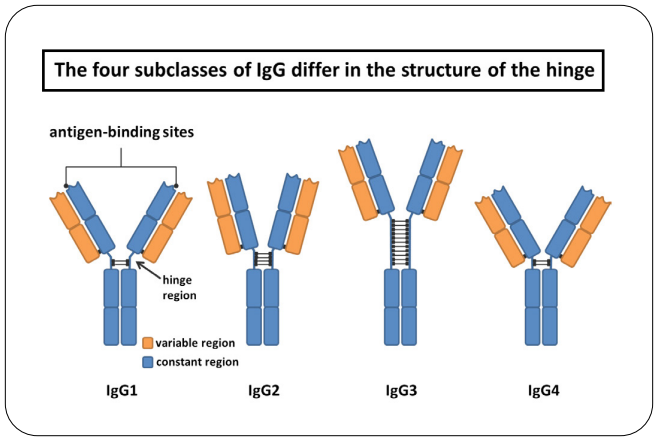



Figure 2: The differences in structures for the four subtypes of Human IgGs are shown. The differences in the hinge regions among the four different subtypes help to explain the differences in binding affinity that the subtypes show for the different FcγRs.

IgGs, captured by a Streptavidin Donor bead (Figure 3A). Untagged antibody is used as an inhibitor of this protein: protein interaction between the chosen FcγRI (CD64) and the biotinylated IgGs. For each subtype of human IgG, an $IC_{50}$ was determined. If the antibodies used as inhibitors have native, non-mutated Fc chains, this assay could also be used to distinguish among some of the different subtypes, as depending on the affinity of the receptor/subtype pairing the $IC_{50}$s determined should be relatively consistent (i.e. in the case of FcγRIIIa/CD16a IgG1 and IgG3 have a higher, almost indistinguishable affinity than IgG2, and IgG4 is the weakest binder of them all).

Alternative assay configurations that could have been chosen, using the same proteins but different AlphaLISA toolbox reagents, are shown in Figure 3B and 3C. For many affinity tags there are multiple toolbox reagents that could be used, and it's generally a good idea to test all possible assay configurations. Also some assay configurations may be inappropriate for certain assay conditions, e.g. Nickel-Chelate beads should not be used in an assay where EDTA or other chelators are necessary, or protein preparations aren't cleared of eluants such as imidazole, as these will interfere with the poly-His: Nickel interaction.

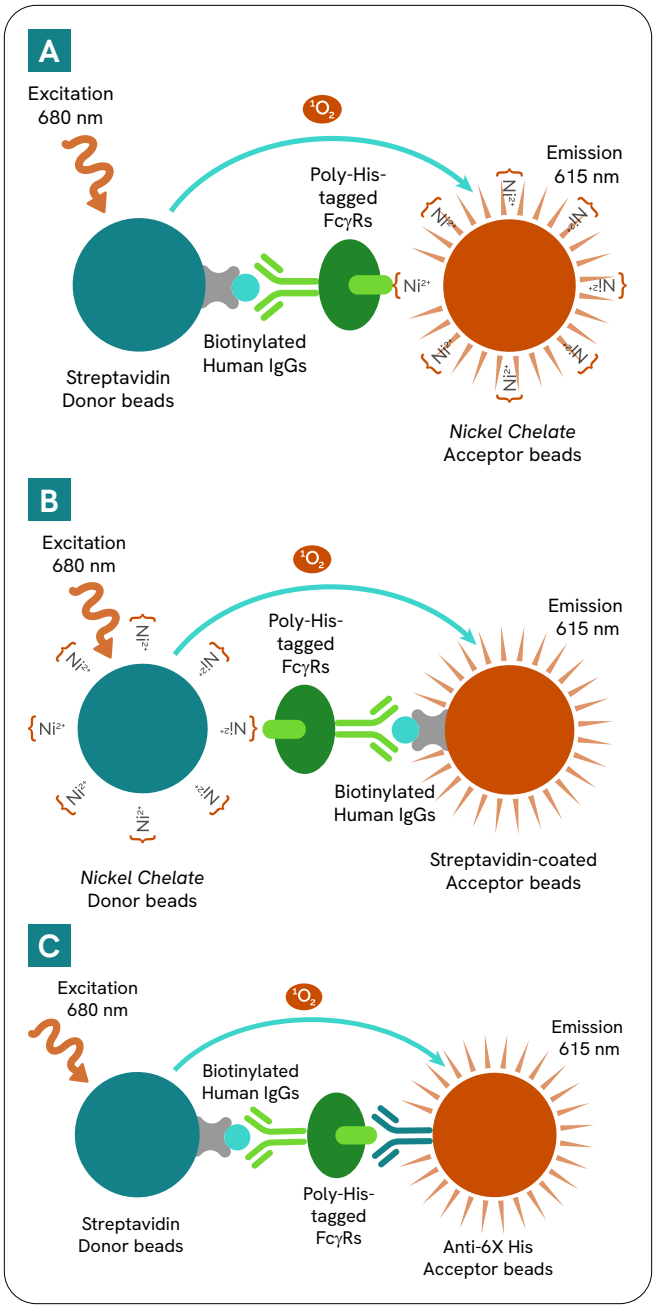



Figure 3: Assay schematics of the AlphaLISA Toolbox binding assay configurations possible to be developed between FcγRI and biotinylated Human IgGs. "A" shows the configuration used here- the poly-histidine tag of the FcγRI protein is captured by the Nickel-Chelate Acceptor beads, and the biotin tag on the Human IgGs is captured by the Streptavidin Donor beads. For the inhibition assay, unlabeled therapeutic IgG is used as a competitor of the binding of FcγRI and biotinylated IgG. "B" and "C" show two other possible assay configurations using toolbox beads. In "B" the affinity tags are captured on opposite beads from "A", whereas in C the same beads capture the same affinity tag, but instead of a nickel-chelate Acceptor bead there's now an anti-Poly-His antibody-conjugated Acceptor bead.

## Materials and methods

AlphaLISA toolbox reagents, Streptavidin Donor beads (6760002), Nickel-Chelate Acceptor beads (AL108C), and OptiPlates (6007290) were from Revvity. Human IgGs, Human Plasma (16-16-090707), and subtypes Human IgG1 (16-16-090707-1), Human IgG2 (16-16-090707-2), Human IgG3 (16-16-090707-3) and Human IgG4 (16-16-090707-4), were from Athens Research Technology. FcγRI, poly-His tagged (1257-FC-050) was from R&D Systems, and ChromaLink™ One-Shot Antibody Biotinylation Kit (B-9007-009K) was from Solulink™.

The ChromaLink biotin labeling of the Human IgGs was performed according to the manufacturer's directions, with a 30:1 labeling ratio of biotin reagent to immunoglobulin. The molar substitution ratio (MSR) for biotinylation of the IgGs was determined using Solulink's™ online calculator.

The buffer utilized in the assay was 50 mM Hepes, pH 7.3 (Affymetrix #16925), 100 mM Sodium Chloride (Sigma #S5150), 0.1% Triton X-100 (Sigma #93443) and 0.1% Bovine Serum Albumin (BSA) (Jackson ImmunoResearch Laboratories, Inc. #001-000-162). The BSA was added fresh the day of the experiment.

The binding assay for FcγRI to biotinylated hIgGs was developed in a 384-well OptiPlate by adding 4 µL of buffer or inhibitor (hIgG subtypes), 4 µL of biotinylated IgGs, and 4 µL of FcγRI, all at 5X final concentration, and allowing them to incubate at 23 °C for one hour. Then 4 µL of Nickel-Chelate Acceptor beads (20 µg/ mL final concentration) were added and allowed to incubate for one hour. Finally 4 µL of Streptavidin Donor beads (20 µg/mL final concentration) were added and allowed to incubate for an additional one hour. The plate was then read on an EnVision™ Multimode Plate Reader (Figure 5) using standard Alpha settings. 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. This protocol was developed for high-throughput characterization of inhibitors, but can be easily adapted to run in a lower-density assay format. As long as all reagents are kept at their respective final concentrations then the assay may be run in 96-well OptiPlates (6005290). Thus for a 50 µL final reaction volume, five additions of 10 µL would be made, all at the same concentrations as the 384-well assay.

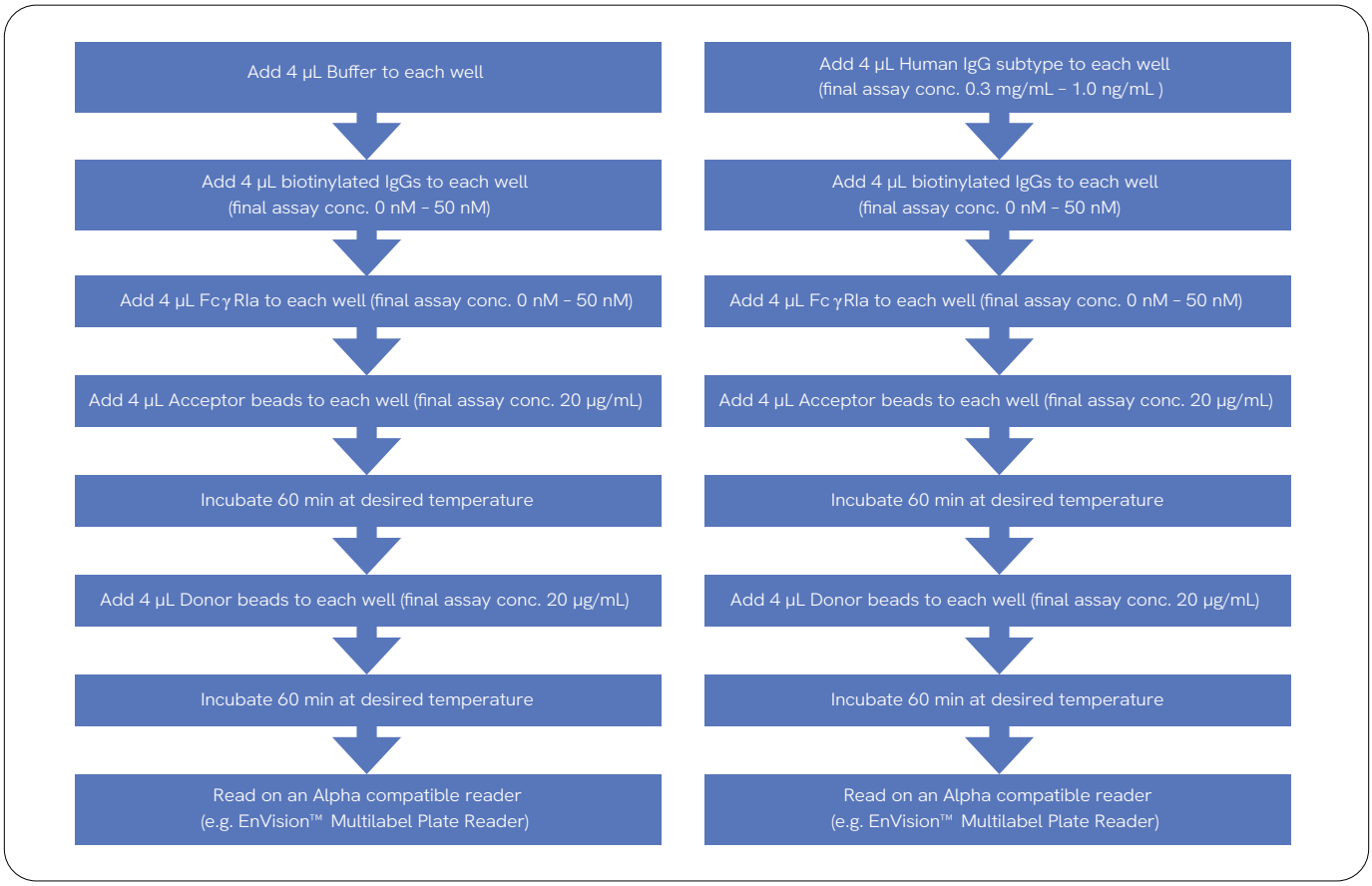



Figure 4: Assay diagram showing the steps used in the titration binding assay (left side) and competition assay (right side).


Figure 5: The EnVision Multilabel plate reader.

## Results

### Biotinylation of human IgGs

Biotinylation of Human IgGs was carried out per the manufacturer's protocol. After the reaction and cleanup was finished the absorbance at three different wavelengths was determined on a NanoDrop (Table 1), and these data were used in the Solulink™ online calculator to determine the adjusted immunoglobulin concentration (the linker for the biotin, which is tested at 354 nM, bleeds through into the concentration determination peak at 280 nM) as well as the MSR or average number of biotins per immunoglobulin molecule. Using these data and a weighted average molecular weight for Human IgGs, the concentration of the labeled IgGs was determined to be 4.06 µM and the MSR to be 12.15 biotins per molecule. Twelve biotins per molecule is a relatively high number, which may affect the binding of the IgGs to the FcγRI molecule, so if no binding were to be seen this could be repeated at a lower ratio of labeling reagent to IgGs. Also, if the protocol used here were to be extended to other FcγRs as well, then further optimizations to tune the MSR to a range of different values is warranted.

Table 1: Absorbance data from the biotinylation reaction used to determine the antibody concentration and degree of biotinylation.

| | A280 | A354 |
|---|---|---|
| Blank | 0 | 0 |
| b-IgGs | 1.2 | 1.43 |

### 2D titration of FcγRI and biotinylated IgGs

To determine the optimal concentrations of FcγRI and biotinylated IgGs to use, eight different concentrations of each were tested in a matrix of all 64 different possible pairings (Figure 6). Additionally, dilutions of each protein were tested without the other protein in the presence of both beads to determine if there were any potential non-specific binding events (i.e. removal of one of the binding partners should result in background signal only, and if there were a concentration dependence of that background signal that would point to some non-specific binding taking place).

The highest concentration of each protein tested was 50 nM, and from there 2-fold dilutions were performed for a total of eight points (down to a final concentration of 390 pM of each), tested in duplicate. The data show that the peak of the binding isotherm was located at 50 nM of biotinylated IgGs and 6.25 nM of FcγRI (the 'X' in Figure 6), so concentrations of 50 nM IgGs and 6 nM of FcγRI were chosen for performing the competition assay.

| 2D Titration | | nM FcyR1a | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.5625 | 0.78125 | 0.390625 | 0 |
| nM b-IgGs | 50 | | | | X | | | | | |
| | 25 | | | | | | | | | |
| | 12.5 | | | | | | | | | |
| | 6.25 | | | | | | | | | |
| | 3.125 | | | | | | | | | |
| | 1.5625 | | | | | | | | | |
| | 0.78125 | | | | | | | | | |
| | 0.390625 | | | | | | | | | |
| | 0 | | | | | | | | | |

Figure 6: Heat Map of the 2D titration generated between poly-His-FcγRI and biotinylated IgGs. The X is located where the pairing produced the largest signal, and values that were just slightly below this were chosen for the inhibition assay.

## Competition binding (inhibition) assay

To determine the binding affinity for a particular antibody, or in this case a particular subtype, an inhibition assay was performed. To perform the inhibition assay, IgG subtypes (the competitors) were added, followed by the biotinylated IgGs, and lastly the FcγRI protein, to give both the labeled and unlabeled immunoglobulins an equal chance to bind the FcγRI protein (Figure 7).

Concentration response curves were produced for the four human subtypes used as inhibitors, IgG1 through IgG4.

All four curves were started at 0.3 mg/mL of antibody, and then half-log dilutions were performed, for a total of 16 points. The $IC_{50}$s generated are shown in Figure 7 plotted using a 4-Parameter logistic fit with variable slope in GraphPad Prism. The $IC_{50}$s determined were 0.95 µg/mL for IgG1, 218 µg/mL for IgG2 (with the bottom of the curve constrained to that of the other three subtypes), 0.82 µg/mL for IgG3 and 12.3 µg/mL for IgG4. Data shown are the average of two replicates.

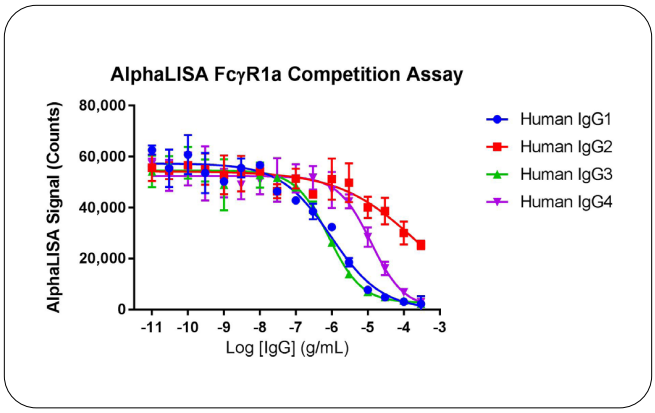



Figure 7: $IC_{50}$s were generated for all four subtypes of Human IgGs. If a native antibody of unknown subtype were used in this assay it would be possible to not only determine its affinity for FcγRI, but also to determine if it were an IgG2, IgG4 or either an IgG1 or IgG3 (since their affinities are so close).

## Conclusions

In this application note we demonstrate the ease with which AlphaLISA toolbox reagents can be used to develop any FcγR binding assay across the various stages of biologics research and development, including therapeutic screening and GMP Lot Release. This assay can be used to characterize and calculate relative binding affinities for FcγR inhibitors. In cases of other Fc receptors for which there are no commercially available assay kits, this same AlphaLISA based assay methodology can be applied, with no limitations other than the availability of the proteins of interest.

## References

Lazar et al., Engineered antibody Fc variants with enhanced effector function, PNAS 2006: 103 (11) 4005-4010.

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