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 (Fig. 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.

Biochemical Binding ADCC Assays Utilizing LANCE Toolbox Reagents for the Characterization of hIgGs and FcγR1A

**Introduction**

Fc Receptors (FcRs) are cell-surface proteins found on a wide variety of cell types - including B
Fc Gamma Receptors (FcγRs) are members of the immunoglobulin superfamily and play a critical role in the function of therapeutic antibodies. The primary mechanism of action (MOA) for many drugs involves effector functions that are FcγR-mediated, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). FcγRs are the largest family of Fc receptors in humans, comprising six members with differing affinities for the different IgG subtypes. 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 (Fig. 1).

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 also that the structure of the hinges is slightly different among all four subtypes of IgGs (Fig. 2). One aim for therapeutic antibody engineering programs is to exploit these differences 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 LANCE® TR-FRET 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 an anti-Poly-His ULight™ Acceptor, binds to biotinylated Human IgGs, captured by a Europylated Streptavidin Donor (Fig. 3A). Untagged antibody was used as an inhibitor of this protein: protein interaction between the chosen FcγR (CD64) and the biotinylated IgGs, and an IC₅₀ 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/isotype pairing the IC₅₀ 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).
Materials and Methods

LANCE toolbox reagents [LANCE Eu-W1024 Streptavidin, 50 µg (AD0062), LANCE Ultra ULight-anti-6xHis, 1 nmole (#TRF0105-D)] and OptiPlates (#6007290) were from PerkinElmer. Human IgGs were from Jackson ImmunoResearch Laboratories [ChromPure Human IgG, whole molecule (#009-000-003), and Biotin-SP-conjugated ChromPure Human IgG, whole molecule (#009-060-003)]. FcγRI, poly-His tagged was from R&D Systems (#1257-FC-050).

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 IgG, and 4 µL of FcγRI, all at 5X final concentration, and incubating at 23 °C for one hour. Then 4 µL of LANCE Ultra ULight-anti-6xHis (50 nM final concentration) was added and incubated for one hour. Finally, 4 µL of LANCE Eu-W1024 Streptavidin (1 nM final concentration) was added and allowed to incubate for an additional one hour. The plate was read on an EnVision® Multimode Plate Reader (Fig. 5) using standard LANCE settings. In addition to fast, sensitive time-resolved fluorescence detection, the EnVision Multilabel microplate reader provides fluorescence intensity, luminescence, absorbance, fluorescence polarization, and Alpha technology detection technologies.

This protocol was developed for high-throughput characterization of inhibitors, but can be easily adapted to a lower-density assay format. The assay may be run in 96-well OptiPlates (#6005290) as long as all reagents are kept at their respective final concentrations. Thus, for a 50 µL final reaction volume, five additions of 10 µL would be made, using the same concentrations as in the 384-well assay.

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

2D Titration of FcγRI and Biotinylated IgG
To determine the optimal concentrations of FcγRI and biotinylated IgG to use, eight different concentrations of each were tested in a matrix of all 64 different possible pairings (Fig. 6). As a control, dilutions of each protein were tested without the other protein in the presence of both detection reagents, to determine if there was any potential non-specific binding (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 indicate some non-specific binding).

The highest concentration of each protein tested was 50 nM. Twofold dilutions were performed for a total of eight points (down to a final concentration of 390 pM), tested in duplicate. The data show that the peak of the binding isotherm was located at 50 nM of biotinylated IgG and 3.125 nM of FcγRI (Fig. 6). Concentrations of 1.5 nM IgG and 1.5 nM of FcγRI were chosen for the competition assay, as these were below the peak and gave a respectable signal-to-background of close to ~7.

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Competition Binding (Inhibition) Assay
To determine the binding affinity of a particular antibody, an inhibition assay was performed. To perform the inhibition assay, unlabeled test antibody was added, followed by the biotinylated IgG, and lastly the FcγRI protein, to give both the labeled and unlabeled immunoglobulins an equal chance to bind the FcγRI protein (Fig. 4).

A concentration response curve was produced for the unlabeled human IgG, starting at 50 µM final concentration, and 2-fold dilutions were performed for a total of 22 points. The IC50 generated is shown in Fig. 7, and was plotted using a four-Parameter logistic fit with variable slope in GraphPad Prism. Data shown are the average of two replicates.

Figure 6. Heat Map of the 2D titration generated between poly-His-FcγRI and biotinylated IgG.

Figure 7. IC50 generated for Human IgGs in the LANCE toolbox assay.
Conclusions
In this application note we demonstrate the ease with which LANCE Ultra 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 LANCE based assay methodology can be applied, with no limitations other than the availability of the proteins of interest.

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