APPLICATION NOTE

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

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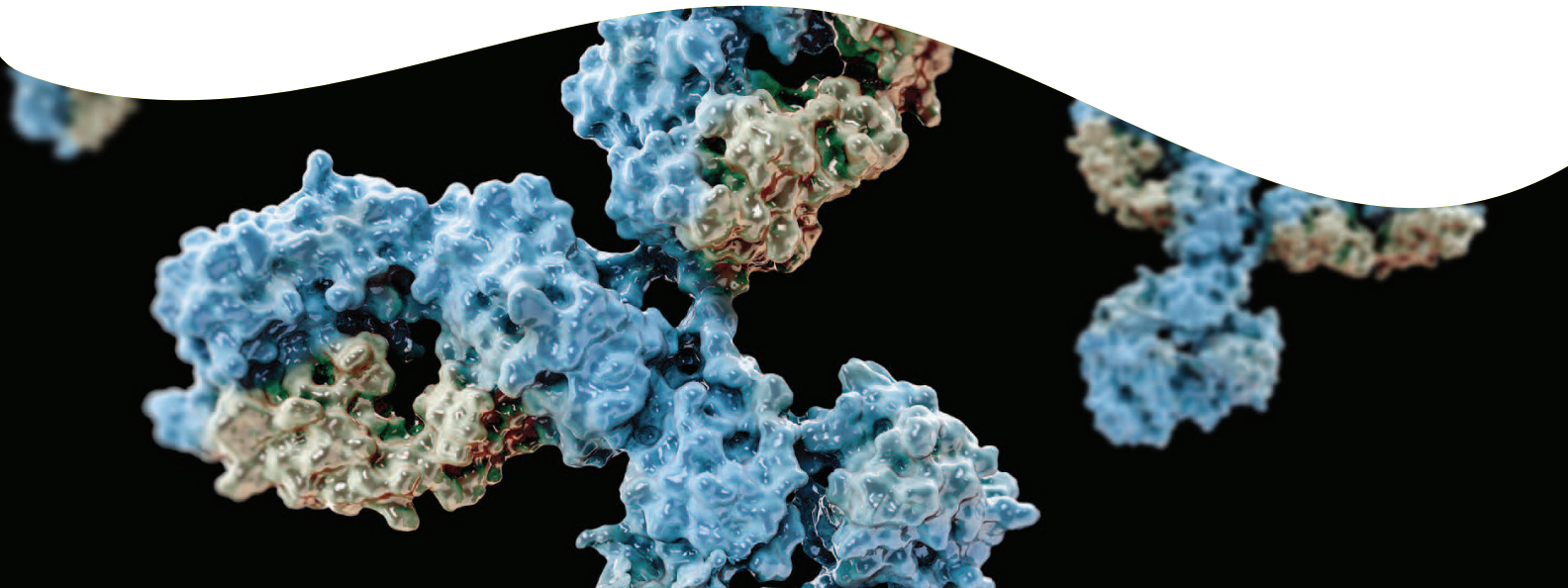
Introduction

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 (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.



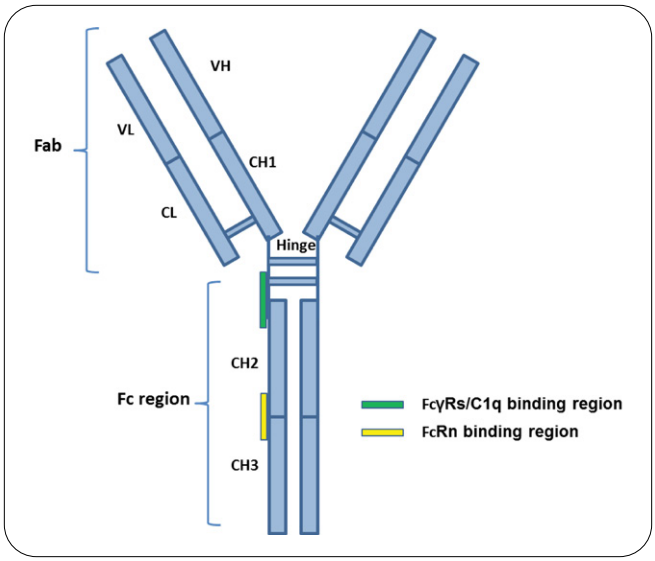


Figure 1: General structure of a Human IgG antibody. N.B. FcγRs bind closely to the hinge region whereas FcRn binds closer to the tail of the Fc region.

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.

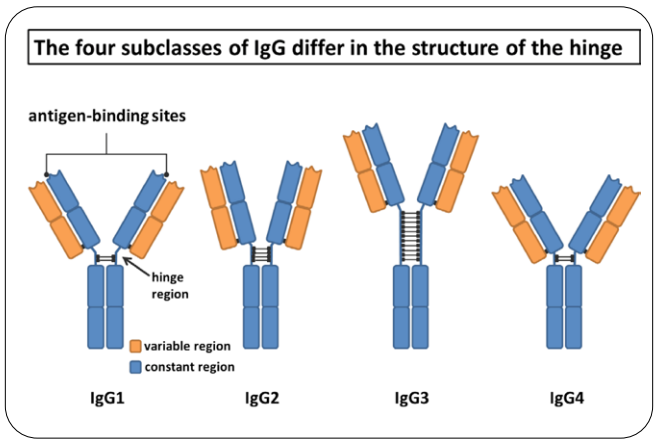


Figure 2: The differences in structures for the four subtypes of Human IgGs are shown. The differences in the hinge regions among the four different subtypes help to explain the differences in binding affinity that the subtypes show for the different FcγRs.

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 U*Light*™ 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 FcγRI (CD64) and the biotinylated IgGs, and 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/isotype 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).

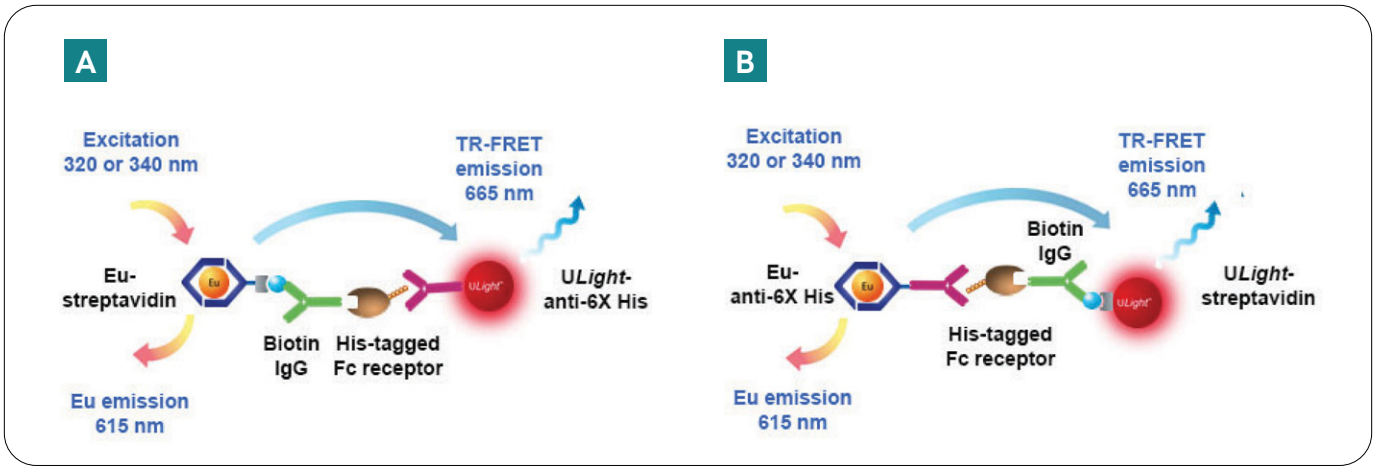


Figure 3: Assay schematic of the LANCE Ultra Toolbox binding assay configuration developed between FcγRI and biotinylated Human IgGs. In the configuration used here (A), the poly-histidine tag of the FcγRI protein is captured by the anti-Poly-His ULight Acceptor, and the biotin tag on the Human IgG is captured by the Europylated Streptavidin Donor. For the inhibition assay, unlabeled therapeutic IgG is used as a competitor of the binding of FcγRI and biotinylated IgG. (B) The affinity tags are captured on opposite reagents.

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 Revvity. 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.

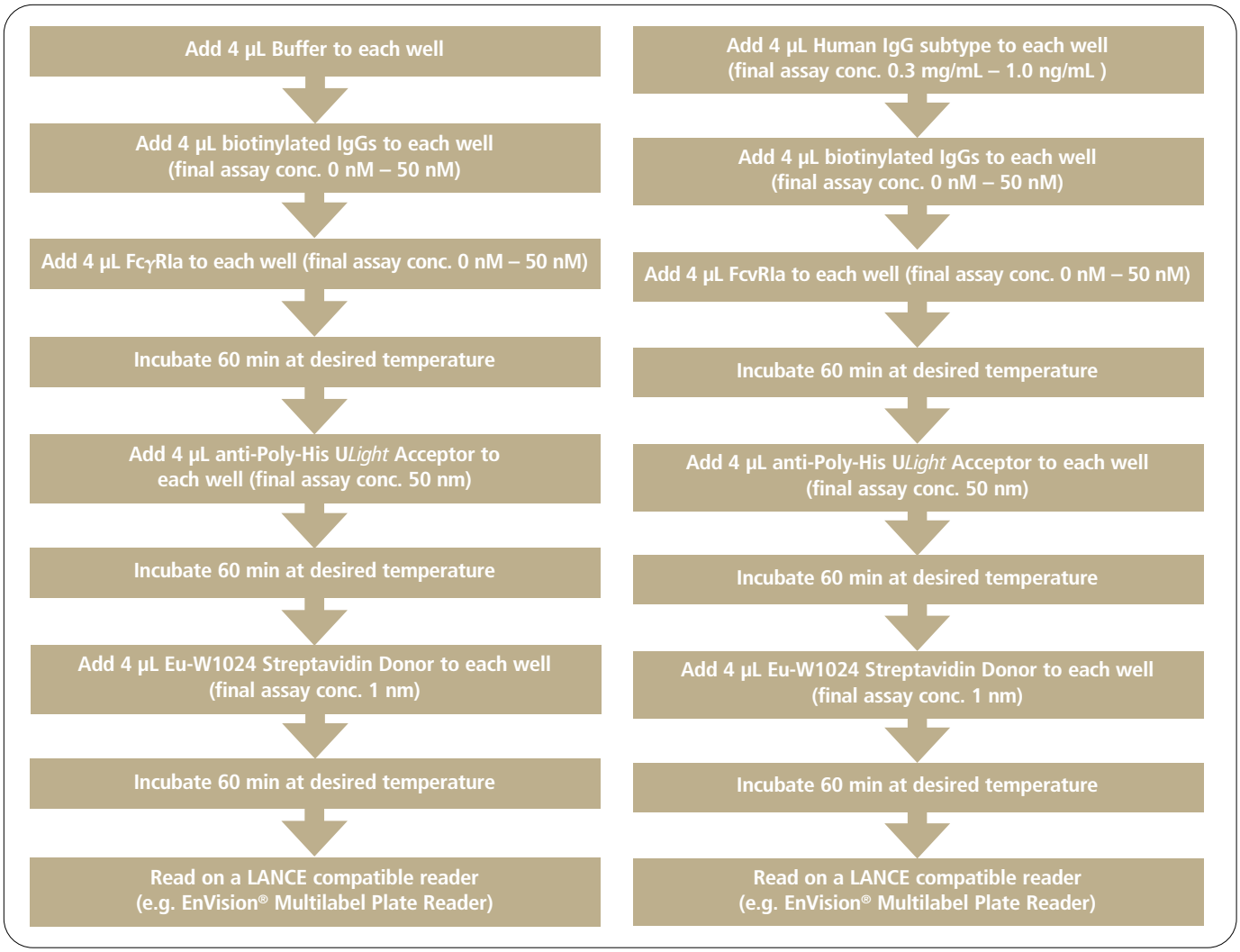


Figure 4: Assay diagram showing the steps used in the titration binding assay (left side) and competition assay (right side).

Figure 5: The EnVision Multilabel plate reader.

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.

		nM b-IgGs								
		50	25	12.5	6.25	3.125	1.5625	0.78125	0.390625	0
nM FcγR1a	50	16544	8642	5582	3729	2811	2473	2056	2055	2051
	25	19414	15169	9025	5806	3773	2748	2301	2321	1981
	12.5	20617	18677	14636	9013	5640	3591	2906	2515	1975
	6.25	20761	19571	18214	15523	8967	5563	3943	3083	2029
	3.125	21172	19654	18121	16186	14279	9181	5859	4045	2125
	1.5625	21134	18916	18837	16671	16013	13165	8876	5586	2335
	0.78125	20908	18455	17222	16363	14999	14271	12393	8700	2277
	0.390625	18542	17181	15120	13854	13149	11908	11512	10738	2521
	0	2528	2688	2384	2257	2045	2037	2106	1861	1803
	S:B									
		6.5	3.2	2.3	1.7	1.4	1.2	1.0	1.1	1.1
		7.7	5.6	3.8	2.6	1.8	1.3	1.1	1.2	1.1
		8.2	6.9	6.1	4.0	2.8	1.8	1.4	1.4	1.1
		8.2	7.3	7.6	6.9	4.4	2.7	1.9	1.7	1.1
		8.4	7.3	7.6	7.2	7.0	4.5	2.8	2.2	1.2
		8.4	7.0	7.9	7.4	7.8	6.5	4.2	3.0	1.3
		8.3	6.9	7.2	7.3	7.3	7.0	5.9	4.7	1.3
		7.3	6.4	6.3	6.1	6.4	5.8	5.5	5.8	1.4
		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

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

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 IC_{50} 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.

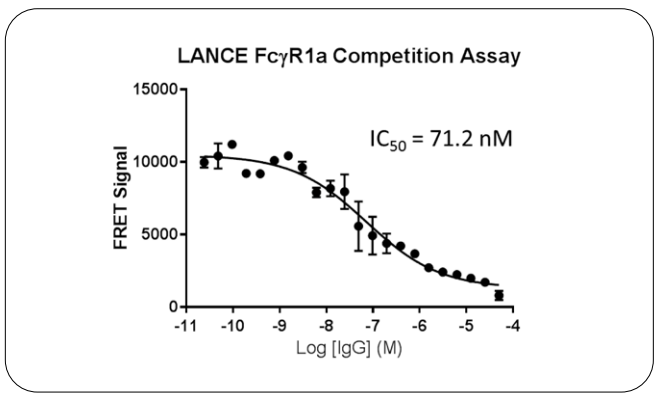


Figure 7: IC_{50} generated for Human IgGs in the LANCE toolbox assay.

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.

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