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High resolution FcRn IC_{50} potency measurements using the Carterra® LSA® platform

Key Takeaways

- Using neonatal Fc receptor (FcRn) as a model system, the versatility of the LSA is demonstrated by measuring binding in three different assay strategies
- Kinetic affinity, steady-state affinity, and IC_{50} potency values were all obtained using the same ligand array
- Multiple conditions and controls, all in triplicate, were included due to the high-throughput nature of the LSA
- The capability to conduct potency assays on the LSA highlights new opportunities for leveraging high-throughput surface plasmon resonance (HT-SPR) in drug discovery and development

Overview

Potency assays have wide applicability in life science research and development. For therapeutic drug candidates, they are essential across the spectrum of discovery and into development. While many potency assay formats are endpoint in nature, such as enzyme-linked immunosorbent assay (ELISA), there are examples where real-time, label-free technologies have been used to determine potency[1]. Here we demonstrate how potency assays can be leveraged as part of an overall characterization strategy via HT-SPR using the Carterra LSA. The versatility of the LSA enables researchers to easily adapt to this assay format in conjunction with more commonly used techniques of measuring binding affinity. Additionally, HT-SPR with the LSA presents opportunities to measure up to hundreds of interactions simultaneously, allowing for inclusion of varied assay conditions, controls, and replicates not typically feasible with other technologies. Using FcRn as a model system, this application note explores a workflow to characterize kinetic affinity, steady state affinity, and potency in a series of experiments that can be completed in a single day.

Materials and Methods

Assay Workflow

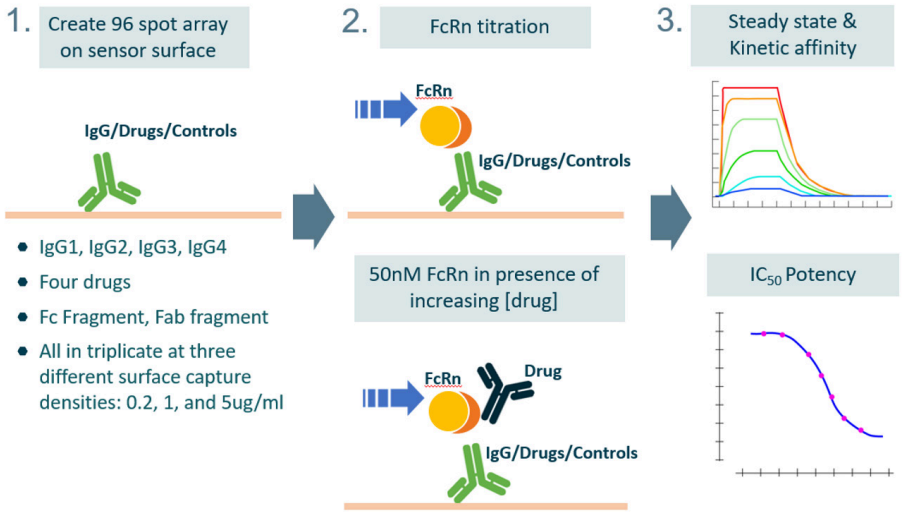

Figure 1. HT-SPR workflowused to measure FcRN kinetic amd steady state affinities, as well as potency, against an array of drugs, IgG subclasses, and controls.

Preparation of the Coupled Ligand Array

A Carterra LSA instrument was primed into 25 mM MES pH 5.5 (Carterra Part # 3625) + 0.05% Tween-20 (Carterra Part # 3631). An HC30M sensor chip (Carterra Part # 4279) was docked in the instrument and the interaction temperature was set to 25°C. Athens Research immunoglobulins IgG1 (Cat. # 16-16-090707-1), IgG2 (Cat. # 16-16-090707-2), IgG3 (Cat. # 16-16-090707-3), and IgG4 (Cat. # 16-16-090707-4) along with MedChemExpress Adalimumab (Cat. # HY-P9908), Pembrolizumab (Cat. # HY-P9902), Trastuzumab (Cat. # HY-P9907),

and Etanercept (Cat. # HY-108847) were prepared in 10 mM sodium acetate pH 4.5 at concentrations of 5, 1, and 0.2 µg/ml. As controls, Jackson Immuno Research F(ab)2 fragment (Cat. # 009-000-006) and human Fc fragment (Cat. # 009-000-008) were prepared at the same concentrations as the IgGs and drugs. Activation solution was prepared by combining 100 µl of 400 mM EDC (Thermo Scientific, Cat. # PG82073), 100 µl of sNHS (Thermo Scientific, Cat. # PG82072), and 100 µl of 0.1 M MES pH 5.5. The surface was activated for seven minutes, then each IgG or drug was coupled to the surface for 10 minutes, and finally remaining active esters were quenched by injection of 1 M ethanolamine (Carterra Part # 3626) for eight minutes. The final array consisted of triplicates of each ligand at three different concentrations, totaling 90 unique protein spots.

Kinetics Analysis of FcRn Binding to Arrayed Ligands

The LSA was then primed into PBS pH 5.8 + 0.05% Tween-20 running buffer (Teknova Cat. # P1193). Human FcRn (BPS Bioscience Cat. # 71285) was prepared as an eight-member titration from 500 to 0.2 nM in buffer, as three-fold dilutions. To ensure the surface was stabilized prior to the measurement of binding kinetics, eight warmup injections of buffer were performed. The FcRn concentration series was then injected starting at the lowest concentration. At the end of each injection cycle, the surface was exposed to PBS pH 7.4 + 0.05% Tween-20 to remove any FcRn still bound to the array. Using Kinetics analysis software (Carterra), data were double referenced, y-aligned, cropped and fit to either a 1:1 Langmuir model or steady state equilibrium model to determine kinetic rate constants and equilibrium binding affinities, respectively.

Measuring FcRn IC_{50} Potency

With the same buffer used for kinetic analysis, 50 nM human FcRn final was prepared in the presence of drugs at the following concentrations: Adalimumab (2.6 µM), Pembrolizumab (2.8 µM), Trastuzumab (2.3 µM), and Etanercept (1.6 µM). Two-fold dilutions of this mixture were then made, with a total of 11 dilutions per drug. This dilution strategy resulted in premixed samples all having 50 nM FcRn, but decreasing amounts of drug. These dilution series were injected across the same array used for kinetics, proceeding from the lowest to highest concentration of drug. At the conclusion of each cycle, remaining FcRn bound to the array was removed by injection of PBS pH 7.4 + 0.05% Tween-20. Data were reference subtracted and y-aligned in Kinetics analysis software. Response unit (RU) signals corresponding to the end of the injection cycles were taken as report points and IC_{50} values obtained in Graphpad Prism 9.2.0 using the log (inhibitor) vs. response non-linear regression equation.

Results

Surface Coupled Ligand Levels

Using the sensorgram phase following ethanolamine quenching, the final levels of amine coupled proteins are shown as triplicate averages including standard error, **Figure 2**. While the ligand concentrations were 5, 1, and 0.2 µg/ml, the spread in signals is less than 25-fold, indicating that saturation was occurring on the surfaces. All replicate ligands coupled in a similar range for their respective concentration, as indicated by the standard error bars.

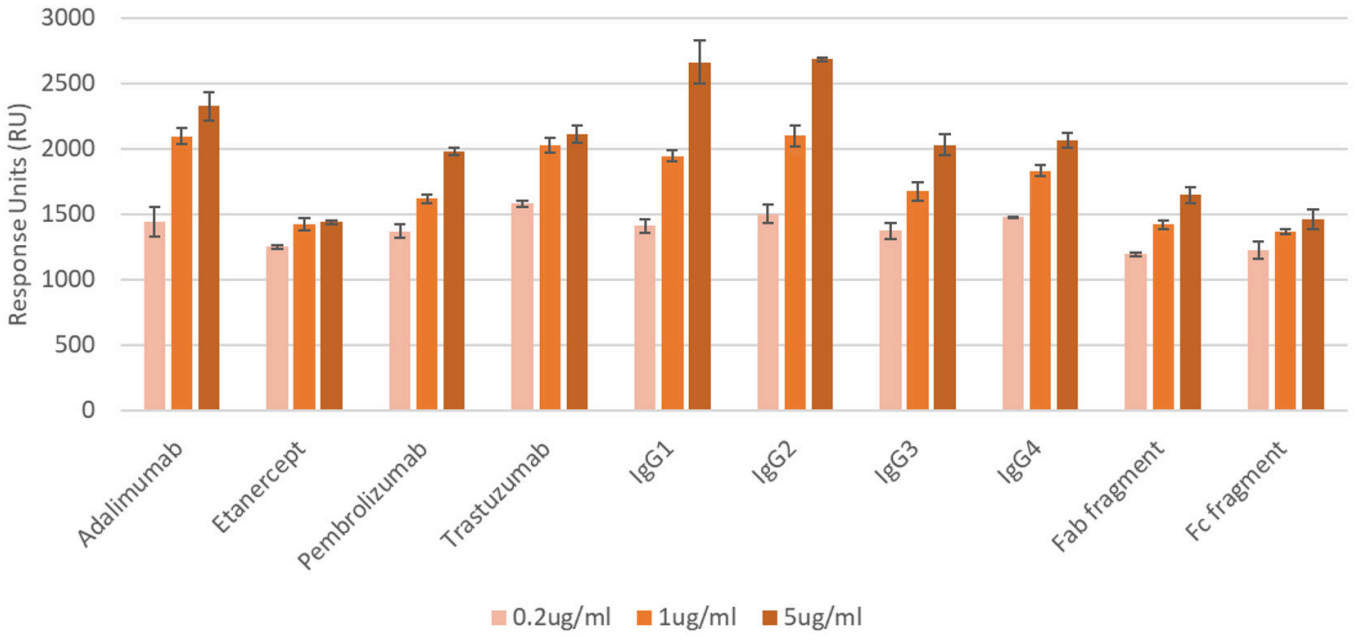

Figure 2. Final levels of ligands amine coupled to the HC30M surface.

Kinetic and Steady State Analysis

Following the arraying of the drugs, IgG subclasses, and controls, a titration of FcRn was injected across the array to assess binding kinetics as well as steady state equilibrium affinity. In **Figure 3**, example sensorgrams are shown for human FcRn binding the drugs and immunoglobulins at 1 µg/ml and fit to a 1:1 Langmuir model (red curves). The sensorgram profiles showing a rapid initial binding, followed by a subtle, but second slower binding phase, are indicative of a biphasic interaction consistent with previous studies[2]. The range of data including for fitting of dissociation was limited due to this biphasic nature and therefore only the initial rapid phase of dissociation was used. Nonetheless, the 1:1 model provides a fair approximation of the association and dissociation profiles and overall, the profiles are very similar amongst both the drugs and immunoglobulins. Similar sensorgram profiles were observed for the 0.2 and 5 µg/ml ligand densities. No meaningful binding was observed for the F(Ab)2 or Fc fragment surfaces (data not shown). For F(Ab)2 this was expected but for the Fc fragment may be due to inactivation or steric hindrance of FcRn binding as a result of amine coupling.

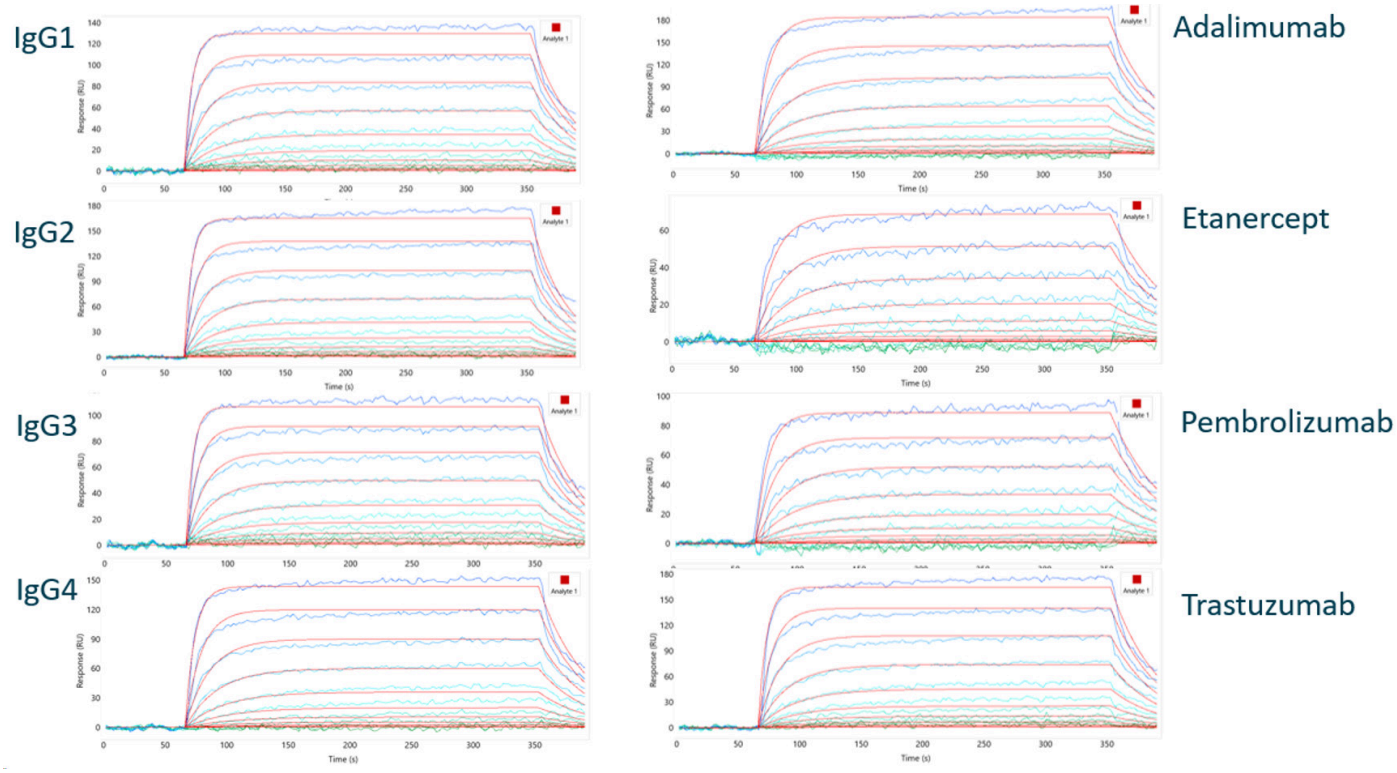

Figure 3. Representative sensorgram fits of FcRn titrations binding IgG subclasses and drugs. Dissociation phase data was collected for 15 min but fitting only performed for the initial 30 second dissociation due to biphasic nature of dissociation phase. Surfaces shown were coupled at 1 µg/ml.

Using the same data from the kinetics experiment, a steady state equilibrium analysis was performed to determine affinity as a function of response over concentration. This was done using a report point from the last 30 seconds of each FcRn injection. An example of these steady state fits is shown in **Figure 4**.

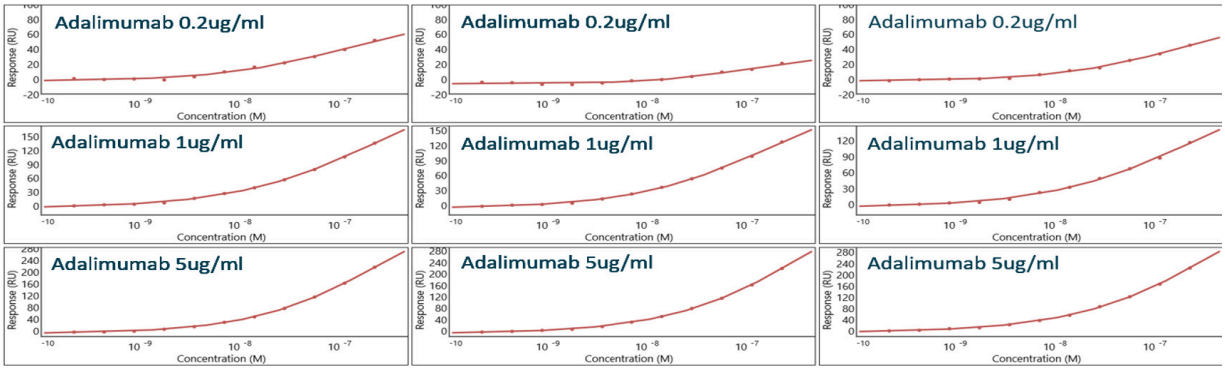

Figure 4. Example of Adalimumab response vs. concentration plots used to calculate steady state affinity. Shown are data for 0.2, 1, and 5 µg/ml amine coupling densities.

Plots of the calculated kinetic and steady state affinities (KD) in triplicate including standard errors are shown in **Figure 5**. Looking across affinities from the two methods, the steady state affinities appear in good agreement with the kinetically determined affinities, suggesting that the slight discrepancies between the 1:1 Langmuir model and the sensorgrams did not materially impact the kinetic affinities. The affinity of FcRn towards the molecules in these studies is highly similar with the potential exception for Etanercept, which appeared slightly weaker than the other drugs and immunoglobulins tested. There were some slight changes in affinity as a function of surface density, for example with Pembrolizumab. Overall, the data suggest that there are no large disparities in affinity across the molecules tested and that using the two approaches to determine affinity yields outcomes in a range around 100 nM.

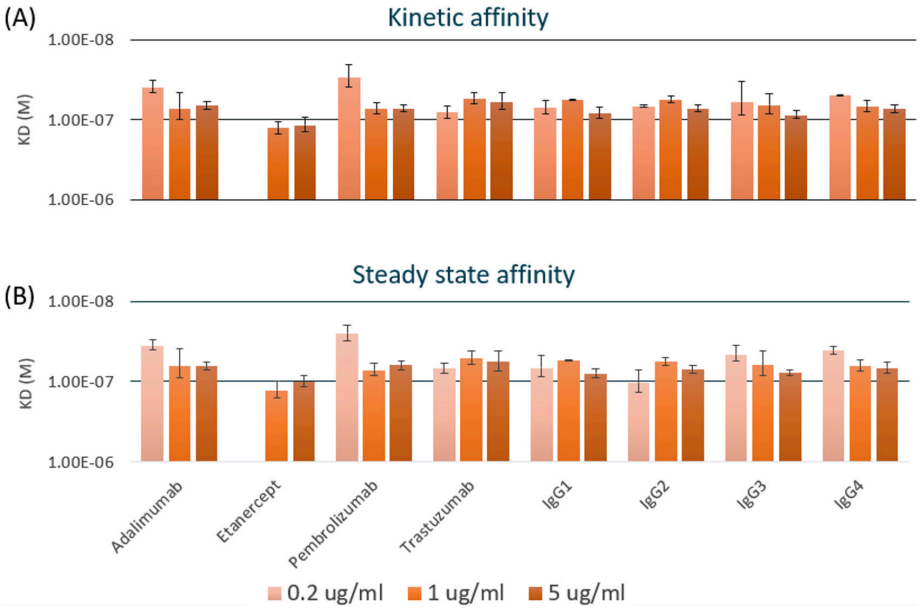

Figure 5. Triplicate mean affinities of FcRn for IgG subclasses and drugs. (A) Kinetically derived affinities. (B) Steady state derived affinities. Etanercept at 0.2µg/ml was excluded due to insufficient signals for fitting.

FcRn Potency

Using the same surface prepared for the kinetic and steady state experiments, the ability of Adilimumab, Etanercept, Pembrolizumab, and Trastuzumab to inhibit binding of FcRn to IgG was then assessed in a dose inhibition assay format. In these experiments, human FcRn at 50 nM was premixed with drug at increasing concentrations and injected across the array. Shown in **Figure 6A** are the binding responses taken for each of the premixtures of FcRn and drug plotted as a dose inhibition curve. The y-scale values are normalized to the maximum response unit observed for FcRn against that IgG surface. Overall, the plotted responses are highly similar across the four drugs against the four IgG subclasses. Slight exceptions include Etanercept which appears somewhat more potent across all IgG surfaces. Additionally Pembrolizumab appears to have a more truncated curve profile and ultimately a modestly higher potency when competed against IgG2. **Figure 6B** lists the IC_{50} values in nM for each interaction.

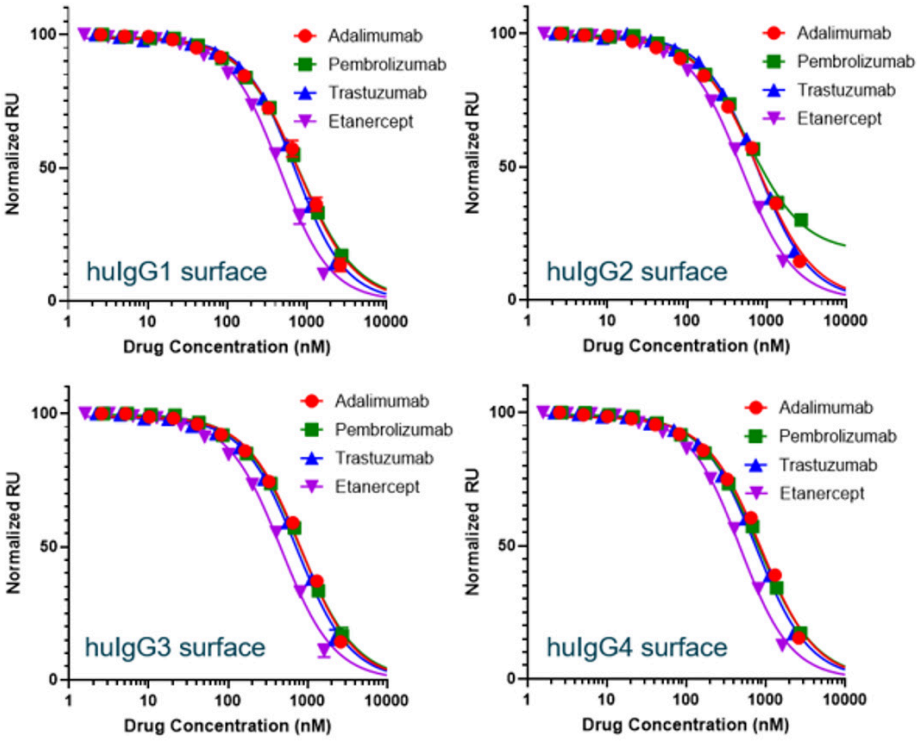

Figure 6A. FcRn IC_{50} plots developed using the LSA against all four 5 µg/ml human IgG subclass surfaces.

Collectively, these data agree with the kinetic and steady state analysis that no major differences in FcRn potency are detected in this format. Interestingly, Etanercept displayed a slightly weaker affinity using both the kinetic and steady state approaches against FcRn compared with the other drugs tested, but showed better potency. While all four drugs maintain affinity and potency within 2-fold, these slight differences offer fascinating insight into differences that could manifest in other assays depending on design, emphasizing the value of that HT-SPR lends to charactertizing these interactions across many different conditions.

Comparison to No-wash Luminescent Assay

To further validate the LSA data suggesting that kinetic affinity, steady state affinity, and IC_{50} potency were highly comparable for FcRn against these drugs and IgGs, the data was compared to an AlphaLISA® FcRn assay (PerkinElmer Cat.# AL3095) using a similar set of reagents, except for the use of biotinylated human FcRn (BPS Bioscience Cat. # 71283). Results are shown in **Figure 7**. The four drugs show similar does response curves and the IC_{50} values are similar as well, except for trastuzumab which is slightly less potent that the others. The absolute values shown here differ from those obtained on the LSA due to differences in assay operating ranges.

Discussion

Using FcRn as a model system, this approach has emphasized the ability of the LSA to rapidly conduct high resolution potency assays. By including varied surface species and controls, all in replicate, a short set of experiments builds a tremendous amount of information around the interaction in question. This approach is readily combined with kinetic and steady state analysis of affinity to further validate potency findings. As most potency assays are endpoint in nature, the ability to monitor signals in real-time gives HT-SPR an advantage in challenging systems where there are concerns about complex binding interactions or unexpected outcomes.

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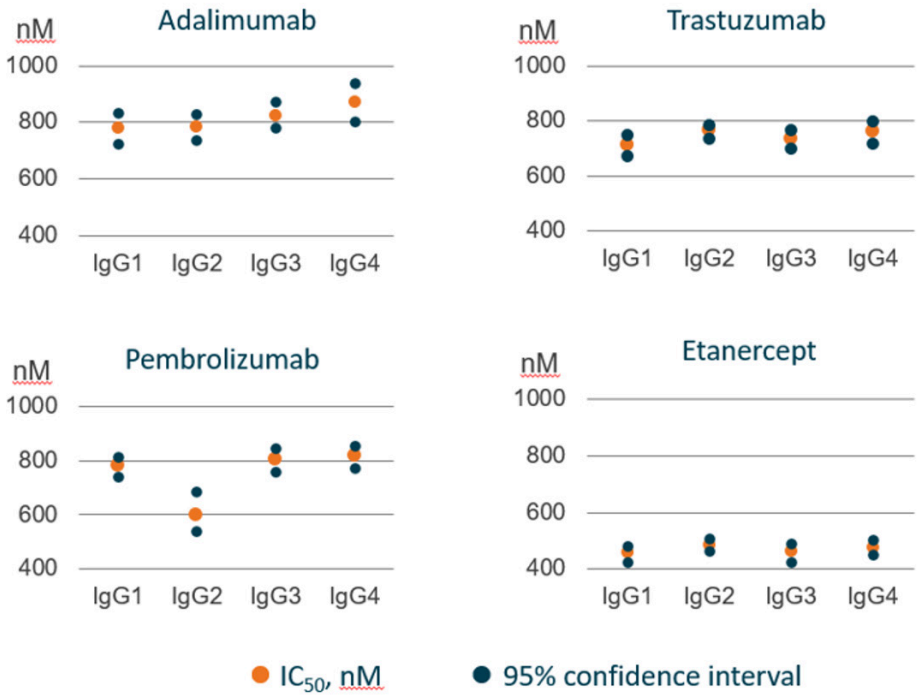
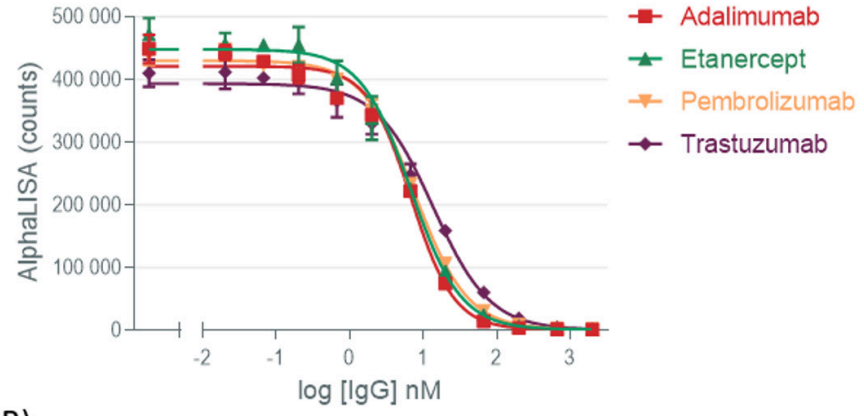


Figure 6B. FcRn IC_{50} potency values in nM. Measurements were performed in triplicate.

(A)



(B)

IC_{50} (nM)	Adalimumab	Etanercept	Pembrolizumab	Trastuzumab
AlphaLISA	6.8	6.7	7.8	13.6

Figure 7. (A) FcRn dose inhibition plots established using an AlphaLISA® FcRn kit. (B) FcRn IC_{50} potency values. Measurements were performed in triplicate. Adapted from: ***www.perkinelmer.com/libraries/APP-high-throughput-fcrn-binding-assays-317271***

Acknowledgments

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References

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