APPLICATION NOTE

High-throughput FcRn binding assays for therapeutic antibody discovery and development

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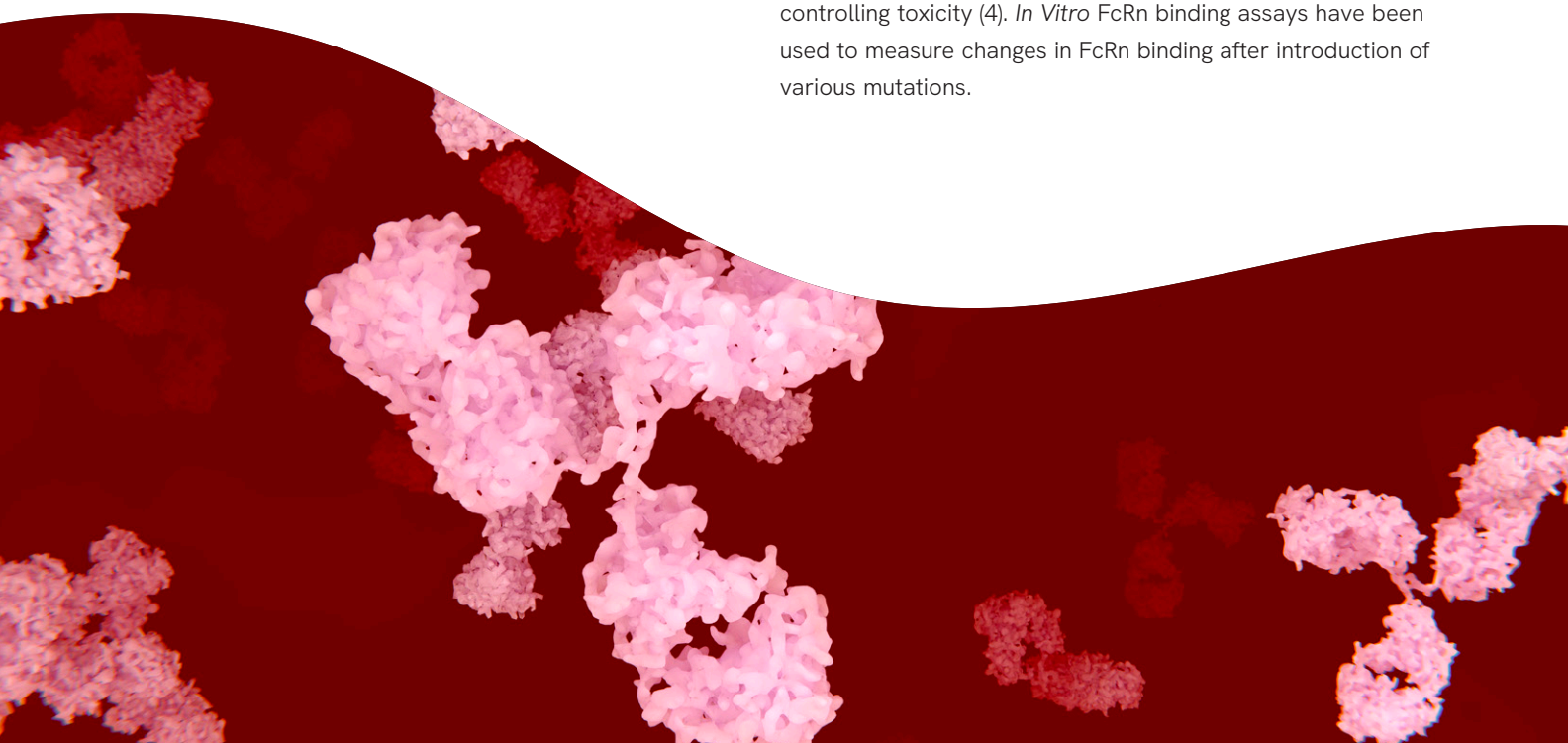
Introduction

The interaction between the Fc portion of therapeutic antibodies and the neonatal Fc receptor (FcRn) is one of the critical factors that determines the antibody serum half-life. FcRn is a heterodimer that contains a transmembrane alpha chain and is associated with beta2-microglobulin. FcRn binds to the Fc portion of an antibody at the CH2 and CH3 domain interface in a pH dependent manner. After pinocytosis, IgGs are internalized into the endosomes of cells and bind FcRn in the acidic environment of the endosome at ~pH 6.0. The bound complexes are then recycled to the cell surface, dissociate at neutral pH, and the antibodies return to the bloodstream, thus increasing their serum half-life. Antibodies that do not bind strongly to FcRn are shuttled to the lysosome for degradation (Monnet *et al*, Pyzik *et al*, Stöppler *et al*.)(Figure 1).

Given the importance of FcRn binding for antibody homeostasis, the Fc portion of therapeutic antibodies has been modified to modulate binding to the FcRn, thereby increasing or decreasing the serum half-life. Increasing binding to FcRn and extending the serum half-life of a therapeutic antibody is important for maintaining longer therapeutic levels and reducing the frequency of dosing. When engineering Fc regions of an antibody for increased serum half-life, it is important to not only increase binding to FcRn at pH 6.0, but it is also important that binding is weak at neutral pH so that it can be recycled. Decreasing the affinity to FcRn reduces the serum half-life which is advantageous if the antibody is used for diagnostic tests or for controlling toxicity (4). *In Vitro* FcRn binding assays have been used to measure changes in FcRn binding after introduction of various mutations.



FcRn binding assays can be used for determining the relative affinity of different therapeutic antibodies to FcRn and can be used to compare biosimilars.

There are many different commercially available FcRn binding assays. Here we present two orthogonal high throughput no-wash assays (AlphaLISA™ and HTRF™) that can be used to compare relative potencies for binding FcRn *In Vitro*. Although both assays use similar homogeneous competition formats, checking relative potencies of FcRn binding in more than one assay format can be beneficial in determining if there are any assay specific biases. We show the specificity of the assays for binding the Fc portion of an antibody and the performance of binding different subtypes (IgG1, IgG2, IgG3, and IgG4). We also show how these assays can be used for testing binding of therapeutic antibodies.

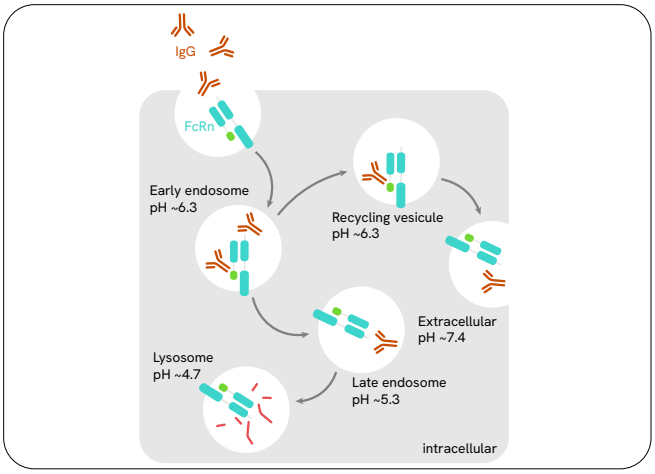


Figure 1: FcRn recycles antibodies to prolong serum half-life. Copyright: this figure was adapted from stöppler *et al.* This figure is covered by the creative commons attribution 4.0 International license.

Materials and methods

Antibody samples

For the FcRn binding assays, the following antibodies were used: whole hIgG (Jackson ImmunoResearch cat# 009-000-003), Fc (Jackson ImmunoResearch cat# 009-000-008), F(ab)2 (Jackson ImmunoResearch cat# 009-000-006), hIgG1 (Athens Research cat# 16-16-090707-1), hIgG2 (Athens Research cat# 16-16-090707-2), hIgG3 (Athens Research cat# 16-16-090707-3), hIgG4 (Athens Research cat# 16-16-090707-4), Adalimumab (MedChemExpress cat# HY-P9908), Etanercept (MedChemExpress cat# HY-108847), Pembrolizumab (MedChemExpress cat# HY-P9902), Trastuzumab (MedChemExpress cat # HY-P9907).

AlphaLISA assays

Alpha technology is a fast, easy, highly sensitive, homogeneous (no-wash) assay platform that can be performed in a microplate format. Alpha assays require two bead types: Donor beads and Acceptor beads. The Donor beads convert ambient oxygen to singlet oxygen upon illumination at 680 nm. The singlet oxygen can then diffuse approximately 200 nm in solution. If an Acceptor bead is within that distance, energy is transferred to the Acceptor bead, resulting in light production. If an Acceptor bead is not in proximity of a Donor bead, little to no signal is produced over background. In the AlphaLISA FcRn binding assay, biotinylated FcRn/B2M complex binds the Streptavidin Donor bead and then binds a human IgG conjugated Acceptor bead, producing maximum signal. Then IgG in the sample binds to the FcRn causing a decrease in signal. If the IgG in the sample cannot bind to FcRn, no decrease in signal will be seen. An example of the competition FcRn AlphaLISA binding assay used in this application note is shown in Figure 2.

The AlphaLISA assays were performed using the AlphaLISA FcRn binding kit (Revvity cat# AL3095). Briefly, 10 µL of IgG sample is added to a 1/2 Area Alpha-96 microplate (cat# 6002350) in triplicate and then 10 µL of biotinylated FcRn/B2M complex (200 ng/mL final) is added. Then 20 µL of a mixture of hIgG AlphaLISA Acceptor beads (20 µg/mL final) and Streptavidin Donor beads (20 µg/mL final) is added and incubated at room temperature for 90 minutes (Figure 4A). Multiple independent experiments were run for the whole IgG sample over several different days. The plates were read on an EnVision 2105 Multimode Plate Reader.

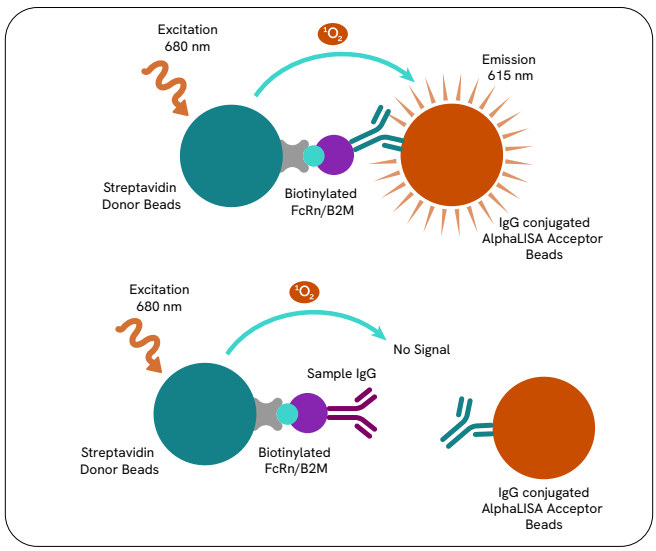


Figure 2: Alpha FcRn binding assay

HTRF assays

HTRF™ (a TR-FRET technology) is a rapid, homogeneous, easy to use and easy to automate assay platform. HTRF is used to assess molecular interactions by coupling two biomolecules with either a donor (long-lived fluorescence) or an acceptor (short-lived fluorescence) fluorophore. When the dyes are in proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). In the HTRF FcRn binding assay (Figure 3), human IgG is detected in a competitive assay by using FcRn - B2M heterodimer labeled with terbium (Tb) cryptate (donor), and human antibody labeled with d2 (acceptor). Antibodies present in the sample compete with the binding between the two HTRF detection solutions and thereby prevents FRET from occurring. The specific signal is inversely proportional to the antibody concentration.

The HTRF assays were performed using the HTRF FcRn binding kit (Revvity cat# 64FCRNPEG). Briefly, 10 µL of IgG sample is added to a HTRF 96-well low volume white plate (cat# 66PL96100) in triplicate and then 5 µL of a d2 labeled IgG acceptor and 5 µL of a Tb cryptate labeled FcRn/B2M complex donor are added and incubated at room temperature for 1 hour (Figure 4B). Multiple independent experiments were run for the whole IgG sample over several different days. The plates were then read on an EnVision 2105 Multimode Plate Reader using a laser with APC 665 nm and Cy5 620 nm filters and the LANCE/DELFIA Dual bias mirror. Both the 620 nm and 665 nm signal were collected, and the HTRF ratio shown in the results was calculated as (665 nm/620 nm) *10^4.

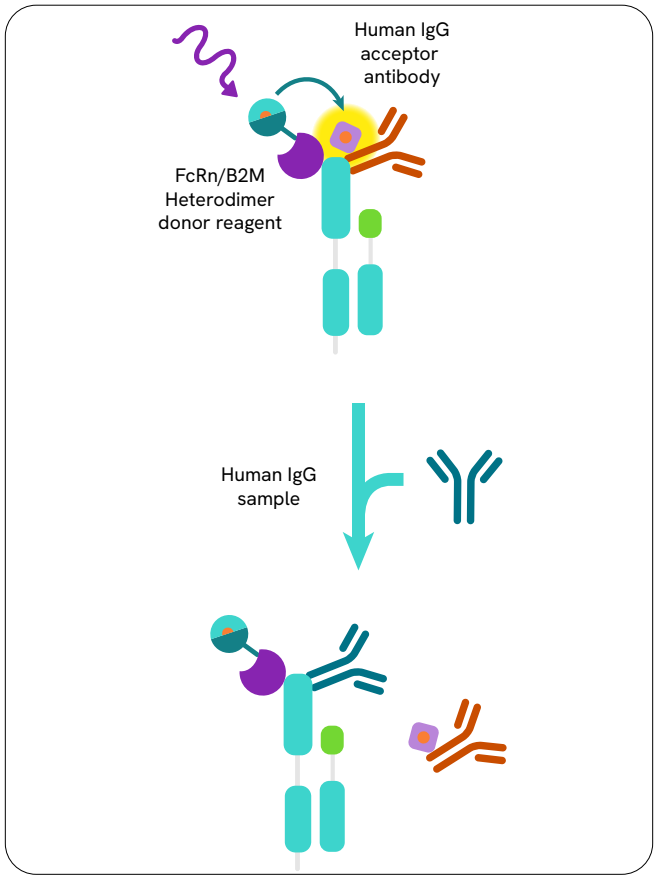


Figure 3: HTRF FcRn Binding Assay

A. AlphaLISA workflow

- Add 10 µL of 4X human IgG or test antibodies
- Add 10 µL of 4X human FcRn (200 ng/mL final)
- Add 20 µL of 2X mix of hIgG conjugated Acceptor beads and SA-Donor beads (20 µg/mL, 20 µg/mL final)
- Incubate 90 minutes at 23°C in the dark
- Read using EnVision-Alpha Reader

B. HTRF workflow

- Dispense 10 µL of human IgG or test sample
- Add 5 µL human IgG acceptor antibody working solution
- Add 5 µL of FcRn donor reagent working solution
- Seal the plate and incubate 1 hour at RT
- Remove the plate sealer and read on EnVision

Figure 4: Workflow for FcRn AlphaLISA binding assays (A) and FcRn HTRF binding assay (B).

Results and discussion

Assay performance and specificity

Antibodies bind FcRn specifically through the Fc portion of the antibody. Although there has been some suggestion that the Fab portion can contribute to the binding affinity, the Fab fragment alone is not expected to bind to FcRn. To validate the assays, whole IgG, an Fc portion, and a Fab fragment were used to test each assay. As shown in Figure 5, both the whole IgG and the Fc portion of the antibody compete in both the AlphaLISA and HTRF assays resulting in a drop in signal with increasing concentration, whereas, as expected, the Fab fragment has minimal effect.

Next, the different human isotypes (IgG1, IgG2, IgG3, and IgG4) were tested in triplicate to show specificity and performance of the assay (Figure 6). In the AlphaLISA assay, very little difference is seen between the different isotypes, which is consistent with values reported in the literature (Abdiche *et al.*). For the HTRF assay, small shifts in the IC_{50} values are seen but are all within approximately 3-fold of the whole IgG control. For both technologies, the IgG1 has slightly less affinity than the other isotypes for FcRn.

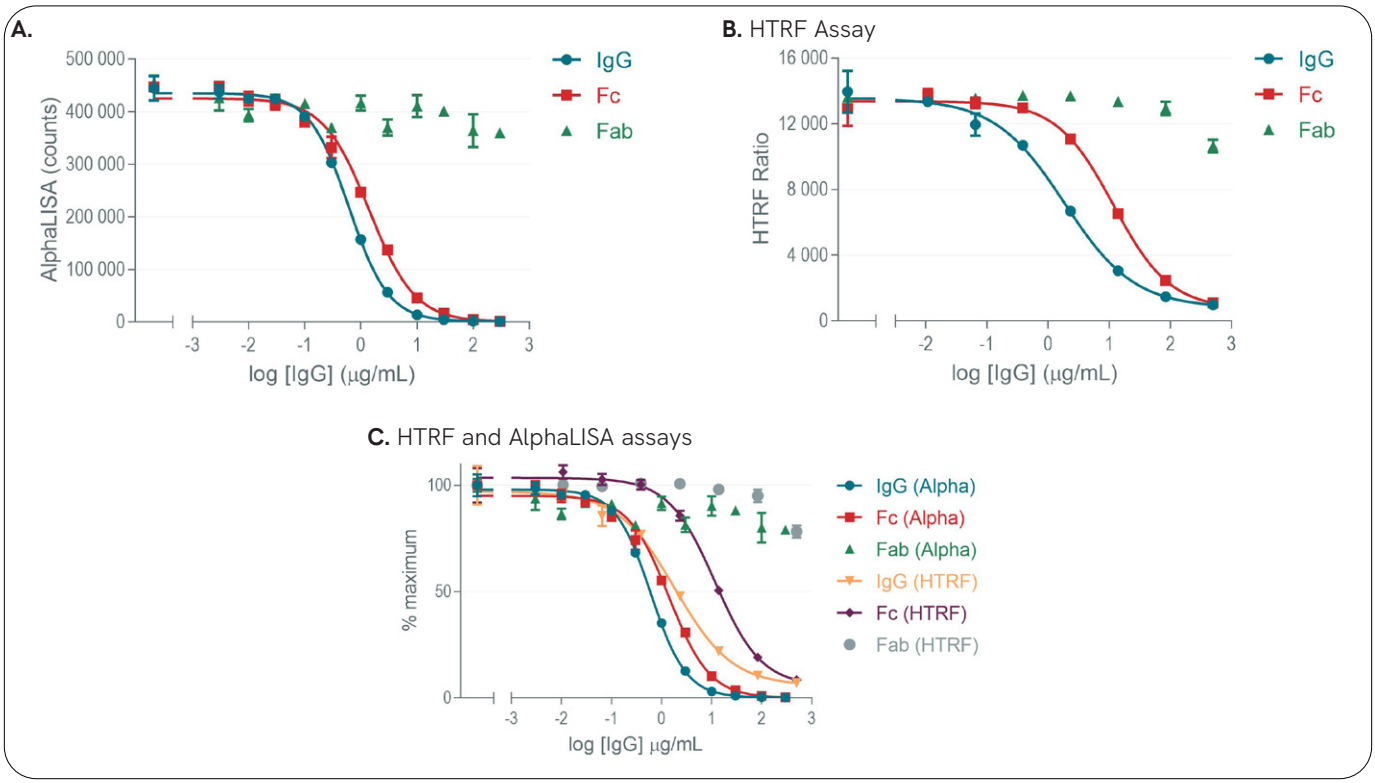


Figure 5: FcRn binding assay for whole IgG (IgG), Fc or Fab binding. The AlphaLISA assay is shown in (A) and the HTRF assay is shown in (B). Overlay of both assays presented as a percent of maximum signal is shown in (C). The IgG concentrations for HTRF were adjusted to final concentration for better comparison.

Table 1: IC_{50} values for antibodies tested in figures 5

IC_{50} (µg/mL)	IgG	FC	Kit Standard
AlphaLISA	0.60	1.24	N/A
HTRF	1.86	11.62	1.87

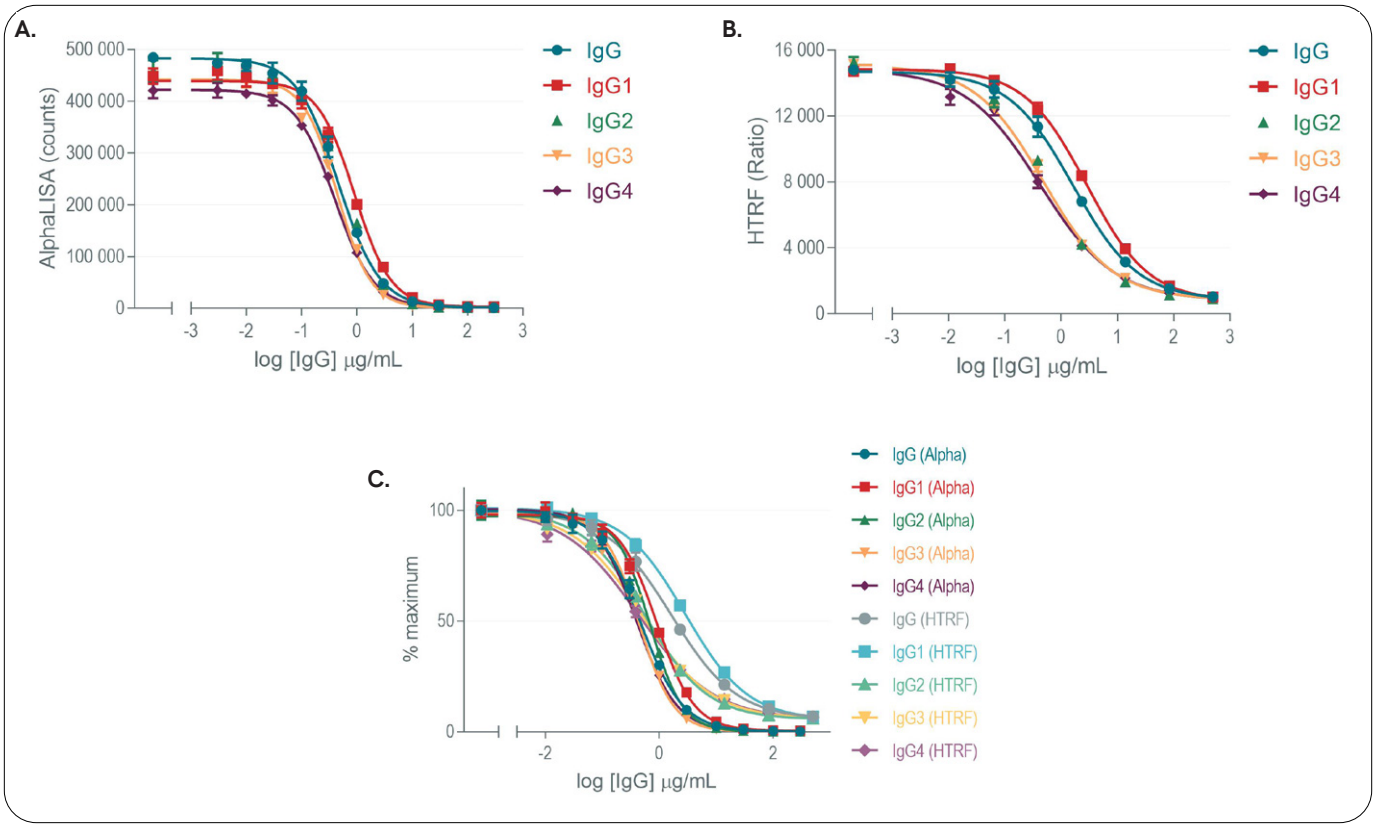


Figure 6: FcRn binding assay for measuring binding of human antibody subtypes in the AlphaLISA assay (A) and the HTRF assay (B). Overlay of both assays presented as a percent of maximum signal is shown in (C). The IgG concentrations for HTRF were adjusted to final concentration for better comparison.

Table 2: IC_{50} values for antibodies tested in figures 6

IC_{50} (µg/mL)	IgG	IgG1	IgG2	IgG3	IgG4
AlphaLISA	0.51	0.79	0.58	0.41	0.42
HTRF	1.66	2.84	0.59	0.54	0.47

Robustness of assays

Since binding to FcRn is highly pH dependent, it is important that the assay reflects binding sensitivity to pH, yet is robust enough that samples with different formulations can be tested. To show this, the assay buffers for AlphaLISA and HTRF were adjusted to pH 7.4 and the whole IgG was run in the competition assay (Figure 7). No signal is seen for the assay run at pH 7.4 which is consistent with the pH dependence of FcRn binding to the antibodies.

There are some commercially available FcRn binding assays that require a buffer exchange and pH adjustment step prior to running FcRn binding assays. For both AlphaLISA and HTRF, this step is not necessary. The whole IgG was diluted into three different buffers and tested in the FcRn binding assays. For the highest concentration in each curve, the sample is in 100% formulation buffer and then diluted into each respective kit assay buffer. Buffer #1 contains 10 mM histidine-HCl, 2% trehalose, 0.01% polysorbate-80, pH 5.5 and buffer #2 contains 10 mM sodium phosphate, 150 mM sodium chloride, 0.001% polysorbate-80, pH 7.2. As shown in Figure 8, the buffering capacity and robustness of the assays allows for testing samples with different formulations.

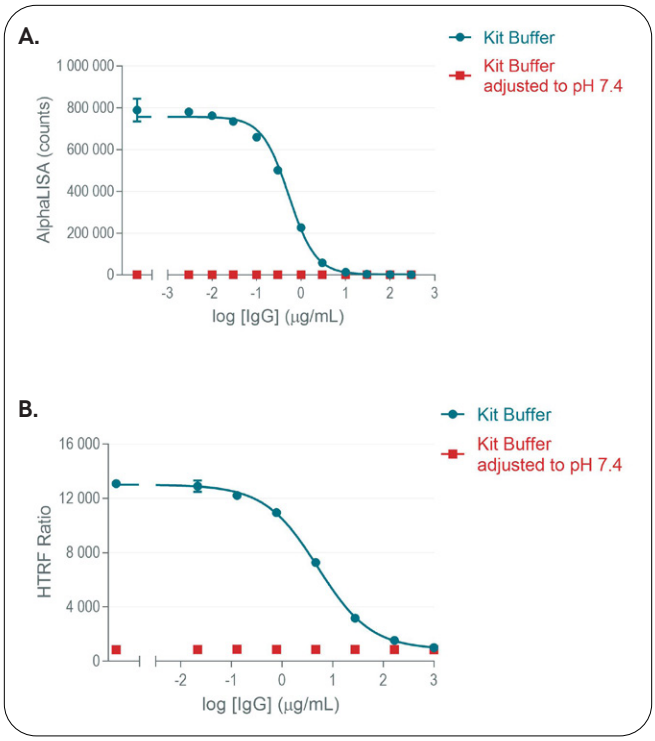


Figure 7: Assay pH dependence. Kit buffers for both assays are pH <6.0. The AlphaLISA assay is shown in (A) and the HTRF assay is shown in (B). The IgG concentrations for HTRF were adjusted to final concentration for better comparison.

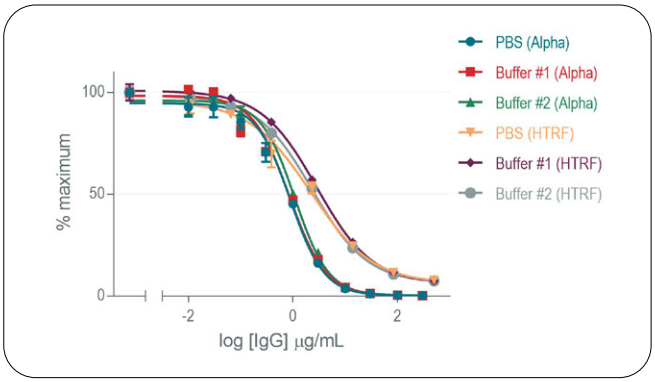


Figure 8: Dependence of assays on formulation of sample buffer. Buffer #1 contains 10 mM histidine-HCl, 2% trehalose, 0.01% polysorbate-80, pH 5.5 and buffer #2 contains 10 mM sodium phosphate, 150 mM sodium chloride, 0.001% polysorbate-80, pH 7.2. The IgG concentrations for HTRF were adjusted to final concentration for better comparison.

These assays can be utilized for screening engineered antibodies for FcRn binding. To show the robustness of these assays for screening, the Z′ value was calculated for each assay. The robustness of each assay was also measured by looking at the day-to-day variation in the IC_{50}. Eight different whole IgG curves were run on five different days. The curves were plotted in Figure 9 as a % maximum to account for day-to-day variations in the maximum counts in the assay. Overall the IC_{50} values are robust, showing excellent reproducibility. The interday variance (%CV) for the AlphaLISA assay is 9.5% and for HTRF is 16.5%, both of which are excellent values.

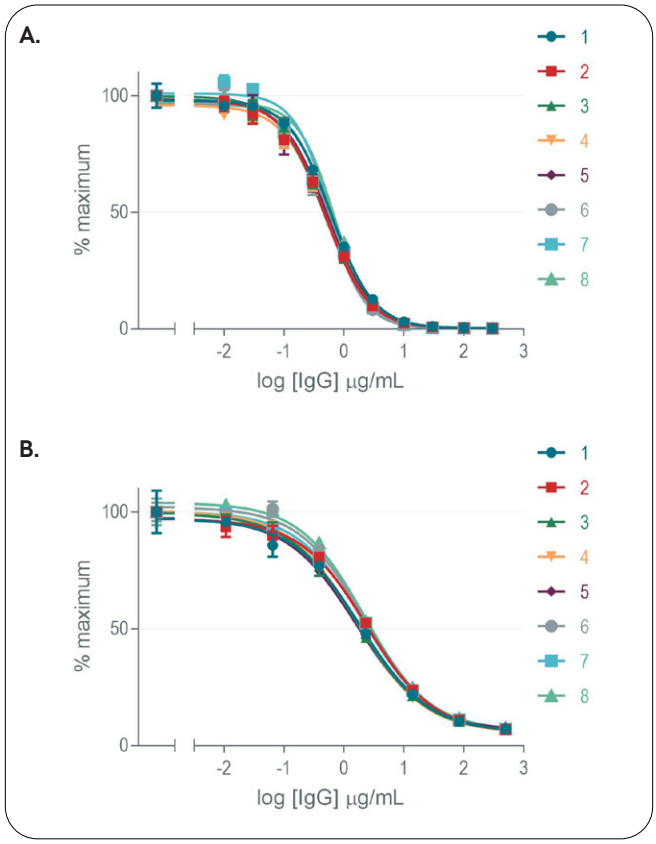


Figure 9: Reproducibility of assays over multiple days. Data used to calculate inter-day %CVs. The AlphaLISA assay is shown in (A) and the HTRF assay is shown in (B). The IgG concentrations for HTRF were adjusted to final concentration for better comparison.

Table 3: Robustness of assays

*variance of independent experiments to determine IC_{50} between subsequent days

	Average IC_{50}	Inter-day Variance %CV IC_{50} *	Z'
HTRF	1.95 µg/mL	16.5	0.72
Alpha	0.51 µg/mL	9.5	0.85

Evaluating assays with therapeutic antibodies

Proving bio-similarity of a monoclonal antibody therapeutic requires many robust assays that show equivalent parameters. One of these parameters is binding affinity to FcRn which is indicative of the serum half-life. To further validate these FcRn assays for use in measuring relative binding affinities for biosimilar research, we looked at FcRn binding of four different commercially available therapeutic antibodies. Adalimumab, which binds to TNFα has an IgG1 format, pembrolizumab is a highly selective IgG4-kappa humanized monoclonal antibody against PD-1 receptor, and trastuzumab is a recombinant humanized IgG1 monoclonal antibody against the HER-2 receptor (6). Etanercept, which also binds TFNα, is a dimeric fusion protein consisting of the extracellular ligand-binding portion of tumor necrosis factor receptor (TNFR) linked to the Fc portion of a human IgG1 (7). All four therapeutics are expected to bind to FcRn. Figure 10 and Table 3 show the relative binding affinities of these four therapeutic antibodies to FcRn using the two orthogonal FcRn binding assays.

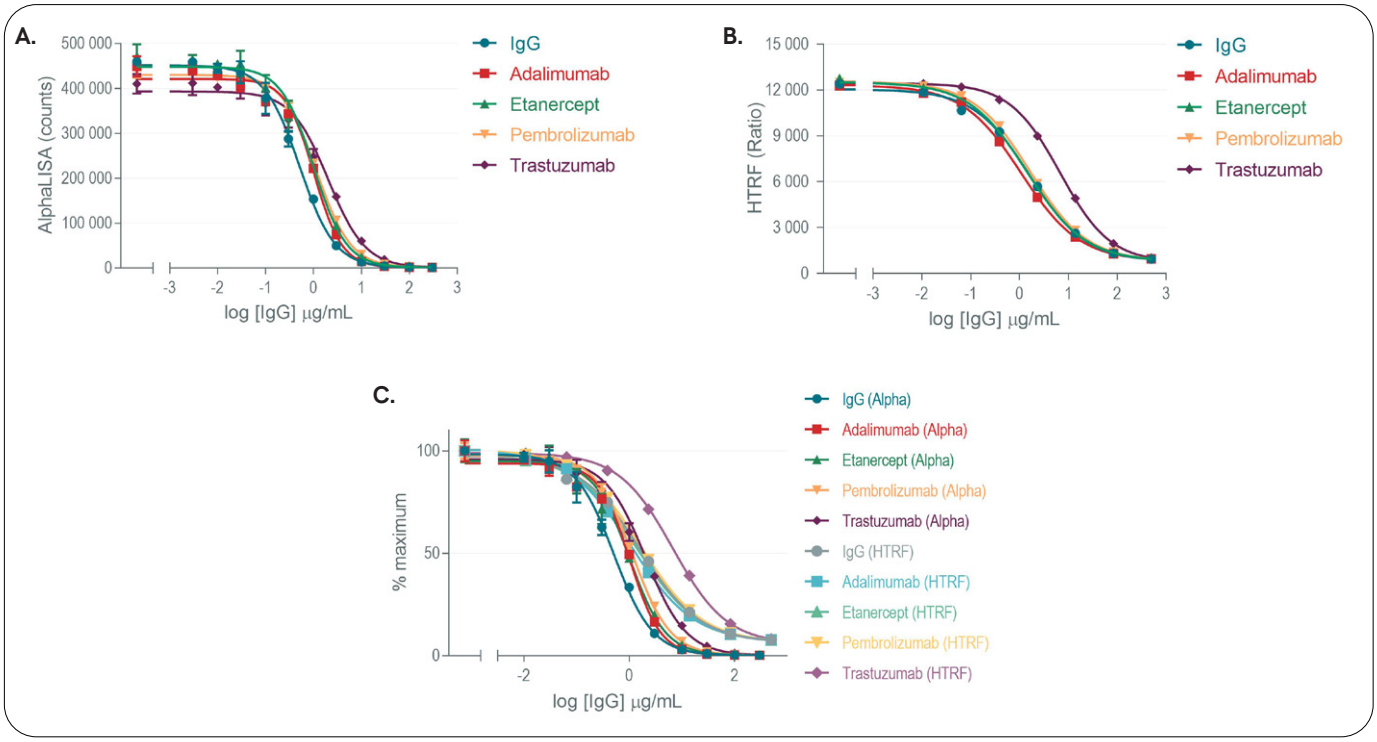


Figure 10: FcRn binding assays for a selection of therapeutic antibodies. The AlphaLISA assay is shown in (A) and the HTRF assay is shown in (B). Overlay of both assays presented as a percent of maximum signal is shown in (C). The IgG concentrations for HTRF were adjusted to final concentration for better comparison.

Table 4: IC_{50} values (µg/mL) for therapeutic antibodies in figure 9

IC_{50} (µg/mL)	IgG	Adalimumab	Etanercept	Pembrolizumab	Trastuzumab
AlphaLISA	0.50	0.95	0.86	1.11	1.71
HTRF	1.59	1.10	1.39	1.65	6.76

Oxidation of methionine 252 and 428 in the Fc portion of IgGs has been shown to decrease binding to FcRn (8). In fact, previous studies show a change in binding of an Fc fusion protein to FcRn using Alpha technology after oxidation with 0.1% hydrogen peroxide (9). Therefore, the AlphaLISA and HTRF assays were tested for detecting oxidation of adalimumab over time. For this, 0.3% hydrogen peroxide was added to an aliquot of adalimumab and samples taken at time 0, 1 hour, 3 hours, and after overnight. As shown in Figure 11, both assays can detect the decreased binding to FcRn in a time dependent manner after incubation with hydrogen peroxide. The overall signal and IC_{50} values at Time 0 are within error of previous measurements, indicating that the hydrogen peroxide is not interfering with either assay. This data shows that significant potency shifts can easily be detected and indicates that these assays are suitable for looking at forced degradation of therapeutic antibodies by oxidation with hydrogen peroxide.

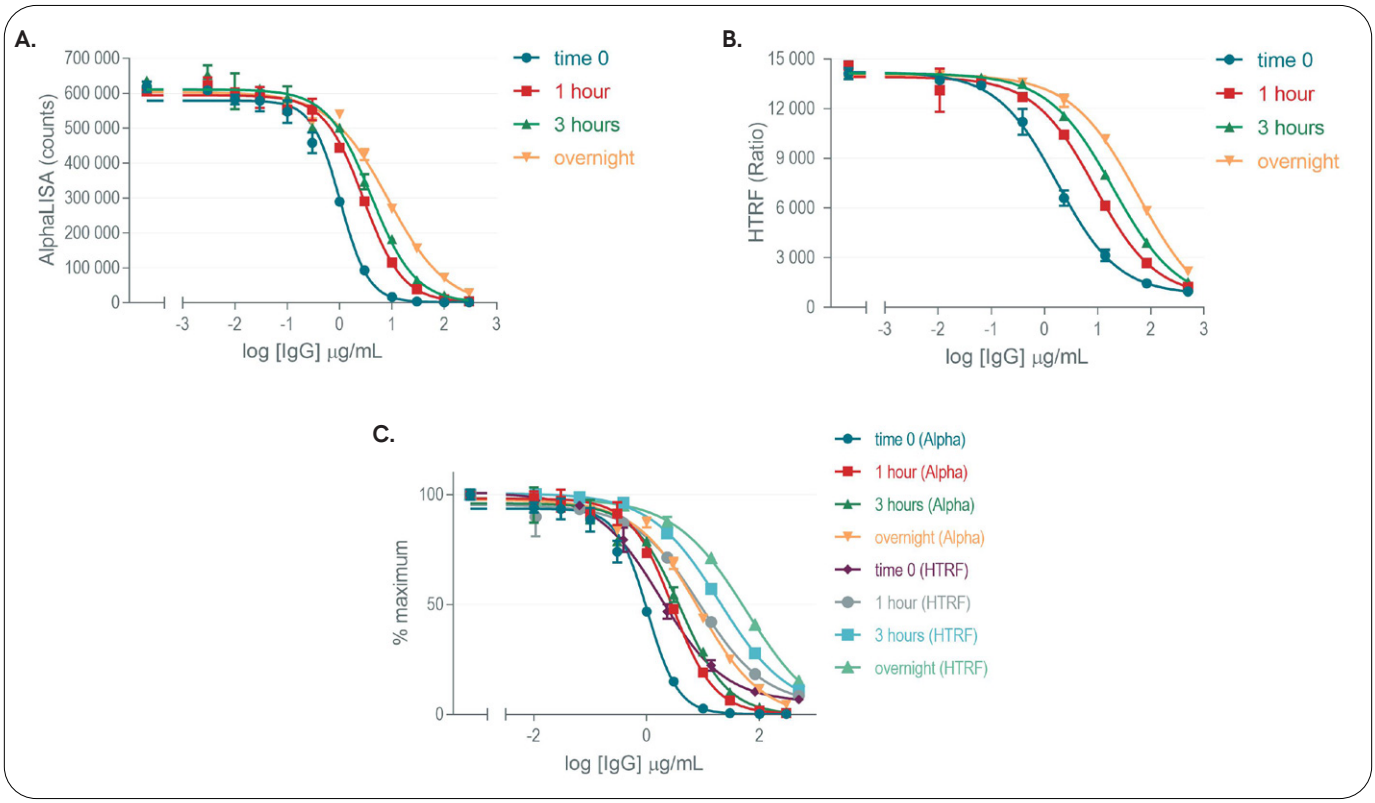


Figure 11: Forced degradation of adalimumab using 0.3% H_2O_2. The AlphaLISA assay is shown in (A) and the HTRF assay is shown in (B). Overlay of both assays presented as a percent of maximum signal is shown in (C). The IgG concentrations for HTRF were adjusted to final concentration for better comparison.

Table 5: IC_{50} values after oxidation with 0.3% H_2O_2

IC_{50} (µg/mL)	Time 0	1 hour	3 hours	overnight
AlphaLISA	0.87	2.77	4.05	8.69
HTRF	1.76	9.01	18.96	~64

Summary

Reliable FcRn binding assays are important in both discovery and development of therapeutic antibodies. These assays can predict the half-life of therapeutic antibodies in *Vivo*. Here, we showed two orthogonal robust high throughput no-wash assays that can be used to measure relative affinities of therapeutic antibodies to FcRn. Both assays are suitable to screening modifications of the Fc portion of a therapeutic antibody for potency shifts in binding FcRn or comparing a biosimilar to a therapeutic antibody drug.

The small total assay volume (20 µL) and minimal overall hands on time of the HTRF FcRn binding assay makes it attractive for routine biologics testing. The high sensitivity of the AlphaLISA FcRn kit and its noteworthy signal to background robustness enables easy assessment of precious and low-concentrated samples. Using more than one assay format to determine the relative potencies of antibodies binding to FcRn can build confidence in the data.

References

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