APPLICATION NOTE

High throughput assays to compare performance and stability of an engineered Fc Silent™ antibody to a therapeutic antibody.

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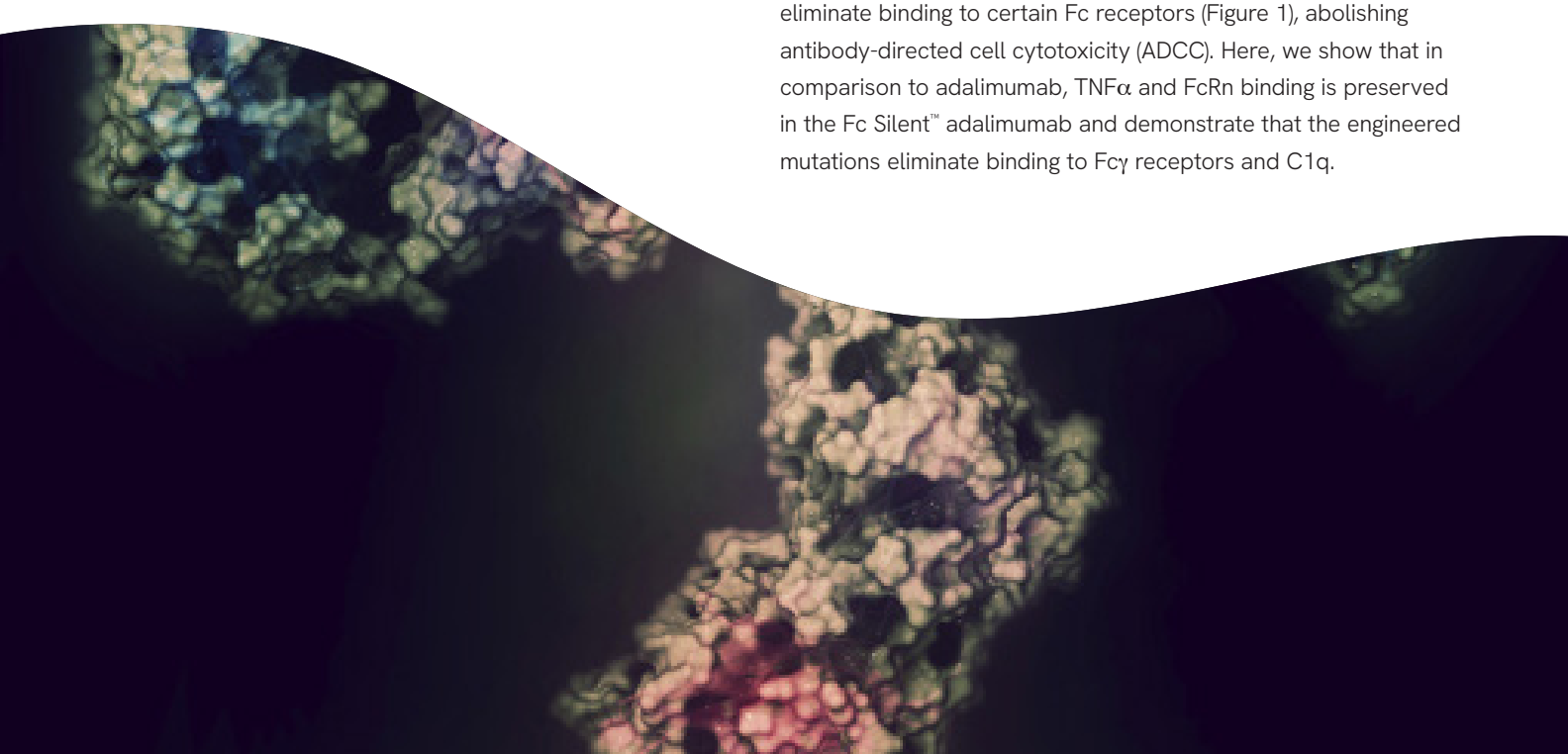
Introduction

Therapeutic antibodies have emerged as the predominant class of new drugs over the last decade, where the world's top-selling therapeutics are antibody drugs such as Keytruda® and Humira®. Fast and accurate methods for functionally evaluating and characterizing antibody therapeutics are necessary during both discovery and development stages. During development, an antibody therapeutic may be further engineered to improve certain characteristics over the original therapeutic, such as increasing half-life or decreasing toxicity. Therefore, it is important to demonstrate that the engineered antibody continues to exhibit similar characteristics as the original therapeutic with respect to efficacy and stability. Fc receptor binding assays, C1q binding assays, target specific binding assays, cell-based neutralization assays, ADCC assays, CDC assays, and apoptosis assays are all techniques that can be used to show functional similarity between the original therapeutic and an engineered or biosimilar antibody drug.[1,2]

For this study, a suite of assays was used to compare Adalimumab, an antibody drug that has been used to treat multiple inflammatory diseases, to an engineered version (adalimumab Fc Silent™) that contains mutations in the Fc region. Adalimumab is a recombinant human IgG1 monoclonal antibody that blocks the interaction of TNFα with its receptors, TNFR1 and TNFR2,3 thereby interfering with TNFα-induced signaling cascades. The "Fc Silent™" Adalimumab antibody has been engineered such that the Fc domain contains mutations that eliminate binding to certain Fc receptors (Figure 1), abolishing antibody-directed cell cytotoxicity (ADCC). Here, we show that in comparison to adalimumab, TNFα and FcRn binding is preserved in the Fc Silent™ adalimumab and demonstrate that the engineered mutations eliminate binding to Fcγ receptors and C1q.



Finally, using forced degradation experiments, we show that the two antibodies have similar thermal stability under the conditions tested.

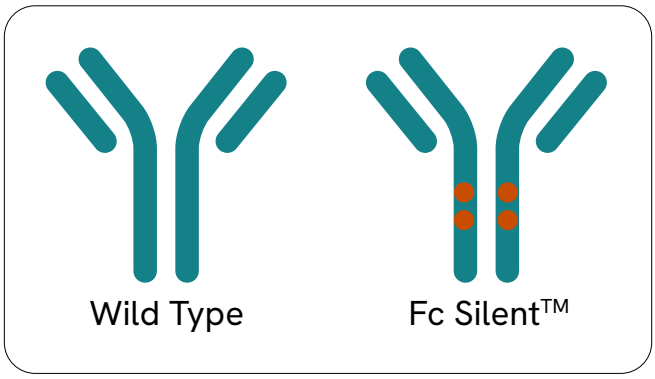


Figure 1: Wild type IgG compared with Fc Silent™ IgG. Fc Silent™ version of adalimumab from Absolute Antibody contains mutations in the Fc region of the antibody.

Materials and methods

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 (Figure 2). The AlphaLISA™ assays used in this application note are the Human FcRn AlphaLISA Binding Kit (cat# AL3095C), FCGR1/CD64 AlphaLISA Binding Kit (cat# AL3081C), IL-8 (human) AlphaLISA Detection Kit (cat# AL224C), and Alpha *SureFire® Ultra™* Multiplex phospho/total NFκB Assay Kit (cat# MPSU-PTNFKB-K500).

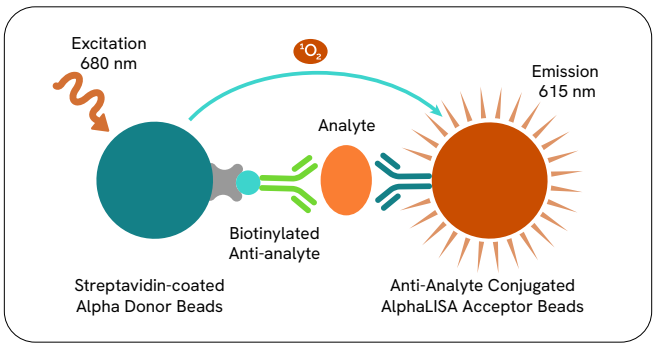


Figure 2: AlphaLISA Schematic for Analyte Detection Assays.

HTRF assays

HTRF™ (a TR-FRET technology) 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 biomolecules are in proximity, energy transfer between the two fluorescent dyes can be measured and is proportional to the number of biomolecules interacting. Upon excitation at 320 nm of the donor, FRET occurs, resulting in light emission at 665 nm (Figure 3). The HTRF assays used in this application note are TNFα-TNFRI binding (cat# 64BDTNFPEG), CD16a (FcgRIIIa, V158) cellular binding kit (cat# 62C16PAG), and C1q binding (cat# 64C1QPET).

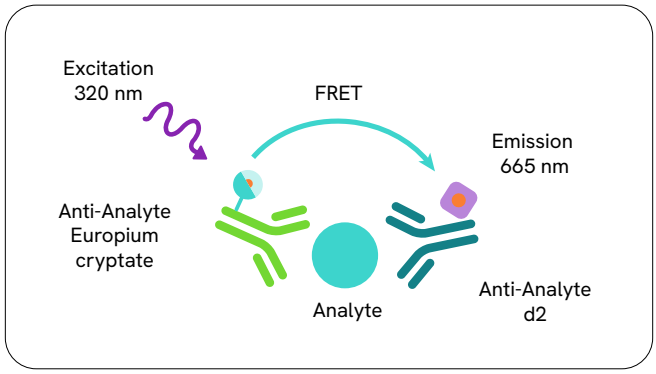


Figure 3: HTRF Schematic for Analyte Detection Assays.

LabChip protein express assay

Fragment assessment experiments performed on a LabChip™ GXII Touch™ HT protein characterization system used microchip capillary electrophoresis-based separation. Molecular weight sizing, concentration analysis, and percent of total area for each species of the antibody sample was evaluated using the LabChip Protein Express chip (cat # 760499) and LabChip Protein Express reagent kit (cat# CLS960008). Samples were prepared following the protocol in Protein Express assay user guide using HardShell PCR Plate-96 microplates (cat# 6008870) and run using default assay parameters on LabChip GXII Touch instrument.

Reagents

The antibodies used in the experiments were purchased from Absolute Antibody (Adalimumab cat# Ab00718-10.0; Adalimumab Fc Silent™ cat# Ab00718-10.3). Recombinant TNFα was purchased from Sigma (cat# SRP3177-50UG).

Cell culture

HCT116 cells (ATCC cat# CCL-247) were cultured in McCoy's 5A medium (ATCC cat# 30-2007) with 10% FBS (ThermoFisher cat# 16140071). Cells were treated with 20 ng/mL (final) of recombinant TNFα overnight (IL-8 release) or for 30 minutes (NFκb activation).

Data analysis

Data were analyzed using GraphPad version 7 software. The binding curves were fitted using nonlinear regression (assuming there is one binding site and none of the signal comes from non-specific binding) with a four-parameter logistic equation (sigmoidal dose-response curve with variable slope) with 1/Y2 weighting. Inhibition curves were generated using log (inhibitor) vs. response -- Variable slope (four parameters) function. HTRF™ data is reported as the ratio of acceptor to donor signal = (665 nm/620 nm)*10,000.

Instrumentation and data collection

Alpha and HTRF assays were measured using a Revvity EnVision™ 2105 multimode plate reader. Barcode #444 mirror (#2101-4010) and AlphaScreen 570/100 filter barcode #244 (cat #2100-5710) was used for the AlphaLISA assay. A laser with APC 665 nm and Cy5 620 nm filters and the LANCE/DELFIA Dual bias mirror was used for the HTRF assays. 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) *104. The terbium barcode #701 filter (cat# 2100-5930), europium barcode #203 filter (cat# 2100-5090), and barcode #605 (cat# 2102-5910) mirror was used for the AlphaPlex assays. For the multiplexing assays, the plate was read sequentially with the terbium channel read first.

Results and discussion

I. Binding Adalimumab and the Fc Silent™ Adalimumab to TNFα and FcRn

After engineering mutations in the Fc region, it is important to show that the binding to the specific target of the antibody is preserved. Adalimumab binds specifically to TNFα and blocks TNFα binding to its receptor. Therefore, the relative binding of adalimumab and adalimumab Fc Silent™ to TNFα was determined using the human TNFα/TNFR1 HTRF binding kit. Each antibody was diluted into the provided assay kit buffer and the ability to block the TNFα/TNFR1 interaction was measured. As shown in Figure 4a, Adalimumab and the Fc Silent™ version can equally compete for the TNFα/TNFR1 interaction (IC50 values of 0.49 nM), indicating a similar binding affinity to TNFα.

Binding to FcRn is important for increased serum half-life of the antibody drug. As the mutations in the Fc region of the Fc Silent™ antibody are not supposed to affect binding to FcRn, relative binding to FcRn was assessed using an AlphaLISA FcRn binding assay. Each antibody was diluted into the provided assay kit buffer and the ability to compete with an FcRn-human IgG interaction was measured. As shown in Figure 4b, Adalimumab and the Fc Silent™ version bind with similar affinity (within three-fold) to FcRn with IC50 values of 0.96 and 0.38 µg/mL, respectively.

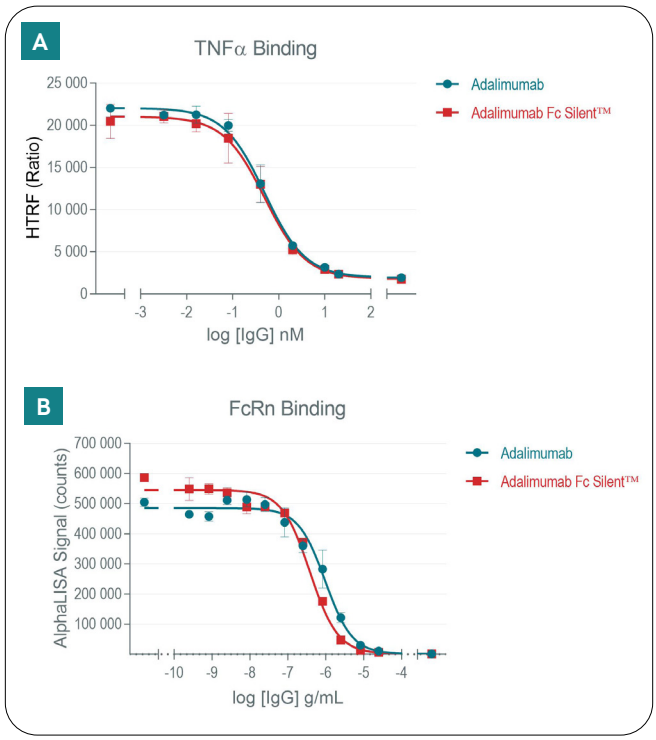


Figure 4: Comparison of binding affinity to TNFα (A) and FcRn (B).

II. Mutations in the Fc region eliminate binding to Fcγ receptors and C1q

Binding to Fcγ receptors is important for ADCC. For some antibody therapeutics, binding to these receptors is critical for their function. However, if the function of the therapeutic is not for cell cytotoxicity, engineering the antibody to eliminate this function can reduce toxicity of the drug. Since the Fc Silent™ version of Adalimumab studied here was engineered to eliminate ADCC, the relative binding of

Adalimumab and the Fc Silent™ version to FcγRIa and FcγRIIIa receptors was measured. To measure FcγRIa binding, an AlphaLISA competition assay was performed. As shown in Figure 5a, the wild type Adalimumab competes with an IC50 of 0.17 µg/mL and, as expected, no competition is seen for the Fc Silent™ version. For assessment of FcγRIIIa binding, an HTRF competition assay was performed. As shown in Figure 5b, Adalimumab competes with an IC50 of 0.3 µM and, as expected,no competition is seen for the Fc Silent™ version

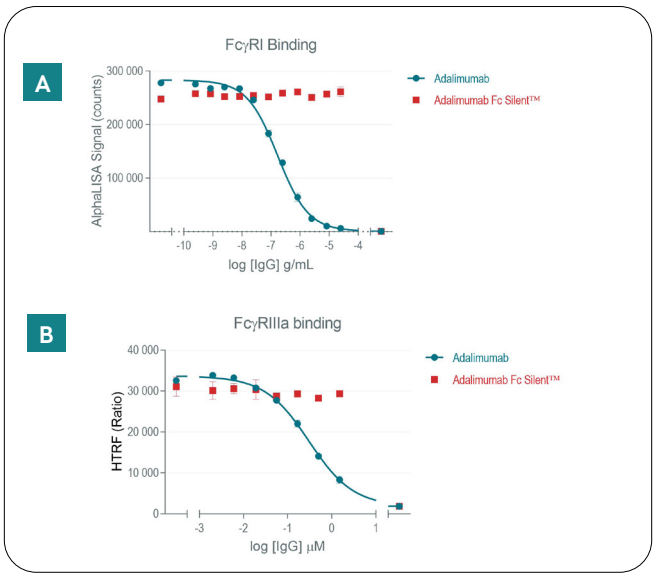


Figure 5: Binding to Fcγ Receptors, FcγRIa (A) and FcγRIIIa (B).

The complement component 1q (C1q) is part of the innate immune system. C1q binds to antibodies that are bound to an antigen on a cell surface and helps clear microbes and damaged cells. The binding of an antibody to C1q is important for complement-dependent cytotoxicity (CDC). Therefore, the relative binding of Adalimumab and the Fc Silent™ version to C1q was compared using HTRF. As shown in Figure 6, Adalimumab binds to C1q and, as expected, the Fc Silent™ version does not since it was engineered to eliminate cell cytotoxicity.

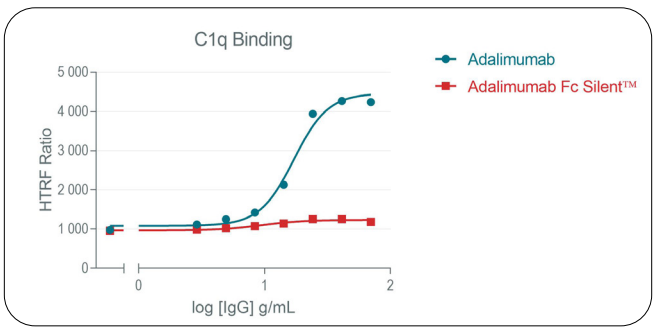


Figure 6: Binding to C1q

III. Neutralization of TNFα is similar between Adalimumab and the Fc Silent™ Adalimumab

To compare the ability of these therapeutic antibodies to function in a cellular system, TNFα neutralization assays were performed. TNFα binding to TNFR1 initiates intracellular signaling pathways that leads to NFκB activation and induces pro-inflammatory cytokine production, such as IL-8 release. To test the ability of these antibodies to suppress TNFα activation of NFκb in cells, HCT116 cells were treated with 20 ng/mL of soluble TNFα in the presence or absence of various concentrations of Adalimumab or the Fc Silent™ version for 30 minutes. The cells were lysed and both phosphorylated NFκb and total NFκb were measured using the Alpha *SureFire® Ultra™* Multiplex phospho/total NFκB Assay Kit. The ratio of the phosphorylated NFκb to total NFκb was plotted as a function of antibody concentration. As shown in Figure 7, TNFα induces an increase in the phosphorylation of NFκb and both Adalimumab and the Fc Silent™ version can similarly suppress the activation of NFκb.

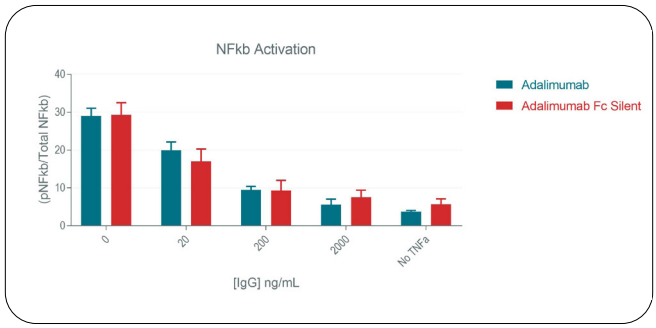


Figure 7. NFκb Activation by TNFα and Neutralization by Adalimumab.

Next, the neutralization assay was performed measuring pro-inflammatory cytokine production. HCT116 cells were stimulated with 20 ng/mL of TNFα overnight in the presence or absence of Adalimumab or the Fc Silent™ version. Then supernatants were collected, and IL-8 was measured using the human IL-8 AlphaLISA assay kit. A recombinant IL-8 standard curve was run in the appropriate media (Figure 8a) and the signal from the supernatants was interpolated from the standard curve to determine the levels of IL-8 secreted in the media. The amount of IL-8 from each well was plotted as a function of antibody concentration. The Adalimumab and the Fc Silent™ version had similar IC50 values (5.9 ng/mL and 6.6 ng/mL, respectively) (Figure 8b) showing that they exhibit similar functionality in the context of cellular signaling.

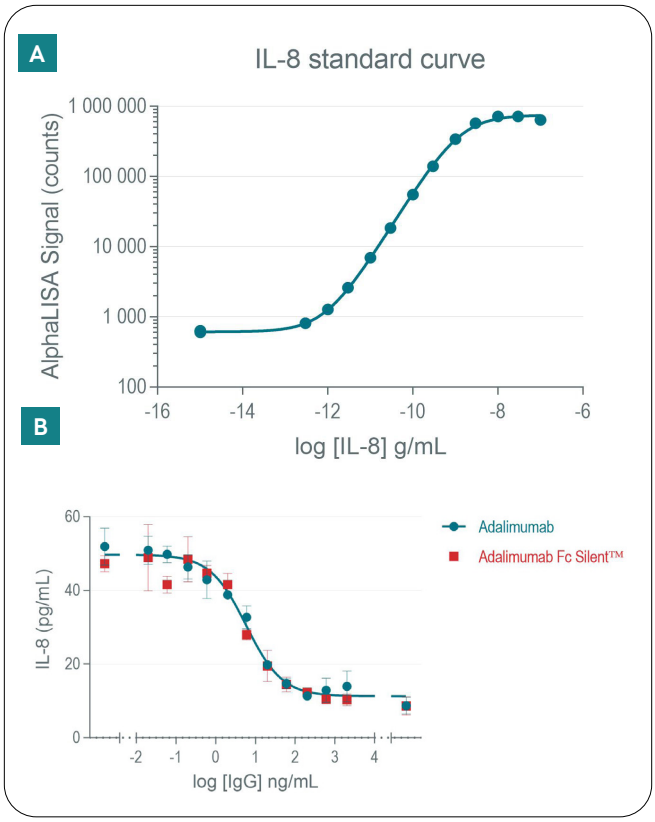


Figure 8. IL-8 release. A) Standard IL-8 curve used to interpolate the amount of IL-8 released into supernatants. B) IL-8 released after TNFα stimulation in the presence or absence of therapeutic antibody.

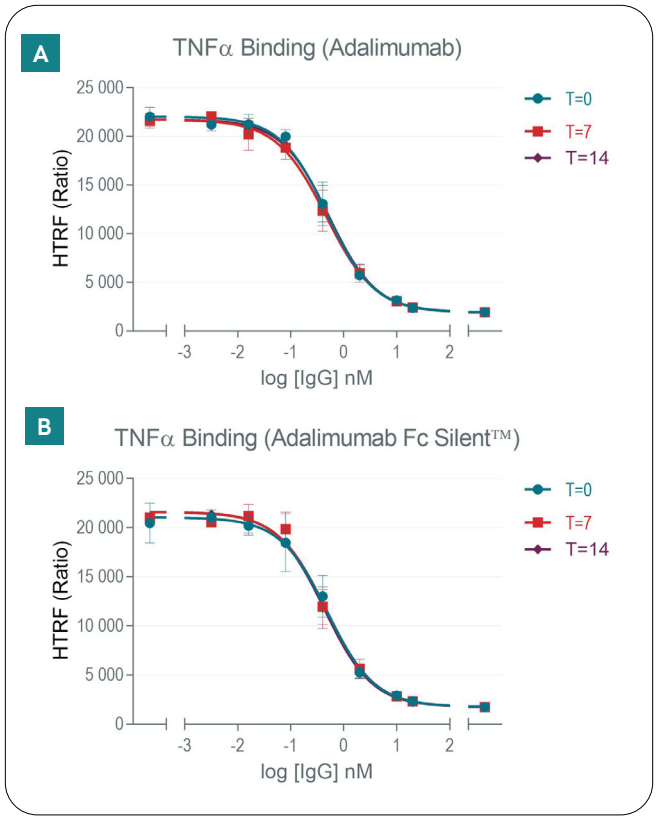
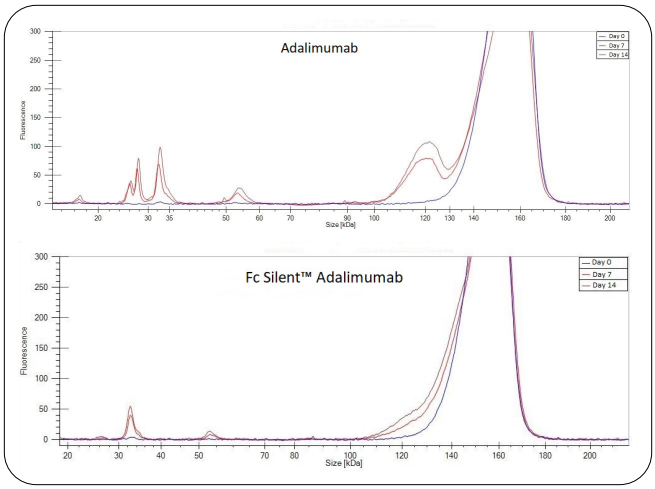


Figure 9. TNFα binding to Adalimumab (A) or Adalimumab Fc Silent™ (B) after thermal stress.

IV. Comparison of antibody stability under thermal stress

Another parameter to compare a biosimilar or engineered antibody to the original therapeutic is stability. The stability of a therapeutic can be assessed using forced degradation studies, such as the effect of thermal stress on a therapeutic. Various methods have been used to study the effects of thermal stress on therapeutic antibodies which include both analytical methods and functional assays. 4 High temperature can accelerate degradation pathways such as aggregation, fragmentation and deamidation. Here, we tested the effect of incubation of the two antibodies at 50°C for 7 and 14 days. Following this, the binding to TNFα was measured using the TNFRI-TNFα HTRF binding assay. As shown in Figure 9, both antibodies retained binding activity to TNFα after 14 days at 50°C. This suggests that the engineered mutations in the Fc region do not affect the stability of the therapeutic under these conditions.

To further verify that the engineered Fc Silent™ version has similar stability as Adalimumab, an orthogonal technology was used to assess fragmentation of the antibody after the thermal stress. For this, the high-throughput microfluidic LabChip™ electrophoretic separation assay was run. LabChip™ assays have previously been used to assess monoclonal antibody degradation from thermal stress.5 As shown in Figure 10, small degradation products increase over time, but contribute to a very low percentage of the total area, leading to a high percent purity of both antibodies. Interestingly, Adalimumab shows an increasing shoulder peak that does not significantly occur in the Fc Silent™ version. The shoulder peak may be a degradation product that can still bind TNFα. Further studies, such as mass spectrometry on the various peaks, would need to be performed to fully understand the differences in the fragmentation seen between the two antibodies.



	Day 0	Day 7	Day 14
Adalimumab			
IgG (Main Peak)	100%	87.1%	83.0%
IgG Shoulder	0%	9%	13%
LMW Peaks	0%	3.9%	4%
Adalimumab Fc Silent™			
IgG (Main Peak)	100%	99.5%	97.4%
IgG Shoulder	0%	0%	0%
LMW Peaks	0%	0.5%	2.6%

Figure 10. Fragmentation after thermal stress measured by LabChip Protein Express assay.

Conclusions

Assays to functionally characterize and evaluate antibodies can be used in the discovery process as well as the development stages of antibody therapeutics. Here we show a suite of assays that can be used to compare an engineered antibody to the original therapeutic antibody. Overall, the ease of use and the minimal sample consumption make Alpha and HTRF assays ideal for quickly comparing binding affinities to the target or Fc receptors or for readouts in functional cell-based assays. In addition, these assays can be used for assessing binding capabilities after thermal stress, giving an insight into the stability of the antibody.

The LabChip Protein Express assay, which leverages the microfluidics capillary electrophoresis technology, is highly sensitive and can separate small degradation products, hence providing further insight into the extent to which the antibody has degraded after stress. Using a variety of orthogonal technologies in a biologics workflow provides critical data to build confidence in a biosimilar or an engineered therapeutic antibody.

References

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