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Combining Binding and Functional Analyses for Comparison of Anti-TNFα Monoclonal Antibody Biosimilars

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Abstract

Generating biosimilars of existing biological medicines can result in lower cost drugs that can be provided to more patients compared to the original product. The continued emergence of novel biosimilars requires high-throughput, reliable techniques for comparing biosimilars during development and manufacture. Here we utilized a combined iQue® Advanced Flow Cytometry and Octet® Biolayer Interferometry workflow to compare the products of an adalimumab clone elution buffer optimization process. Binding and Fc function assays performed using the iQue® platform indicated a general enhancement of activity of antibodies eluted at high pH and in the presence of L-Arginine. These same conditions led to increased kinetic rate constants for binding to antigen (TNFα) and FcγRIIIa receptor (CD16a) as measured using Octet® BLI assays. These data display the advantage of a dual platform approach to generate a full profile of biosimilar antibody activity *in vitro*.

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Introduction

Biosimilars are biological medicines that are generated to be highly similar in structure and function to an already approved product. They can be released after patent expiration of the reference product on which they are based.[1] Since biosimilars are based upon an existing product, the cost of their development is much lower than the original, resulting in a drug that can be marketed at a lower price. This can reduce healthcare costs and provide greater access of the drug compared to the reference product.[2] Biosimilars are now available as treatments in a range of therapeutic areas, from inflammatory diseases to cancers, for example monoclonal antibody (mAb) biosimilars of rituximab and trastuzumab.[3,4]

Adalimumab (Humira®) is a TNFα-targeting mAb that is used to treat a wide range of inflammatory diseases, such as rheumatoid arthritis and Crohn's disease.[5] Despite receiving regulatory approval in 2016, the Humira® biosimilar Amjevita (Amgen) only launched in February 2023, which marked the end of Humira® market exclusivity.[6,7] In coming years, there is expected to be an increase in biosimilars entering the pharmaceutical market, meaning techniques to quantify their function relative to their reference medicinal product (RMP) will be essential.[8]

It is accepted that the inherent variability that can occur in biologics, due to differences in the source or manufacturing process used, can result in small differences between a biosimilar and its RMP. Rigorous testing is performed during biosimilar development and manufacturing to ensure that these differences do not result in clinically meaningful differences in product quality, safety or efficacy.[1] A crucial part of this testing process is to compare performance of the biosimilar to the RMP in a range of *in vitro* functional assays.[9] Traditional methods for comparing the activity of biosimilars may be limited due to:

- Use of instrumentation with low-throughput acquisition (e.g., traditional flow cytometry).
- Requirements for large volumes of precious antibody samples.
- Laborious and time-consuming workflows, requiring steps such as protocol optimization, fixation, and multiple washes.
- The need for complex and lengthy data analysis.

In this application note, the iQue® Advanced Flow Cytometry Platform and Octet® Biolayer Interferometry (BLI) System have been used to compare the binding and function of anti-TNFα (adalimumab) mAbs. The iQue® platform was used to perform high-throughput measurement of mAb binding to live cells and quantification of *in vitro* functional activity. This data was complemented with real-time assessment of mAb binding kinetics to the TNFα target and FcγRIIIa receptor CD16a V176 using the Octet® BLI system.

Methods

Antibodies

An adalimumab clone was prepared by Cellca GmbH using the primary amino acid sequence of Humira® (adalimumab). A single clone from a perfusion process was prepared and affinity column buffer optimization performed. Samples of adalimumab were captured on a Protein A column and eluted using 100 mM glycine buffer at pHs 3.0, 3.4, 3.6, 3.8. The pH 3.6 and 3.8 buffers were also used with the addition of 1 M L-arginine. Eluates were neutralized with Trizma pH 9.0 and further diluted using PBS (1:1) before sterile filtration and storage at 4 °C. Humira® RMP was purchased from WEP Clinical. An anti-β-Gal-hIgG1 antibody was used as a control (Invivogen).

Cell Lines

CHO-K1-mTNFα cells from a line that is engineered to stably express membrane bound TNFα were used to assess adalimumab binding and Fc function. Puromycin was used to select for transfected cells during culture. MCF-7 cells, from a breast cancer line, were used as a negative TNFα-expressing control in these experiments. THP-1 cells, from a monocytic leukemia cell line, were used for apoptosis experiments.

iQue® Live-Cell Antibody Binding

Test antibodies (10 µL) were incubated with 10 µL of CHO-K1-mTNFα mixed with MCF-7 cells (10 K/cell type/well) in a 384-well V-bottom plate (Costar 3656) for 30 minutes on ice, to inhibit internalization. MCF-7 cells were pre-labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye (97054) (1:2500) and the CHO-K1-mTNFα cells left unlabeled so the two cell types could be distinguished in-well. Following incubation, plates were washed with PBS + 2% FBS, the media removed, and cells resuspended by shaking on the iQue® plate shaker (2000 RPM).

Fluorophore-conjugated secondary antibody (R-Phycoerythrin-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, Jackson ImmunoResearch) was mixed with iQue® Cell Membrane Integrity (R/Red) Dye (97057) and added to the plate. After a further 30 minutes on ice, plates were washed and run on the iQue® with a 3 - 5 second sip time. Binding was quantified using the iQue Forecyt® software as an increase in the median fluorescence intensity (MFI) for the secondary antibody or as the percentage of cells positive for secondary antibody binding above a defined threshold.

Octet® BLI Analysis of Antigen (TNFα) and FcγRIIIa Receptor (CD16a V176) Binding Kinetics

Octet® SAX Biosensors (18-5117), which are pre-immobilized with Streptavidin, were hydrated for at least 30 minutes at room temperature prior to use in 1x Kinetics Buffer prepared by dilution of Sartorius 10x Kinetics buffer (18-1105) 1:10 with PBS, pH 7.4. 96-well, black, flat bottom microplates (Greiner Bio-One, 6552091) and a temperature of 25 °C were used throughout. Recombinant AviTagTM biotinylated human TNFα and AviTagTM biotinylated human CD16a V176 were purchased from ACRObiosystems. All antibodies and proteins were diluted in 1x Kinetics Buffer. Analysis was performed using an Octet® BLI R8 using Octet® BLI Discovery and Analysis Studio Software version 13.0.1. Data was fitted using Octet® Analysis Studio software to either a 1:1 model (TNFα) or 2:1 model (Heterogenous Ligand) (CD16a V176).

A load scout experiment was performed to determine a concentration of biotinylated TNFα and CD16a V176 that induced a ~0.6 nm and ~1.0 nm loading response, respectively, over the load period. Concentration range testing experiments used a 3-fold serial dilution of Humira® RMP from 300 nM for experiments with TNFα and 5000 nM for CD16a V176. In each case a biotinylated protein-loaded background reference sample was analyzed simultaneously, using 1x Kinetics Buffer alone as the analyte solution. The Humira® RMP concentration used for the kinetic screen was tested across seven simultaneous measurements in triplicate across three experiments (i.e., 63 replicates in total) to confirm low CVs for inter- and intra- assay variability (these were also across multiple days and by multiple users). In the kinetic screen, each adalimumab biosimilar was tested at the defined concentration and compared to a QC sample of Humira® RMP that was included in each kinetic run to confirm assay validity.

iQue® Apoptosis Assay

THP-1 cells were seeded at 15 K/well (20 µL) in a 384-well V-bottom plate (Costar 3656) with 0.3 µg/mL soluble TNFα (20 µL, Sartorius RUO, CYK-0025-1004) and test antibodies (20 µL). After 72 hours incubation (37 °C, 5% CO_2), 20 µL samples were taken and analyzed using the iQue® Human 4-Plex Apoptosis Kit (90053). This kit contains four mix-and-match reagents that can be used for fluorescence-based quantification of multiple apoptosis endpoints per well, using the iQue® platform. These readouts include cell viability, caspase 3/7 activity, annexin V binding and mitochondrial membrane integrity.

iQue® Complement-Dependent Cellular Cytotoxicity (CDC)

Test antibodies (10 µL) were incubated with 10 µL of CHO-K1-mTNFα cells mixed with MCF-7 cells (10 K/cell type/well) in a 384-well plate for 15 minutes. All cells and antibodies were prepared in serum free media. The MCF-7 cells were pre-labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye (1:2500) and the CHO-K1-mTNFα cells left unlabeled so the two cell types could be distinguished in-well. Human serum was added to a final assay concentration of 15% for 45 minutes. Following incubation, plates were washed with PBS + 2% FBS, the media removed, and cells resuspended by shaking on the iQue® plate shaker (2000 RPM). iQue® Cell Membrane Integrity (R/Red) Dye was used to label cells to enable quantification of cell death as a measure of the CDC activity induced by test antibodies.

iQue® Antibody-Dependent Cellular Cytotoxicity (ADCC)

CHO-K1-mTNFα cells (5 K/well) were labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye (provided with the iQue® Human Natural Killer (NK) Cell Killing Kit (97082)) and seeded in 96-well flat bottom plates (Corning® 3595) for 6 hours to allow them to adhere. Test antibodies were added at a range of concentrations alongside unlabeled natural killer (NK) cells (purchased frozen from StemCell Technologies, 20 K/well). After 20 hours, 10 µL samples of supernatant were transferred to V-bottom plates for analysis of IFNγ and Granzyme B cytokine concentrations using 2-plex iQue Qbeads® from the iQue® Human NK Cell Killing Kit. Cells were lifted using Accutase® and transferred to V-bottom plates for labeling using the antibody cocktail provided in the kit. The kit contains antibodies for phenotyping of NK cells with markers CD3, CD56, CD16a, CD25 and CD69 alongside iQue® Cell Membrane Integrity (B/Green) Dye. The gating and analysis template included with the kit was imported into iQue Forecyt® software for quantification of cytokine concentrations using a standard curve and analysis of NK marker expression.

Results & Discussion

To exemplify the use of a combined iQue® Advanced Flow Cytometry and Octet® BLI workflow for biosimilar characterization we compared a range of binding and functional characteristics of adalimumab antibodies. As described in the methods section, a single adalimumab clone was subject to an elution buffer optimization process, with the resulting antibodies assessed here. These were eluted at a range of pH (3.0, 3.4, 3.6, 3.8) and the pH 3.6 and 3.8 buffers were tested with and without the inclusion of L-arginine.

Typically, for IgG purification from Protein A columns, the IgG is first bound to the Protein A-charged column under physiological pH conditions, then a low pH is used for the elution process. However, for adalimumab, low pH conditions have been shown to lead to the elution of aggregated species and driven a need to optimize the elution process. Moreover, the addition of L-arginine to the buffer eluent has been shown to increase the recovery of monomeric, correctly folded antibodies, with a further decrease in aggregation. Here, we assessed samples from an affinity purification of a single clone of adalimumab, and compared them to Humira® RMP, to determine the effects of Protein A column elution buffer on the properties of the final antibody product.

Target Antigen Binding

When developing biosimilar antibodies, it is important to test that binding to the target antigen has not been disrupted and to ensure it is comparable to the RMP. Here we used two complementary techniques to evaluate binding of the adalimumab antibodies to TNFα. The first utilized the iQue® Advanced Flow Cytometry Platform to assess binding to live cells. This provides important information about how the antibodies bind to the native target antigen. An antigen negative cell type can also be included to gain additional information on binding specificity. The second method used to evaluate binding was to assess binding kinetics using the Octet® BLI System, which provides real-time information on antibody binding to biosensor immobilized target proteins. This supplies additional data on the kinetics of the binding interaction between the antibodies and their target, which can have critical effects on its pharmacokinetics *in vivo*.

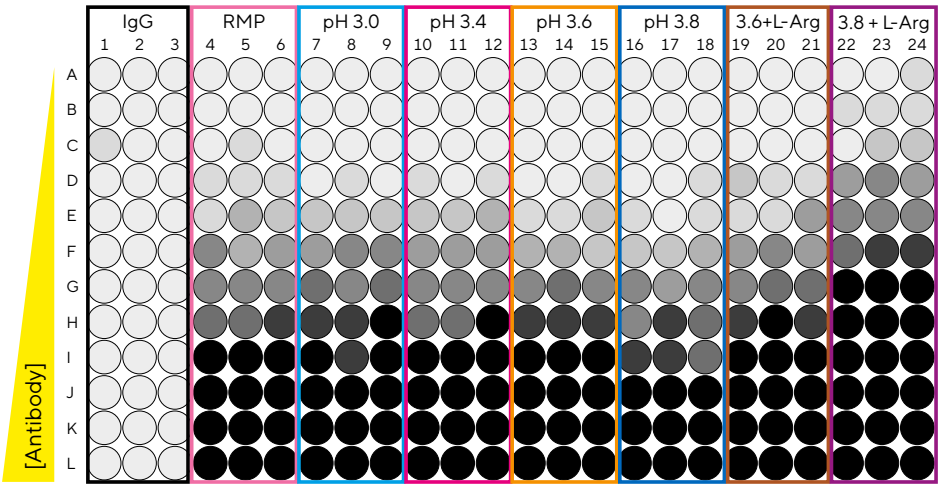
Live-Cell Binding

For the live cell antibody binding assay, CHO-K1 cells that were engineered to express membrane bound TNFα (CHO-K1-mTNFα) were mixed with encoder dye labeled, TNFα negative MCF-7 cells. Cells were combined with a range of concentrations of each adalimumab biosimilar eluted at different pH, as well as the Humira® RMP and an IgG control. A second labelling step, containing secondary antibody and iQue® Cell Membrane Integrity (R/Red) Dye, enabled quantification of the percentage of live cells of each type above a defined median fluorescence intensity (MFI) threshold for antibody binding (% positive).

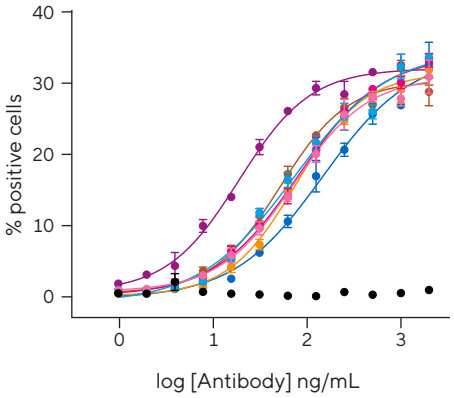
There was a concentration dependent increase in live cell binding of all the adalimumab antibodies (Figure 1A). This increase was specific to the CHO-K1-mTNFα cells and was not observed with the negative control MCF-7 cell type, suggesting high binding specificity (Figure 1B and C). Binding and specificity of the column-eluted biosimilars were highly comparable to the Humira® RMP, suggesting their binding activity has been maintained. Both biosimilars eluted in the presence of L-arginine yielded the lowest EC_{50} values for CHO-K1 binding, of 51.5 and 18.9 ng/mL, for the antibodies eluted at pH 3.6 and 3.8, respectively (Figure 1D). This is lower than the EC_{50} for the Humira® RMP (72.1 ng/mL). In contrast, the EC_{50} for the antibody eluted at pH 3.8 in the absence of L-arginine was 2-fold higher than the Humira® RMP, at 147.4 ng/mL. These findings suggest that the combination of a higher pH buffer and L-arginine induces lower aggregation of the IgG product during elution and results in an increase in correctly folded antibody available for binding.

Figure 1: *Comparison of Live Cell Binding Across Biosimilars*

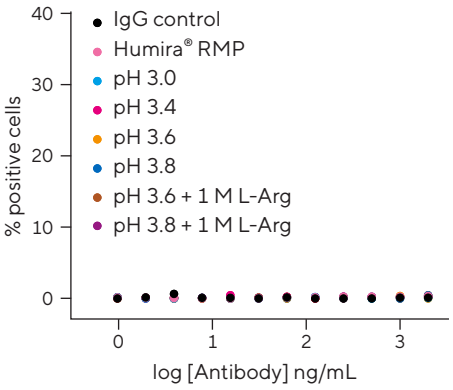
A. CHO-K1 (% positive)



B. CHO-K1



C. MCF-7



D. CHO-K1 binding

Antibody	EC_{50} (ng/ml)	95% CI
Humira® RMP	72.1	57.25 - 92.22
pH 3.0	73.4	54.64 - 107.6
pH 3.4	80.9	67.97 - 98.04
pH 3.6	76.1	63.44 - 92.59
pH 3.8	147.4	102.7 - 273.9
pH 3.6 + 1 M L-Arg	51.5	41.57 - 64.05
pH 3.8 + 1 M L-Arg	18.9	14.60 - 23.54

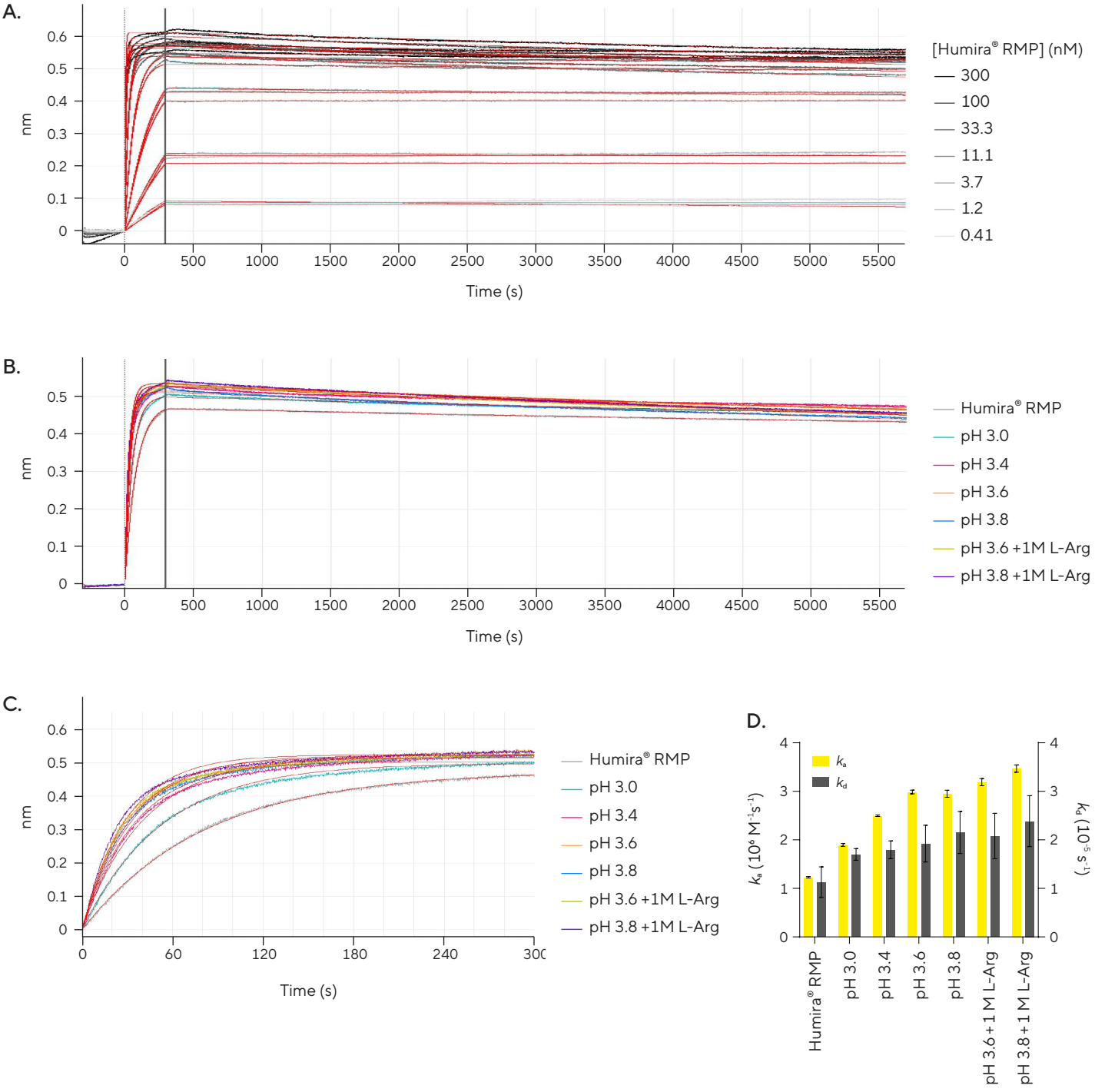
Note: Unlabeled CHO-K1-mTNFα cells were mixed with MCF-7 cells (pre-labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye, negative control) (10 K/cell type/well) and incubated with test antibodies in a 384-well plate. Secondary antibody and iQue® Cell Membrane Integrity (R/Red) Dye were then added before samples were acquired using the iQue® platform. The % positive cells defines the % of cells above a defined MFI threshold for secondary antibody labelling (n=3). (A) Heat map exported from iQue Forecyt® shows % positive CHO-K1-mTNFα cells. Concentration response curves compare adalimumab binding to (B) CHO-K1-mTNFα and (C) MCF-7 cells. (D) Table shows EC_{50} values for antibody binding to CHO-K1 cells with 95% confidence intervals (CI).

Binding Kinetics

Octet® BLI assays were performed to investigate the kinetics and affinity of antigen binding. Biotinylated AviTagTM TNFα was captured on to Octet® SAX Biosensors and real-time association and dissociation of the antibodies quantified. Prior to assessment of the full panel of adalimumab biosimilars, a kinetic screen was set up using the Humira® RMP (full details can be found in the Octet® Application Note: Fcγ Receptor and Antigen Kinetic Screening for Rapid Biosimilar Downstream Processing Assessment Using Octet® BLI).[10] Initially, a range of Humira® RMP concentrations binding to the captured TNFα (Figure 2A) were measured. A single concentration with a 'desirable' binding kinetic profile for the subsequent screening experiments was then selected.

In short, this is considered to be an analyte concentration that showed sufficient curvature for binding association, as is required to generate accurate association kinetic rate constants, but that isn't so high as to have reached surface saturation, as this can lead to an increase in non-specific binding events. As shown in Figure 2A, an increase in deviation from a simple 1:1 kinetic model (indicated by the red line data fit) is observed at high analyte concentrations (Humira® RMP 300, 100 and 33.3 nM). Conversely, the binding association curves for the lowest concentrations in Figure 2A (1.2 and 0.41 nM) are linear and do not exhibit the curvature required for kinetic rate constant calculations.

Figure 2: *Association and Dissociation Rates for Adalimumab Biosimilars Increased With Elution Buffer pH and the Addition of L-Arginine*



Note: Soluble, biotinylated TNFα was captured onto Octet® SAX biosensors and binding kinetics of adalimumab antibodies to the TNFα was quantified using an Octet® R8 BLI system. Red lines indicate 1:1 fit from Octet® analysis software as used to calculate kinetic parameters. (A) Association and dissociation of a range of concentrations of Humira® RMP to TNFα (n=3). (B) Single concentration (10 nM) kinetic screen of binding of adalimumab biosimilars compared to Humira® RMP (n=3, 1 replicate shown). (C) Association only for data in (B) (n=3, 1 replicate shown). (D) Association and dissociation rates for each antibody binding to TNFα in the single concentration screen.

Both the 11.1 and 3.7 nM Humira® RMP concentrations displayed the requisite binding characteristics for determination of accurate association kinetics, and a concentration between these two (10 nM) was chosen for the kinetic screen. Binding of this concentration of Humira® was assessed as seven simultaneous measurements in triplicate across three experiments (i.e., 63 replicates in total) to confirm low CVs for inter- and intra- assay variability (data not shown). This repeatability was used to qualify the kinetic screen, with a QC sample of Humira® RMP included in each kinetic run to confirm assay validity.

The single concentration kinetic screen, which measured the rate of association (k_a) and dissociation (k_d) of the adalimumab antibodies, showed that the adalimumab biosimilars had both faster k_a and k_d compared to the Humira® RMP antibody. There was a general increase in both k_a and k_d with increasing elution buffer pH for the biosimilars, which increase further with the addition of L-arginine (Figure 2D). Traces in 2B and 2C show that the increase in binding of the adalimumab clones eluted at high pH and in the presence of L-arginine has resulted in a deviation from the 1:1 model applied to the Humira® RMP molecule, suggesting an over-saturation of the biosensor.

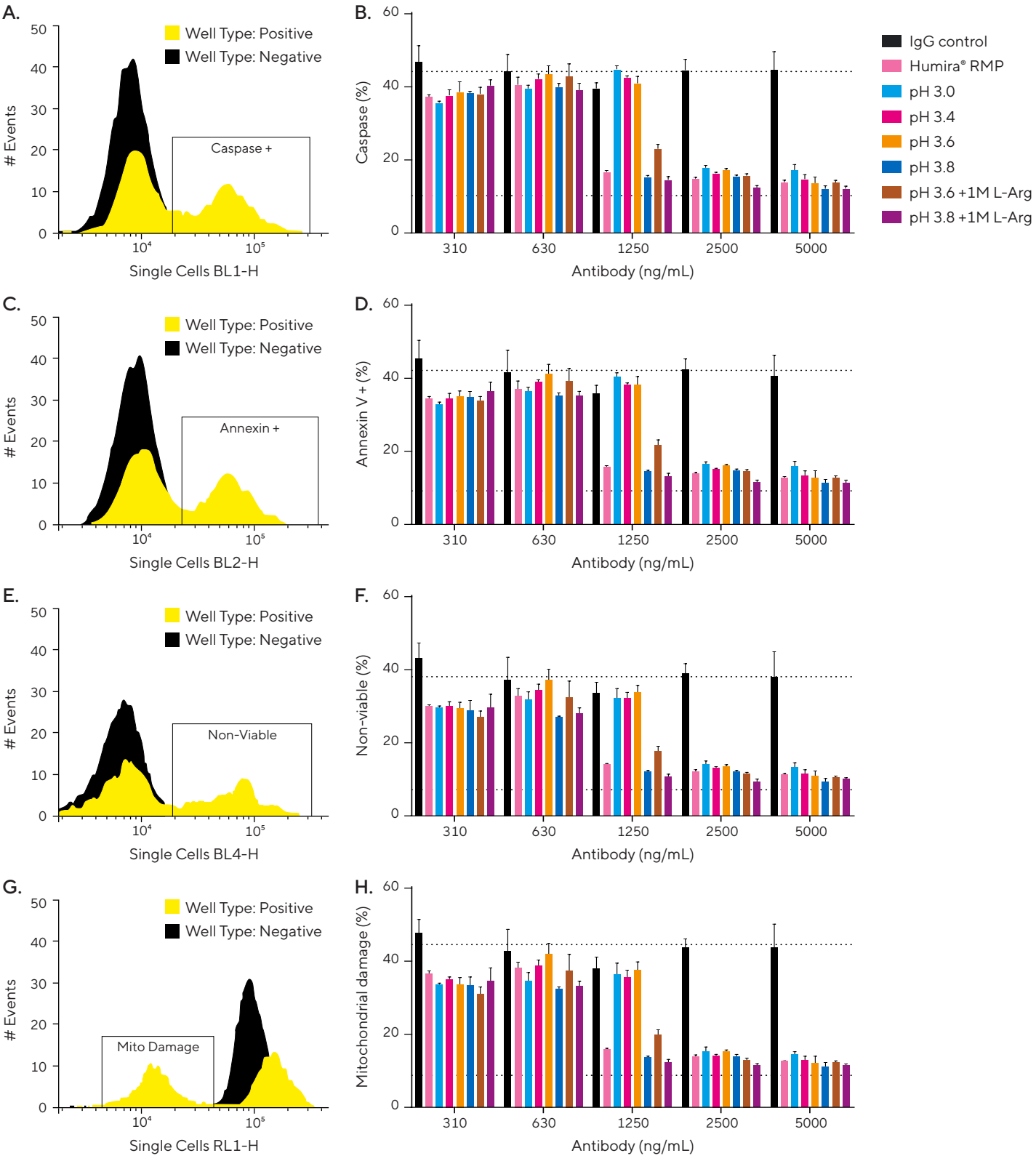
Inhibition of TNFα-Induced Apoptosis

TNFα is a critical cell-signalling molecule within the body, with a plethora of functions, generally working to promote inflammation and induce apoptosis.[11] The TNFα receptor is expressed to varying degrees on most cell types, reinforcing the potential breadth of its activities.[12] A key mechanism of action of adalimumab during the treatment of inflammatory diseases, is to bind to TNFα molecules and to inhibit their interaction with anti-TNFα receptors on the surface of cells.[13] To model this inhibition of TNFα activity by the adalimumab biosimilars *in vitro,* soluble TNFα was added to THP-1 cells to induce their apoptosis. The biosimilars were also added at a range of concentrations to neutralize the TNFα activity and inhibit apoptosis. Apoptosis was measured using the iQue® Human 4-Plex Apoptosis Kit, which simultaneously measures cell viability (via a non-membrane permeable membrane integrity dye), caspase 3/7 activity, annexin V binding and mitochondrial membrane integrity in each well of a 384-well plate.

Histograms in Figure 3A, C E and G represent the level of apoptosis of THP-1 cells in the presence (yellow) and absence (black) of TNFα. These control populations aided positive gating for apoptotic populations, with TNFα inducing a clear increase in all four markers of apoptosis. Mitochondrial membrane potential is determined by sequestration of a small fluorescent molecule inside the lumen of intact mitochondria with an active membrane potential. Upon mitochondrial depolarization the dye leaks into the cytoplasm and the cell exhibits a decrease in fluorescence, hence the positive gate for this population has the dimmest peak.

Bar charts in Figure 3B, D, F and H show that high concentrations of adalimumab (2500 and 5000 ng/mL) reduced the level of apoptosis towards negative control levels (i.e., the lower dotted line on the graphs). Comparatively, the concentration of the IgG control had no impact on apoptosis induced by TNFα. The level of apoptosis in the presence of 1250 ng/mL of antibody, split the biosimilars into two clear groups in terms of their potency of apoptosis inhibition. At this concentration, only the Humira® RMP and the biosimilars eluted with pH 3.8 buffer (both with and without L-arginine) and at pH 3.6 with L-arginine cause a reduction in apoptosis compared to the positive control. This provides further evidence to suggest that elution is favorable at higher pH and in the presence of L-arginine. These data also show that, at this TNFα concentration, the potency of adalimumab inhibtion of apoptosis through sequestration of TNFα is much lower than the potency of it's direct cell-based MoAs, such as binding and CDC activity (Figure 4).

Figure 3: *Biosimilars Eluted at High pH and | or With L-Arginine Inhibit TNFα-Induced Apoptosis With Highest Potency*



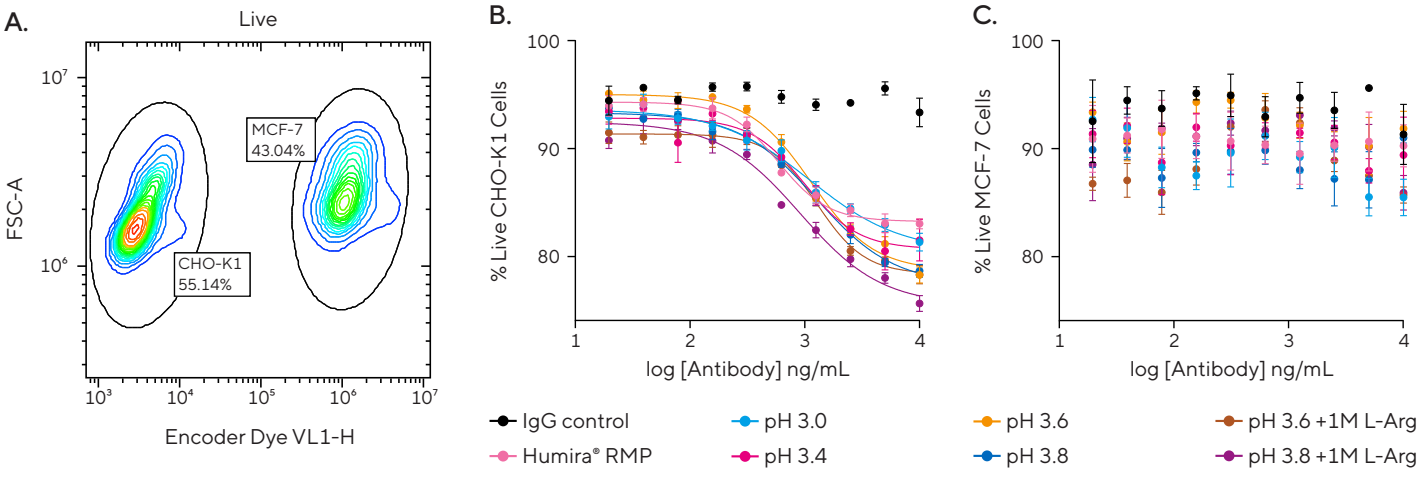
Note: THP-1 cells were seeded (15 K/well, 20 µL) in a 384-well plate with soluble TNFα (0.3 µg/mL, 20 µL) and test antibodies (20 µL). After 72 h, samples were analyzed using the iQue® Human 4-Plex Apoptosis Kit. This kit determines multiple apoptosis endpoints per well, including cell viability, caspase 3/7 activity, annexin V binding and mitochondrial membrane integrity. Histograms show positive (yellow, with TNFα) and negative (black, no TNFα) control populations, in the absence of test antibody, that were used to confirm gate positioning. Bar charts compare inhibition of apoptosis between adalimumab antibodies and to the IgG control. Upper and lower dotted lines in each graph represent the average of the positive and negative control populations, respectively (n=3). Metrics analyzed are (A, B) Caspase, (C, D) Annexin+, (E, F) Non-viable and (G, H) Mitochondrial damage.

Complement-Dependent Cellular Cytotoxicity (CDC)

Aside from the direct inhibition of TNFα, adalimumab activity towards some indications may also be promoted via Fc-mediated functions, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).[14] Here, to assess the CDC activity of the adalimumab biosimilars *in vitro*, they were mixed with a combination of CHO-K1-mTNFα cells and iQue® Cell Proliferation and Encoding (V/Blue) Dye labeled, TNFα negative MCF-7 cells. This enabled separation of the two cell types based on the level of encoder dye fluorescence, as displayed in the contour plot in Figure 4A. Human serum was added to induce CDC activity through the recruitment of complement proteins to adalimumab bound to surface TNFα. The resulting cell lysis was quantified by labelling cells with iQue® Cell Membrane Integrity (R/Red) Dye.

Graphs in Figures 4B and C show that there was a concentration-dependent increase in death of the CHO-K1 cells in the presence of Humira® RMP and all the adalimumab biosimilars. This was not observed with the IgG control or with the antigen negative cell type, highlighting that this is a target antigen-dependent response. Curves suggest a slightly higher potency of response for CHO-K1 cell death for the antibody eluted at pH 3.8 in the presence of 1M L-arginine. This provides evidence that elution of antibodies at high pH and with L-arginine can lead to more correctly folded protein available to induce an enhanced functional response.

Figure 4: *Induction of CDC Activity is Greatest by the Biosimilar Eluted at pH 3.8 With L-Arginine*



Note: Unlabeled CHO-K1-mTNFα cells were mixed with MCF-7 cells (pre-labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye, negative control) (10 K/cell type/well) and test antibodies in a 384-well plate. Human serum (15%) was added to induce CDC activity. Following incubation, plates were labeled with iQue® Cell Membrane Integrity (R/Red) Dye to enable quantification of cell death. (A) Contour plot to show separation of encoder dye labeled MCF-7 cells from unlabeled CHO-K1-mTNFα cells. (B) Concentration response curves compare cell death induced by adalimumab biosimilars and an IgG control with CHO-K1-mTNFα target cells and (C) MCF-7 targets.

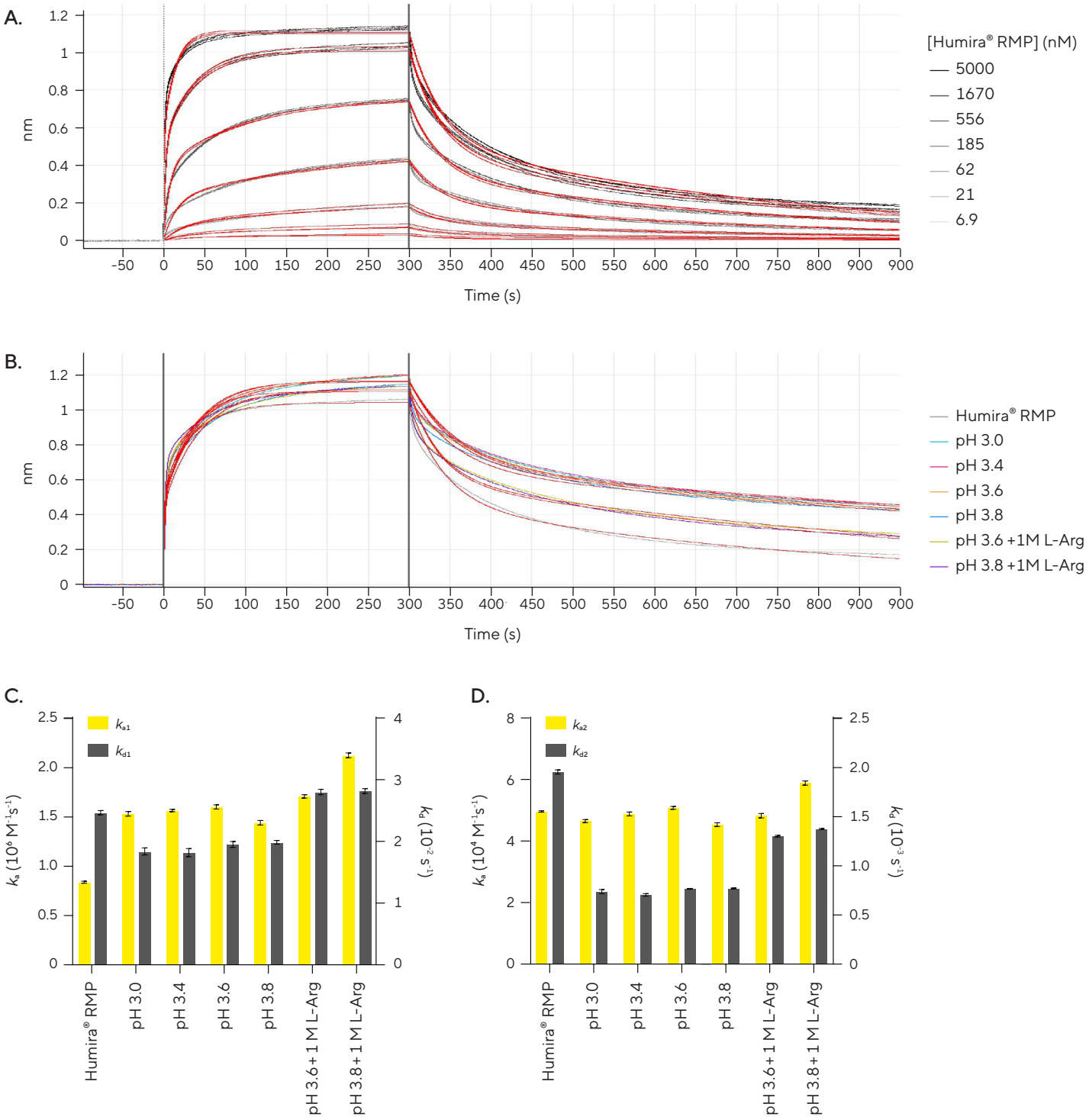
CD16a V176 Binding

CD16a is an FcγRIIIa receptor found on the surface of NK cells and plays a key role in facilitating ADCC activity. In these experiments, Octet® BLI was used to test the binding kinetics of the adalimumab antibodies to CD16a V176 via their Fc region. This was performed by using the same kinetic screening method as described above for TNFα, except a 2:1 heterogenous ligand binding model was used to fit the data, suggesting CD16a has two independent binding sites for IgG. This yields two k_a and two k_d values from each binding trace. Figure 5A shows the initial Humira® RMP binding test with a range of antibody concentrations, from which it was determined that binding of the top two Humira® RMP concentrations (5000 and 1670 nM) were too rapid to generate accurate kinetic parameters during the screen.

Binding of the lowest concentrations (between 185 and 6.9 nM) were decided to be too low as they didn't fully reach equilibrium over the time period and so lack the required kinetic information. A concentration of 500 nM of each antibody was chosen as optimal for the biosimilar kinetic screen.

The kinetic screen data (Figures 5B to D) shows a different profile of the Humira® RMP compared to the adalimumab biosimilars, particularly evidenced by the slower k_{a1} and faster k_{d2}. The remaining antibodies split into two groups based on their dissociation rates, with the antibodies eluted in the presence of L-arginine yielding faster k_{d1} and k_{d2} values, closer in value to the Humira® RMP. The antibody eluted at pH 3.8 in the presence of L-arginine exhibited the fastest rate of association (both k_{a1} and k_{d2}).

Figure 5: *Adalimumab Biosimilars Displayed Different Binding Kinetics to the Humira® RMP*



Note: Soluble, AviTagTM CD16a V176 was captured onto Octet® SAX biosensors and binding kinetics of adalimumab antibodies was quantified using an Octet® R8 BLI system. Red lines indicate a 2:1 heterogenous ligand binding model. (A) Association and dissociation of a range of concentrations of Humira® RMP to CD16a (n=3). (B) Single concentration (500 nM) kinetic screen of binding of adalimumab biosimilars compared to Humira® RMP (n=3, 1 replicate shown). Kinetic parameters from adalimumab kinetic screen fit with a 2:1 heterogenous ligand binding models yields two k_a and two k_d values shown in (C) k_{a1} | k_{d1} and (D) k_{a2} | k_{d2}.

Antibody-Dependent Cellular Cytotoxicity (ADCC)
Functional testing of the ADCC activity of the adalimumab biosimilars was performed by adding them to co-cultures of CHO-K1 and NK cells. After 20 hours, cells and supernatants were analyzed for NK marker expression (CD3, CD56, CD16a, CD69 and CD25) and cytokine concentrations (IFNγ and Granzyme B) using the iQue® Human NK Killing Kit. During ADCC, it is expected that expression of key FcγRIIIa receptor for ADCC, CD16a, will decrease. The data in Figure 6A shows that with increasing concentrations of adalimumab, there is a decrease in CD16a expression, which is not observed with the IgG control. The Humira® RMP consistently induced a larger decrease in CD16a expression than the biosimilars, suggesting that it's induction of ADCC was greater.

It is also expected that activation of NK cells will increase during ADCC, which can be measured through the expression of activation markers (e.g., CD25 and CD69). The data in Figure 6B shows that, compared to the IgG control, there was elevated expression of CD25 in the presence of all the antibodies.
At lower concentrations of antibody (2.3 to 21 ng/mL), the Humira® RMP induced greater CD25 expression than the biosimilars. Release of IFNγ is also a key indicator of NK activation. IFNγ release was greater with higher concentrations of antibody and was increased in the presence of the Humira® RMP compared to the biosimilars. Granzyme B is a protease which is directly involved in the killing mechanism utilized by NK cells. Like the other activation markers, at most concentrations, Granzyme B release was greatest in the presence of the Humira® RMP. There was also evidence of increased Granzyme B release in the presence of the antibodies eluted at pH 3.8 (both in the presence and absence of L-arginine).

Together, these data suggest that ADCC functional activity of the Humira® RMP may be enhanced compared to the adalimumab biosimilar clones. This antibody also displayed unique CD16a binding properties compared to the biosimilars, in Figure 5, suggesting that these may have been favorable for induction of ADCC activity.

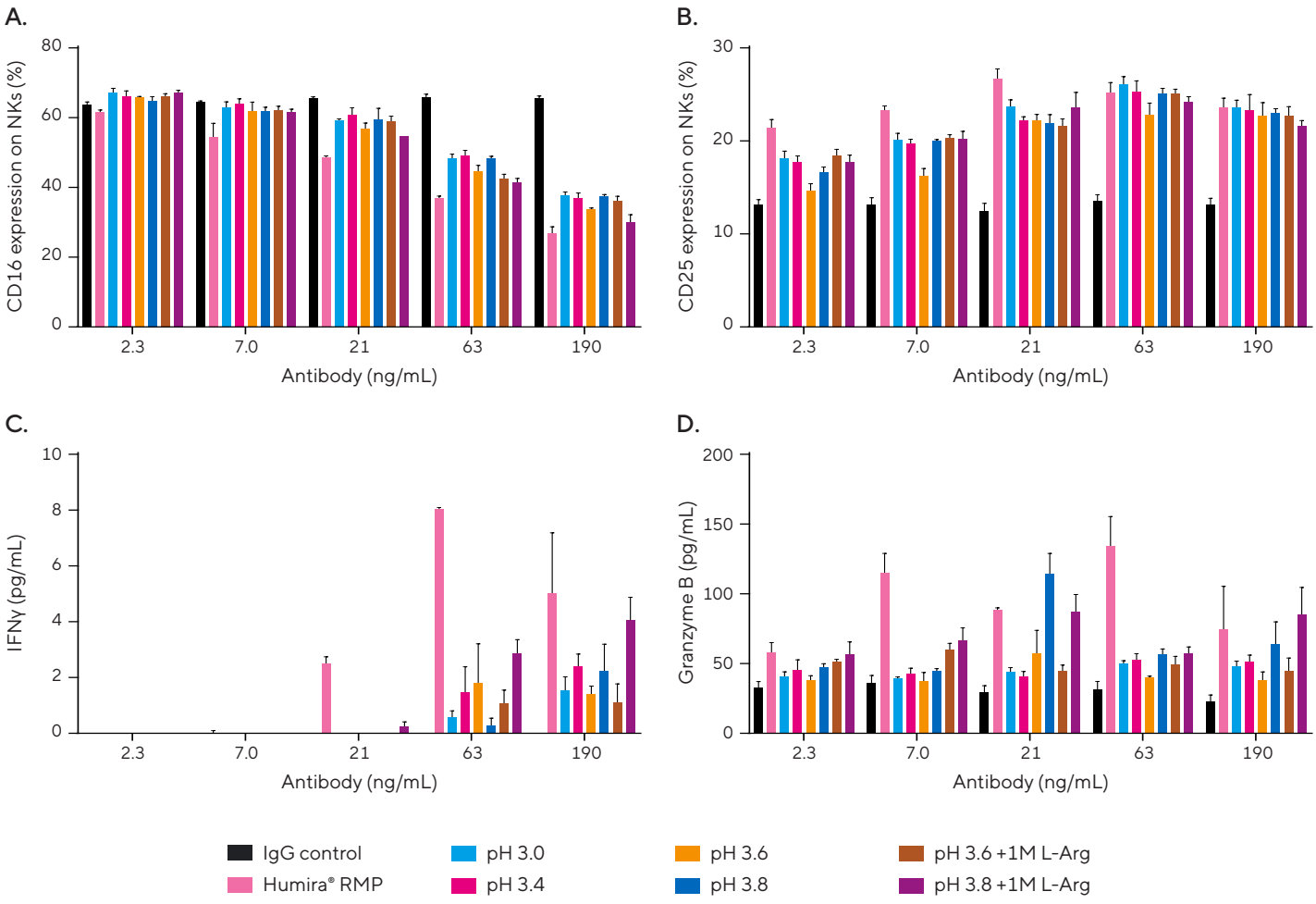
Conclusions

This application note exemplifies the use of the iQue® Advanced Flow Cytometry Platform in combination with the Octet® BLI System to perform comprehensive analysis of biosimilar antibodies, easily allowing them to be compared both to each other and to an RMP. Live cell antibody binding and a range of functional analyses were performed using the iQue®, whilst a kinetic assessment of antibody binding to both the target antigen and to FcγRIIIa receptors was performed using Octet® BLI. The advantages of this workflow include:

- Live cell antibody binding measures binding to the target as it is found on live cells and gives an indication of off-target specificity. Combining this with Octet® BLI provides additional information on binding kinetics which can inform on what the pharmacokinetic profile of the drug might be *in vivo*.
- High-throughput data acquisition using the iQue® platform facilitates rapid profiling of a range of antibodies and/or concentrations, requiring a minimum of only 10 minutes to run a full 96-well plate or 30 minutes for a full 384-well plate.
- Utilizing a kinetic screening method with the Octet® BLI system can reduce both the time and consumable cost required to compare the binding properties of multiple biosimilars to each other and to an RMP.
- The broad range of lasers and detection channels in the iQue® platform allows for measurement of many dyes and markers to be multiplexed in each well, yielding information-rich data from every plate.

These advantages enable fast, broad assessment of antibody characteristics, with the potential to enhance biosimilar testing both during development and manufacturing workflows.

Figure 6: *Humira® RMP Induced Greater NK ADCC Activity and Activation Than the Biosimilars*



Note: CHO-K1-mTNFα cells (5 K/well) were labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye and seeded in 96-well flat bottom plates. Test antibodies were added at a range of concentrations alongside unlabeled natural killer (NK) cells (25 K/well). After 20 hours, 10 µL samples of supernatant were transferred to V-bottom plates for analysis of IFNγ and Granzyme B cytokine concentrations using 2-plex iQue Qbeads® from the iQue® Human NK Cell Killing Kit. Cells were lifted and analyzed using the antibody cocktail provided in the kit. Markers assessed using the NK Killing Kit included: (A) CD16a (% expression on CD3-CD56+ NKs), (B) CD25 expression (% expression on CD3-CD56+ NKs), (C) IFNγ concentration (pg/mL) and (D) Granzyme B concentration (pg/mL).

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