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Fcγ Receptor and Antigen Kinetic Screening for Rapid Biosimilar Downstream Processing Assessment Using Octet® BLI

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Abstract

Octet® Bio-Layer Interferometry (BLI) systems have high-throughput capabilities and allow high-quality kinetic data to be directly generated from a range of drug development processes, from small scale supernatants generated during upstream cell line development (CLD) through to column elution fractions in process development and other downstream processes.

This application note describes how throughput can be increased even further on the Octet® R series by use of a kinetic screening technique, which rather than utilizing a range of analyte concentrations to generate kinetic data, instead uses a single concentration of analyte to determine a kinetic parameter range (k_a, k_d) of an interaction with the desired target (ligand). This then allows rapid screening of multiple analytes and/or ligands in a single run. Combining kinetic screening with the high-throughput capabilities of Octet® BLI allows users to either bypass orthogonal assay formats, such as ELISAs, or allows users to triage molecules so that only those with desired characteristics are progressed downstream for further assessment.

Introduction

There is a continuing need for affordable drugs that increase the number of treatment options and access to lifesaving medications whilst potentially lowering health care costs. Recent years have seen an increase in competition for blockbuster biologics in part due to the increase in the number of biosimilars, which are biological medicines that are highly similar to an already approved biopharmaceutical drug (originator or reference medicinal product (RMP)), which can be manufactured when the original product's patent expires. Following the launch of the first biosimilar in 2015, there are now 33 FDA approved biosimilars with 21 available on the market as of January 2022[1]. Between 2022 and 2027, 31 biologics come off patent, and with potential sales of up to $20 billion per annum for each biosimilar and a total market predicted to be worth more than $100 billion by 2028 there is a large interest in instrumentation and assay formats that allow rapid progress in narrowing development funnels[2].

Unlike small-molecule generics, biosimilars are not classified as a generic of the RMP as the inherent variability and complexity in the manufacturing of biological medicines does not allow exact replication. For this reason, no clinically meaningful differences in safety, purity, and potency between the biosimilar and the RMP are allowed but minor differences in clinically inactive components are allowed[3].

Initial development of a biosimilar begins with creating multiple cell-clones that contain the primary amino acid sequence of the desired biologic. Expression of the desired biologic is then assessed and optimized upstream bioprocessing; specifically during cell line and process development. These upstream processes can create critical rate-limiting steps as choosing the wrong clone at this stage can result in reselection being required. Typically, yield is the most important attribute determined during CLD and other critical quality attributes (CQAs) are corrected during process development before proceeding to downstream processing (bioprocess development).

Downstream bioprocessing is an essential series of techniques in biologics development, which involves the isolation, purification, and characterization of the target biologic from a complex mixture of biological or chemical components. This is performed to maximize yields and obtain a pure and potent biologic product that is suitable for clinical use or commercialization while minimizing the costs and risks associated with impurities or contaminants.

Downstream bioprocessing typically includes stages such as cell harvesting, cell disruption, extraction, purification by chromatography, filtration, and formulation with the aim of ensuring that the final drug product meets the required purity, potency, and safety standards.

Biosimilarity needs to be demonstrated throughout the entire upstream and downstream production processes. The use of standard analytical methods to screen all potential candidates can be prohibitive; as a result, an alternative high-throughput analytical approach is desirable.

Traditionally, the main pathway to demonstrate similarity between a biosimilar and its RMP has been through a comparability study as outlined by the regulatory agencies EMA, FDA & WHO, where the binding of the proposed biosimilar must fall within 80-125% of the RMP for a variety of interactions including Fcγ receptors and its intended antigen.

Octet® BLI systems can be used at multiple points upstream and downstream to assess several key biologics attributes including target binding characterization, Fcγ receptor binding, glycan screening and assessment of impurities and host cell proteins along with other CQA, which helps simplify the development process.

This application note demonstrates how combining a single analyte kinetic screen of a monoclonal IgG1 antibody against the Fcγ receptors CD16a V176 and CD64 in addition to the antigen TNFα can be used to rapidly assess column elution fractions of a biosimilar under development. This process can be simply adapted to assess multiple CQAs and requires very small amounts of protein (<1 µg per well is used here).

When kinetic screening is combined with the high-throughput capabilities of the Octet® BLI systems; a facile orthogonal approach is possible where multiple attributes, such as Fcγ receptor binding and glycan screening can be rapidly assessed directly from supernatants with or without purification.

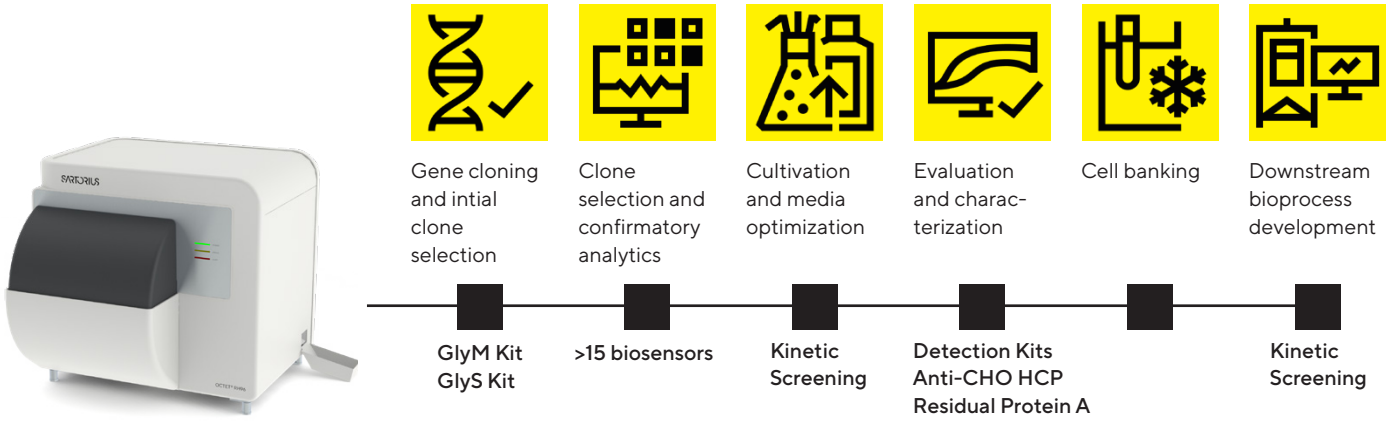

Figure 1: Octet® BLI systems can be used at multiple points upstream and downstream to assess several key attributes including target binding characterization. Octet® BLI Biosensors and kits allow users to use multiple assay formats and to assess and analyze a wide range of biological information.

Kinetic Screening – Analyte Response Optimization

In a classical kinetic assessment, several concentrations of an analyte binding to a ligand are tested to ensure that the kinetic space is covered and accurate kinetic rate constants can be determined. Binding of an analyte to a ligand to form an analyte-ligand complex is a second order binding process where the observed response is dependent on the concentration of the two reactants. The concentration of the ligand can be viewed as a constant and therefore, accurate association kinetics (concentration dependent) can be determined from a single concentration of analyte[4]. In general, for all kinetic assessments, in order for association (k_a) kinetic parameters to be accurate, sufficient curvature must be present in the observed response as curvature is kinetics (Figure 2). Assuming that avidity effects are minimized and the association phase not run for too long so that weaker affinity interactions may occur then the dissociation rate constant (concentration independent) does not require optimization.

In a kinetic screen, the concentration of analyte required to obtain sufficient curvature during the binding responses is determined empirically based upon the K_D of the proposed interaction. Initially for analyte response optimization it is recommended to test a wide range of analyte concentrations to determine the optimal single concentration for use in a kinetic screen. As shown in Figure 2 at high analyte concentrations the association response is too rapid and inaccurate kinetics would be determined from a single concentration, whereas the opposite is true for lower analyte concentrations where the binding response is essentially linear and therefore, lacks sufficient information about kinetic events. The optimum single analyte concentration shows sufficient curvature to determine accurate kinetic rate constants but does not reach steady equilibrium for extended periods of time, which

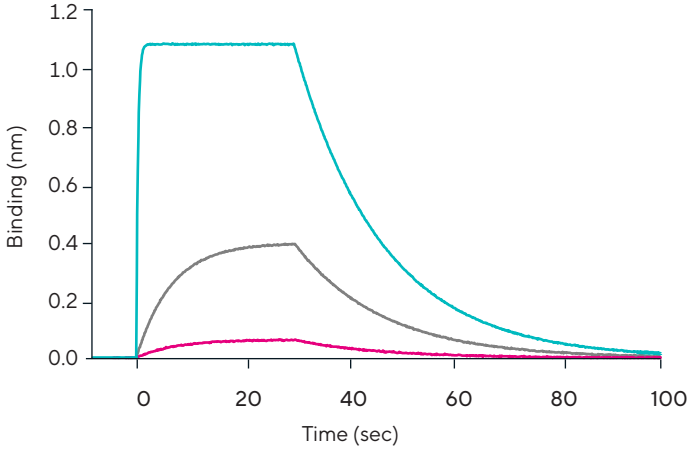

Figure 2. Curvature is Kinetics. When using a single concentration, it is important that sufficient curvature is observed to determine reliable kinetics. Here, the teal curve shows rapid association and equilibrium, which would result in inaccurate kinetics determination. The magenta curve shows a low level of response and curvature, which would also result in poor kinetics determination. The gray curve shows a good level of curvature during the association phase and also reaches equilibrium and should generate acceptable estimates of kinetics and affinity from a single concentration assessment. It is important to avoid running the association step for too long and allowing binding curves to reach equilibrium for a long period of time, as this leaves opportunity for weaker, non-specific interactions to occur and may result in inaccurate determination of kinetic parameters.

reduces binding events that may occur due to low-affinity interactions. Although dissociation is a concentration independent event, it is important to note that surface saturation can lead to an increase in surface crowding and non-specific binding events.

An important consideration when setting up a high-throughput kinetic screen is assay orientation, the expected stoichiometry of the interaction and whether the simplest kinetic model (1:1 binding) can accurately fit the data. If the analyte is bivalent, as in this study, there is an increased risk of avidity effects (See Application Note: **Optimizing Kinetics Assays to Avoid Avidity Effects**). Therefore, assay design must be considered when performing a kinetic screen and, where necessary, the assay should be performed in an orientation as to remove avidity effects.

Materials and Methods

Instrument and Reagents

All assays were performed using an Octet® R8 using Octet® BLI Discovery and Analysis Studio Software version 13.0.1. Octet® SAX Biosensors (18-5117 (tray), 18-5118 (pack),18-5119 (case) and 96-well, black, flat bottom microplate, Greiner Bio-One Cat. No. 655209 were used in all assays. Biosensors were pre-hydrated for at least 30 minutes at room temperature prior to use in 1X Kinetics Buffer (pH 7.4) prepared by dilution of Sartorius 10X Kinetics Buffer (18-1105) 1:10 with PBS. Unless indicated, all assays were performed at 25 °C.

Recombinant AviTag™ biotinylated human TNFα, AviTag™ biotinylated human CD64 and AviTag™ biotinylated human CD16a V176 were purchased from ACROBiosystems. Humira® RMP was purchased from WEP Clinical.

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, 3.2, 3.4, 3.6, 3.8, pH 3.6 + 1 M L-Arginine and pH 3.8 + 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.

Analyte Response Optimization

Biotinylated ligand loading onto SAX Biosensors was optimized using the highest proposed analyte concentration for each receptor such that a response of ~0.6 nm was observed for TNFα, CD64 and ~1.0 nm for CD16a V176 (data not shown, see **Biomolecular Binding Kinetics Assays** and **Octet® BLI Kinetics Assay: Method Development Guideline** for further information) on the Octet® Platform. Humira® was prepared to a final concentration of 300 nM (TNFα and CD64) and 5 µM (CD16a V176) in 1X Kinetics Buffer, and a 3-fold serial dilution performed. A background reference sample was analzyed simultaneously, using a SAX biosensor loaded with the relevant ligand and 1X Kinetics Buffer as the analyte solution.

Data was fitted using Octet® Analysis Studio software to either a 1:1 model (TNFα and CD64) or 2:1 (Heterogenous Ligand) (CD16a V176).

Kinetic Screen Qualification - Repeatability

Biotinylated ligand loading levels determined for analyte response optimization were used during the kinetic screen repeatability assessment. A stock concentration of ligand was prepared for each repeatability assessment and a threshold limit applied to the ligand loading level in the assay method file. A new biosensor was used for each assessment. Humira® was prepared in 1X Kinetics Buffer to the concentration determined during analyte response optimization and intra- and inter-assay variability determined by measuring seven binding measurements performed in triplicate over multiple days by multiple users.

Data was fitted using Octet® Analysis Studio software to either a 1:1 model (TNFα and CD64) or 2:1 (Heterogenous Ligand) (CD16a V176).

Results and Discussion

Analyte Response Optimization

CD64

Initially it is recommended to test a wide range of analyte concentrations to determine the optimal single concentration for use in the kinetic screen. As shown in Figure 3A for CD64, an initial concentration series of 300, 100, 33.3, 11.1, 3.7, 1.23 and 0.41 nM was assessed. At high analyte concentrations (300, 100 and 33.3 nM) the association response is too rapid , meaning an inaccurate association kinetic rate constant would be determined from a single concentration. The opposite is true for lower analyte concentrations (1.24 and 0.41 nM) where the binding responses are essentially linear and therefore lack sufficient information about kinetic events.

The single analyte concentrations of 3.7 nM and 11.1 nM show sufficient curvature to determine accurate kinetic rate constants and a value between this range of 10 nM was chosen for the kinetic screen. Although dissociation is a concentration independent event, it is important to note that surface saturation can lead to an increase in surface crowding and non-specific binding events. As shown in Figure 3B, the highest concentration (300 nM) exhibits a markedly different dissociation phase to the other concentrations tested and therefore, visual inspection of the response curves is an important quality check before proceeding with a full kinetic screen.

It is generally accepted in the kinetics community that determining accurate dissociation kinetics requires a > 5% decrease in response during dissociation to determine accurate K_D values[5]. It is preferable that a visual drop in the response is also observed in addition to a mathematical decrease of 5% and this must be considered when determining the optimum concentration and dissociation time for a kinetic screen (See: **A Compendium for Successful BLI and SPR Assays)**.

TNFα

A critical factor in biosimilar development is the ability of the biologic to bind the antigen with similar kinetics and affinity to the RMP. As discussed above, assay design and the expected stoichiometry of the interaction must be considered when performing a kinetic screen and where necessary, the assay should be performed in an orientation as to minimize or remove avidity effects. This allows data to be assessed by the simplest kinetic model (1:1 binding) and ensure an accurate fit to the data.

Here, where the IgG1 antibody Humira® was assessed as the analyte, there is a risk of avidity effects during the assay and use of a sufficiently low ligand density and an analyte concentration that shows sufficient curvature was adequate to remove any avidity issues.

As shown in Figure 4A, where the analyte is a monoclonal antibody, and is therefore bivalent, as the analyte concentration increases the resultant data fit for a 1:1 model worsens due to a potential increase in avidity effects (See: **Application Note: Optimizing Kinetics Assays to Avoid Avidity Effects)**.

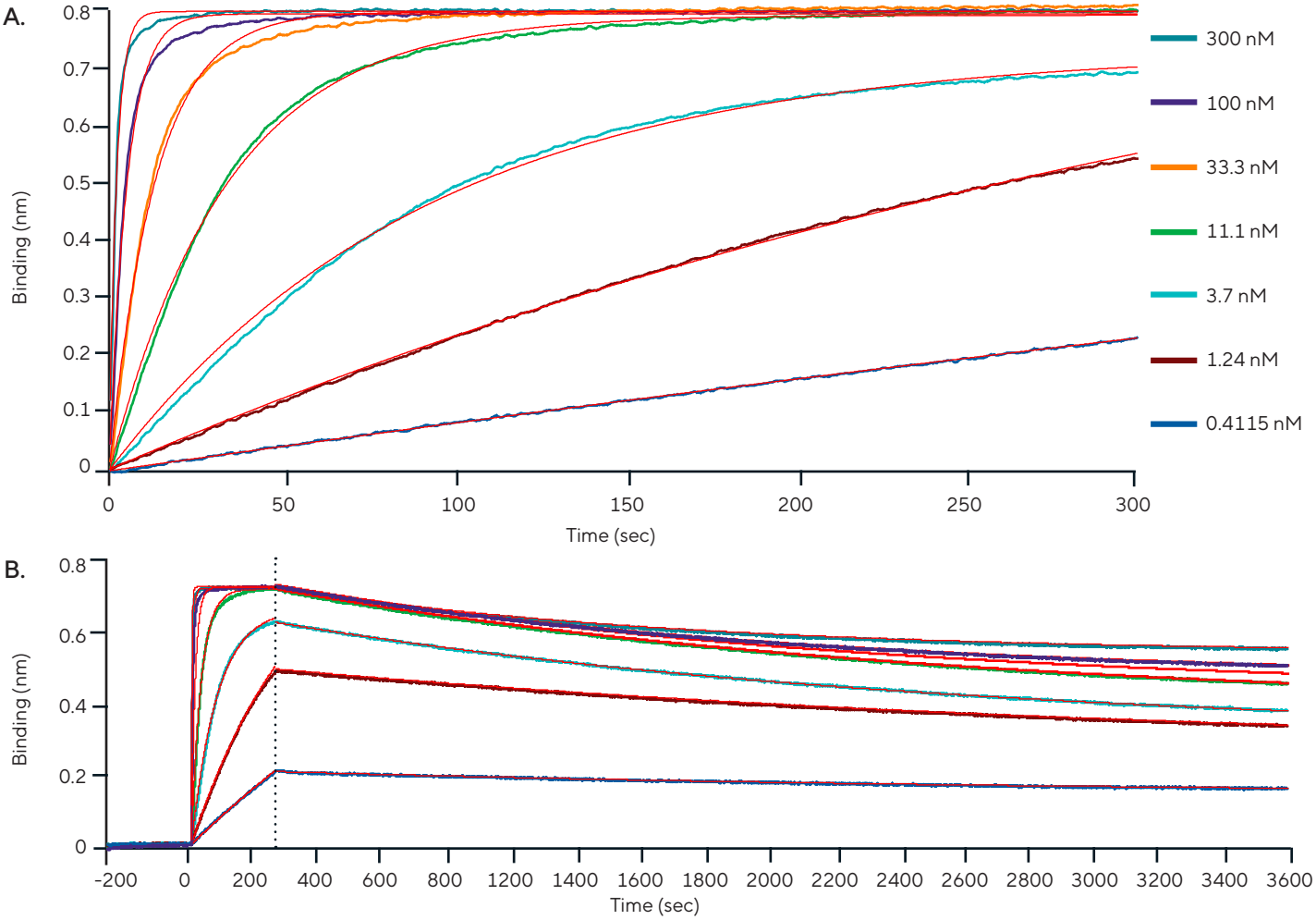

Figure 3: Humira® CD64 Analyte Optimization Concentration Series. (A) Association kinetics are too rapid for the highest analyte concentrations while too linear for the lowest concentrations. Teal (3.7 nM) and green (11.1 nM) show a good range of curvature and a suitable analyte concentration around this range would be suitable for a kinetics screen. (B) Dissociation kinetics in general do not require optimization but deviations such as that observed for the 300 nM analyte concentration may imply additional effects at the biosensor surface.

As observed with CD64, at high analyte concentrations (300 and 100 nM) the association response is too rapid and inaccurate association kinetic parameter would be determined from a single concentration, the opposite is true for lower analyte concentrations (1.24 and 0.41 nM) where the binding response is essentially linear and therefore lack sufficient information about kinetic events. The single analyte concentrations of 3.7 nM and 11.1 nM show sufficient curvature to determine accurate kinetic rate constants and a value of 10 nM was chosen for the kinetic screen.

As discussed below for CD64, it is generally accepted in the kinetics community that determining accurate dissociation kinetics requires a > 5% decrease in response during dissociation to determine accurate K_D values. The interaction of TNFα with Humira® is a high affinity interaction (pM) and a slow dissociation rate constant (10^{-5} s^{-1}) was observed during the 90-minute dissociation phase. Assays were performed at 25 °C for this kinetic screen but where desired, a higher assay temperature of 37 °C may be used to increase the observed change in response and accuracy in the calculated dissociation rate constant.

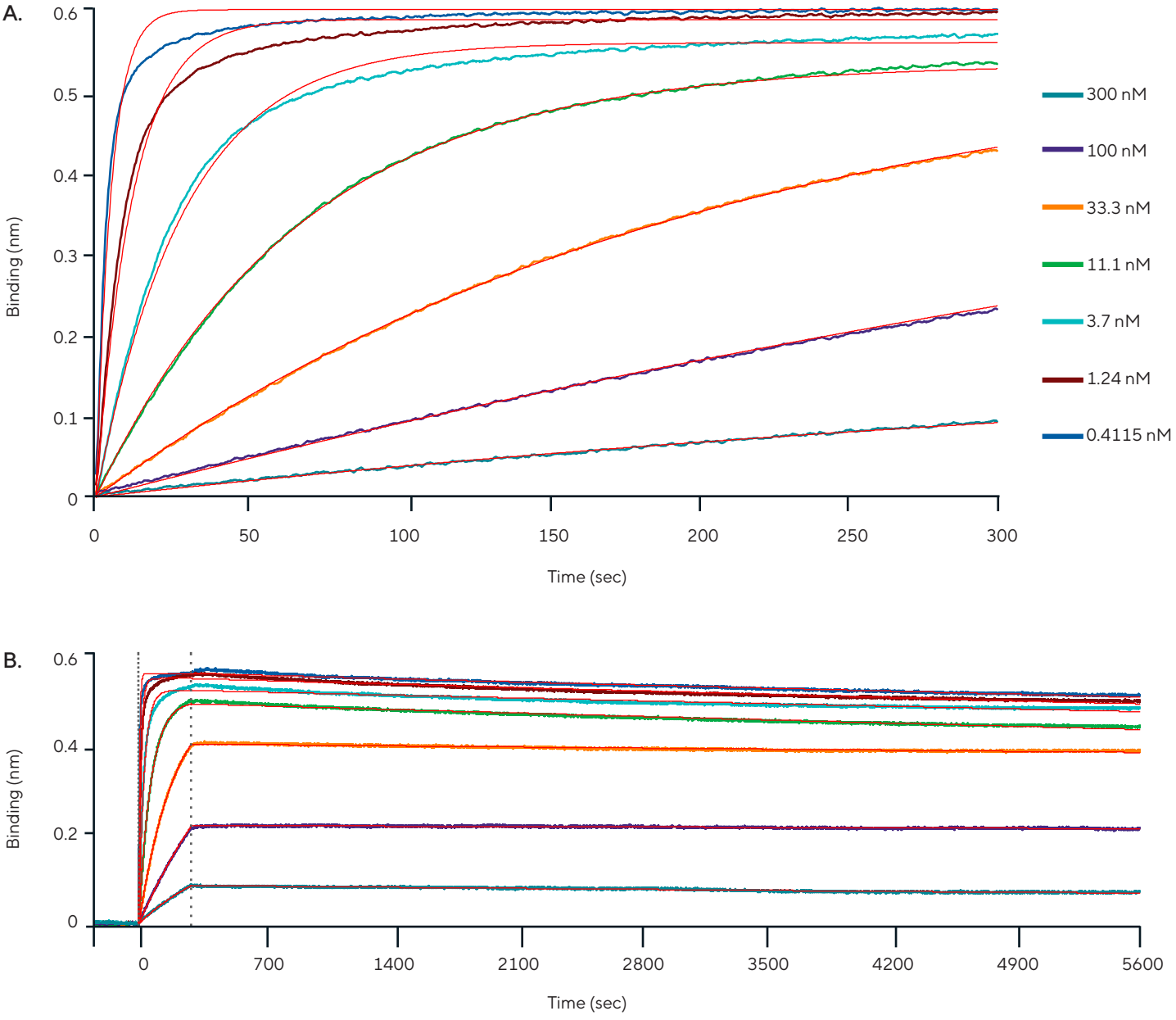

Figure 4: TNFα Analyte Optimization Concentration Series. (A) In assays formats where the analyte is bivalent, avidity issues must be considered. Here, as analyte concentration increases, the observed curvature also increases but the risk of avidity effects also increases and move the assay away from a simple 1:1 binding model. As shown here, at lower concentrations the observed binding data (colored lines) fits a 1:1 kinetic model (red line) well but at higher analyte concentrations, the 1:1 does not fit the observed data, potentially due to an increase in avidity effects and biosensor surface crowding due to saturation. (B) Minimal decrease in the binding response is observed for Humira® binding to TNFα is observed at 25 °C.

CD16a V176

CD16a (FcγRIIIa) V176 (aka V158) is a medium affinity Fcγ receptor that unlike the high-affinity Fcγ receptor CD64 (FcγRI) can display significant heterogeneity in its five glycosylation sites and therefore, is not amenable to a 1:1 kinetic analysis [6,7,8].

As CD16a is known to have a lower affinity for IgG1 antibodies than CD64[9] an initial concentration series of 5000, 1667, 555.6, 185.2, 61.7, 20.6 and 6.86 nM was assessed (Figure 5A). At high analyte concentrations (5000 and 1667 nM) the association response is too rapid and inaccurate kinetics would be determined from a single concentration. The opposite is true for lower analyte concentrations (6.86, 20.6 and 185.2 nM) where the binding response is low and does not approach equilibrium, therefore lack sufficient information about kinetic events.

The single analyte concentration of 555.6 nM shows sufficient curvature to determine accurate kinetic rate constants and approaches equilibrium. Therefore, a value of 500 nM was chosen for the kinetic screen. Unlike a 1:1 interaction, a short plateau (steady state) is acceptable for a heterogenous interaction to ensure both sites are saturated.

Unlike CD64 and TNFα, which display slow dissociation rates that require extended periods to determine an accurate dissociation constant, CD16a V176 shows a rapid dissociation in line with its medium affinity for IgG antibodies and a greater than 5% decrease in binding is seen within 60 seconds of the start of the dissociation phase (Figure 5B). Where desired, the dissociation period can be curtailed to decrease the overall assay time and increase throughput during the kinetic screen.

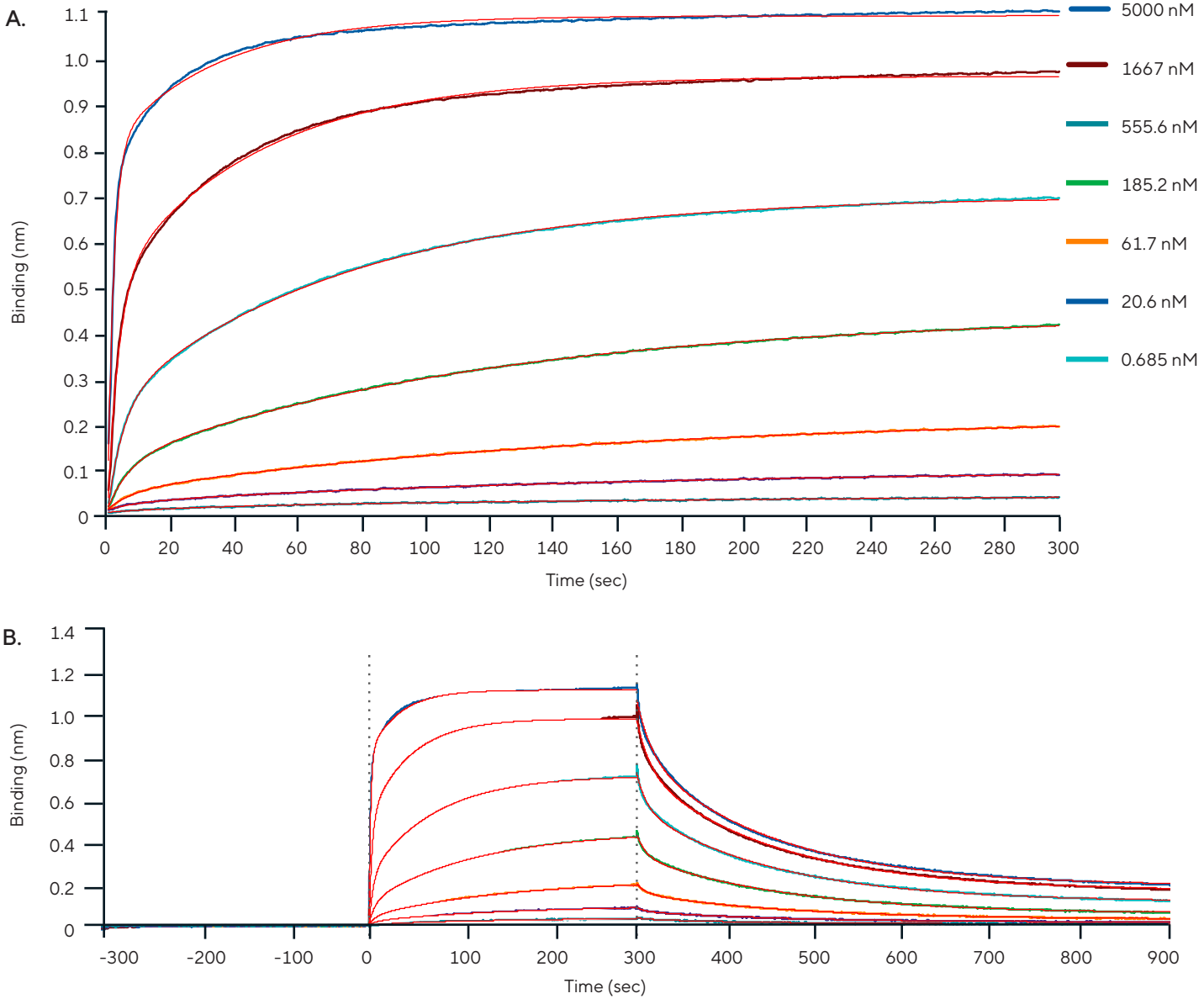

Figure 5: Humira® CD16a Analyte Optimization Concentration Series. (A) CD16a V176 shows a typical binding response for a heterogenous ligand with rapid association phases that plateau at later time points. (B) Medium affinity interactions such as CD16a V176 show rapid dissociation that ensures an observable decrease in binding response.

Kinetic Screen Qualification - Repeatability

As discussed in **Materials and Methods**, qualification of the kinetic screen allows the natural assay variability between users and sample preparation to be assessed. For all kinetic screen qualification assays (CD64, TNFα and CD16a V176) three assays were performed with triplicate measurements performed in each run. A reference biosensor was included for each replicate so a total of 21 replicates (3 * 7) were assessed per assay for a total number of 63 replicates (Figure 6). The Octet® R8 makes this process simple as 8 responses can be measured simultaneously, meaning assay duration is minimized for triplicate assessment but can also be optimized where multiple different samples are to be assessed.

Ligand Loading and Response Results

All assays show good coefficient of variation (CV) for ligand loading of the AviTag™ biotinylated proteins across all assays performed. CD64 ligand loading (threshold 0.25 nm) exhibited intra-assay variability of <3 %CV and inter-assay variability of <1.5 %CV (Figure 7A, Table 1). TNFα (loading threshold 0.12 nm) showed intra- and inter-assay variability of <6 % and 3.5 %, respectively (Figure 7B, Table 1) and CD16a V176 (loading threshold 0.25 nm) showed intra- and inter-assay variability of <5 % and 3 %, respectively (Figure 7C, Table 1).

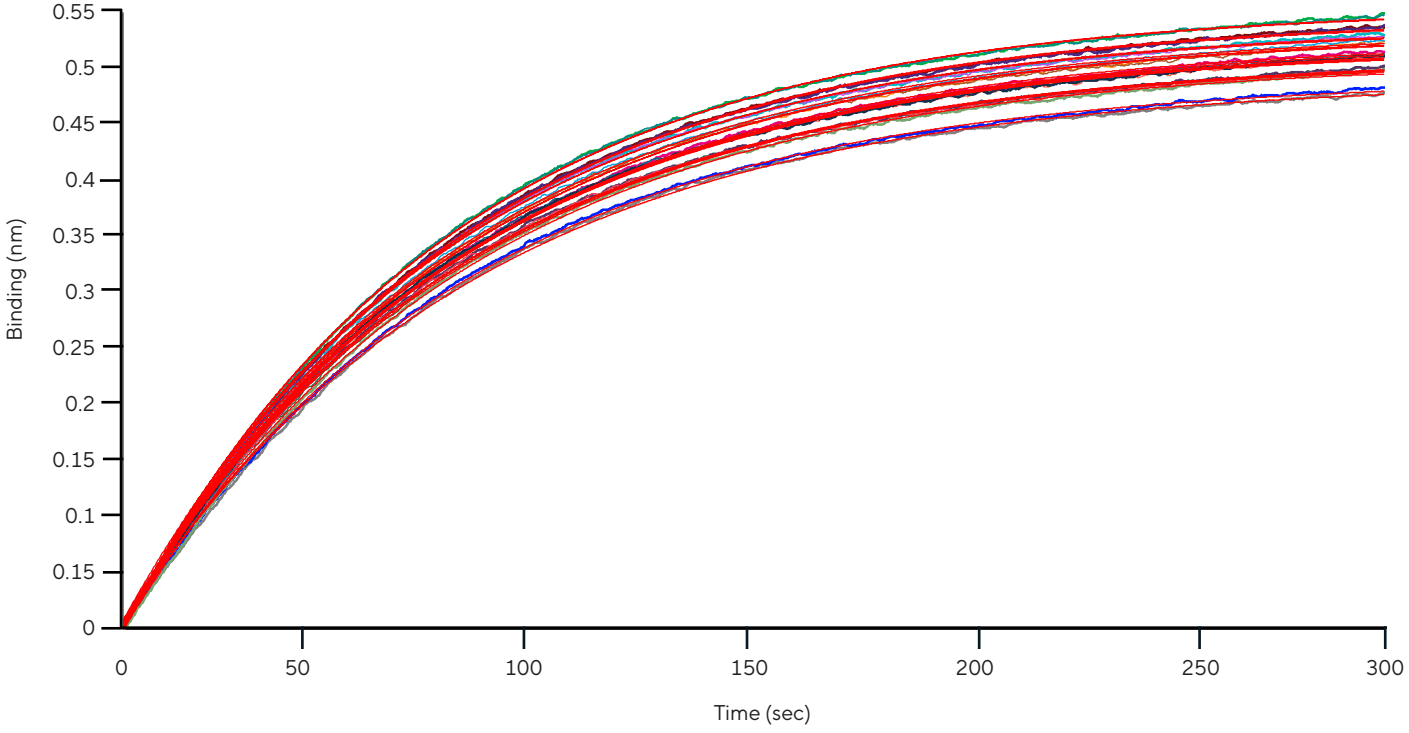

Figure 6: Example of Humira® – TNFα Single Concentration Binding (k_a). Triplicate of seven measurements were performed across three separate assays to determine the natural k_a, k_d, and K_D range from multiple users and sample preparation.

	Intra-assay 1	Intra-assay 2	Intra-assay 3	Inter-assay 1, 2 and 3
CD64	2.89	2.50	2.76	1.02
TNFα	5.64	5.39	3.72	3.48
CD16a V176	3.79	4.67	3.73	2.37

Table 1: Ligand Loading %CV During Kinetic Screen Qualification

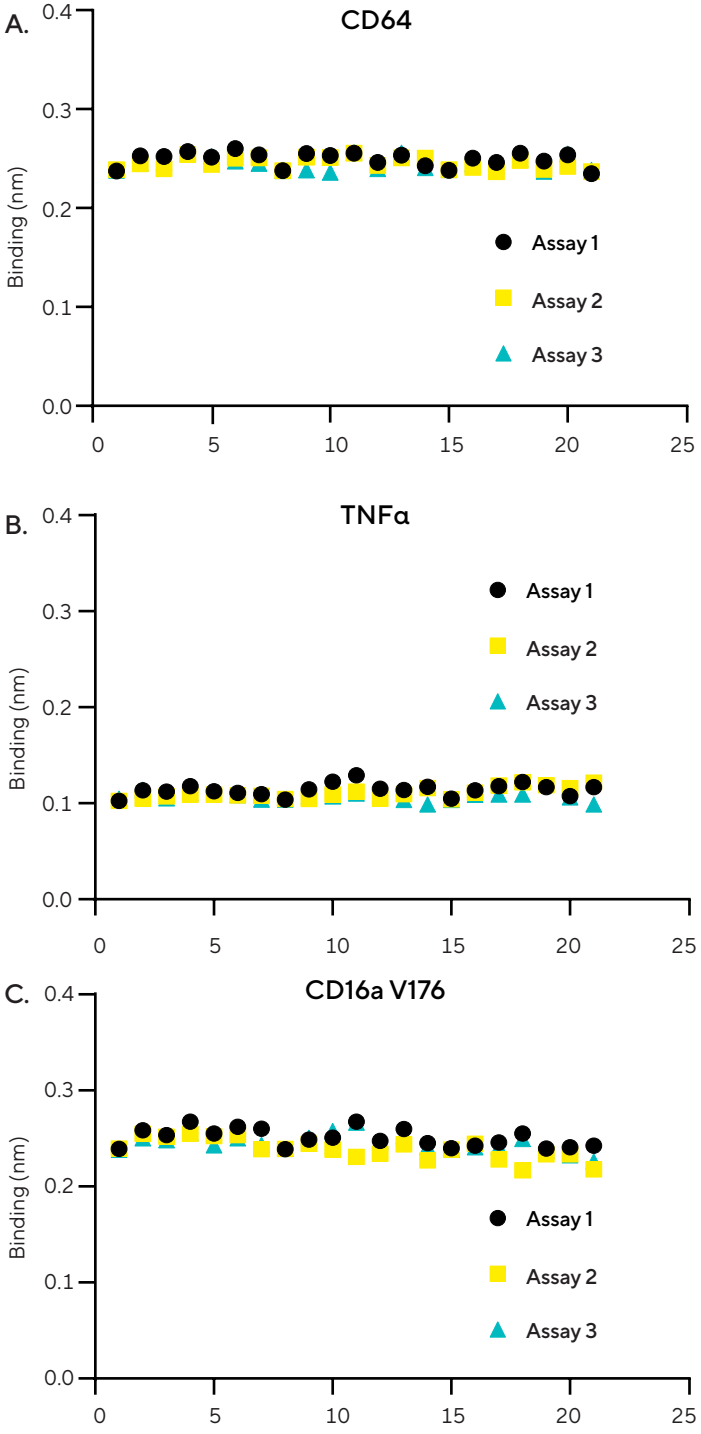

Figure 7: Ligand Loading. *(A) CD64 (loading threshold 0.25 nm), (B) TNFα (loading threshold 0.12 nm) and (C) CD16a V176 (loading threshold 0.25 nm) show good precision across the kinetic screen* qualification.

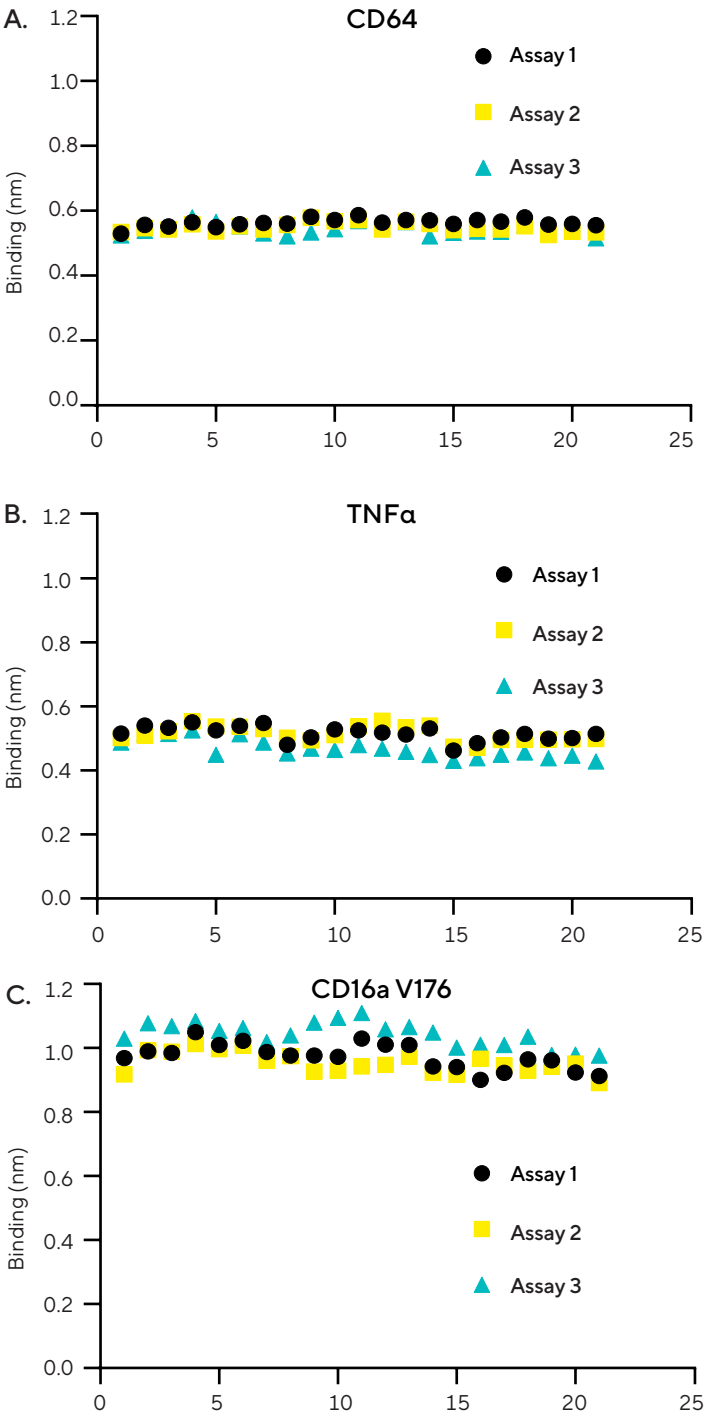

Figure 8: Analyte Response During Kinetic Screen Qualification *Repeatability. (A) CD64 (B) TNFα and (C) CD16a V176.*

As expected from the ligand loading values, the analyte binding response values are dependent upon the analyte assessed. Kinetic screening of CD64, which exhibited precise ligand loading levels, using Humira® showed an intra- and inter-assay CV of ~3 % and < 2 %, respectively (Figure 8A, Table 2). TNFα, showed an intra- and inter-assay CV of ~5 % when assessing Humira® in kinetic screening. (Figure 8B, Table 2). CD16a V176 intra- and inter-assay analyte responses were <5 % when assessing Humira® and kinetics screening of the biosimilars showed an intra-assay analyte response of <5 % and overall inter-assay variability of <5 % (Figure 8C, Table 2).

	Intra-assay 1	Intra-assay 2	Intra-assay 3	Inter-assay 1, 2 and 3
CD64	2.20	2.53	3.23	1.81
TNFα	4.38	4.76	6.53	5.15
CD16a V176	4.12	3.46	3.75	4.65

Table 2: *Analyte Response %CV During Kinetic Screen Qualification.*

CD64

Across all single concentration replicates assessed during assays 1, 2 and 3 Humira® bound to CD64 with an average global affinity of 780 pM (Range: 699 – 915 pM) which shares good agreement with literature values. Association kinetic rate constants were well defined with an intra-assay CV of <4 % and inter-assay value of <6 %, with an average value of $6.24*10^5$ $M^{-1}s^{-1}$ and a range of $5.82 – 6.52*10^5$ $M^{-1}s^{-1}$. Dissociation kinetics exhibited an intra- and inter-assay CV of <9 % and 10%, respectively with an average value of $4.81*10^{-4}$ s^{-1} with a range of $4.46 – 5.34*10^{-4}$ s^{-1}.

TNFα

Humira® bound to TNFα with an average global affinity of 268 pM (Range: 197 – 385 pM) across the three single concentration assays, which is in good agreement with literature values. Association kinetic rate constants were well defined with an intra-assay CV of <4 % and inter-assay value of <3 %, with an average value of $1.22*10^6$ $M^{-1}s^{-1}$ and a range of $1.20 – 1.26*10^5$ $M^{-1}s^{-1}$. As discussed above (See: Analyte Response Optimization), during a 90-minute dissociation period a linear decrease with a change in binding level of ~5 % was observed. This is reflected in an increase in the intra- and inter-assay CVs. The dissociation constant (k_d) displayed an average value of $3.23*10^{-4}$ s^{-1} with a range of $2.49 – 4.55*10^{-4}$ s^{-1}.

CD16a V176

Binding of Humira® to CD16a V176 was assessed using a heterogenous ligand model and therefore, two sets of kinetic and affinity values are generated. An average global affinity of 180 nM and 129 nM was observed across the three single concentration assays (Range: 177 – 183 and 121 – 133 nM, respectively). Association kinetic rate constants were well defined with an intra-assay CV of <4 % and inter-assay value of <3 % for both k_a, with an average value of $3.7*10^4$ and $5.6*10^5$ $M^{-1}s^{-1}$, and a range of $3.61 – 3.79*10^4$ and $5.53 – 5.73*10^4$ $M^{-1}s^{-1}$, respectively. Dissociation kinetics were well-defined with an intra- and inter-assay CV of <5 % and 5 % for both dissociation (k_d) constants, respectively. Dissociation kinetic values displayed an average value of $6.68*10^{-3}$ and $7.26*10^{-2}$ s^{-1} with a range of $6.63 – 6.71*10^{-3}$ s^{-1} and $6.96 – 7.60*10^{-2}$ s^{-1}, respectively.

Kinetic Screen – Biosimilar Assessment

The aim of the kinetic screen qualification - repeatability study above is to set parameters that allow additional bioprocess samples of either known or unknown status to be rapidly assessed against a multitude of receptors, antigens or CQA of choice. As the range here is for a single analyte concentration it allows users to make decision based on kinetic data as to whether a clone, production process or any other parameter can generate data that is within a user-specified range. It is important to note here that kinetic screening should not be used in place of a fully qualified or validated assay but merely as a facile way to assess and triage large libraries of drug candidates or CLD processes. IgGs are typically purified using low pH conditions off Protein A affinity columns. The IgG is first bound to the Protein A charged-column under physiological pH conditions before elution is carried out at low pH. For Adalimumab however, low pH conditions may lead to the elution of aggregated species hence the need for optimization of the elution process. In addition to elution process optimization, it has also been previously shown that the addition of L-Arginine to the buffer eluent can greatly increase the recovery of monomeric correctly folded antibodies and a subsequent decrease in aggregation. Here we assessed samples from an affinity purification of a single clone of adalimumab to the determine the effects of Protein A column elution buffer on kinetics and affinity to CD64, TNFα and CD16a V176 and to assess whether further orthogonal processes such as size exclusion may be needed to generate products with the desired kinetics attributes.

Ligand Loading and response results

As with the kinetic screen qualification – repeatability, all assays show good coefficient of variation (CV) for ligand loading of the AviTag™ biotinylated proteins across the biosimilar column eluates assessment. CD64 ligand loading (loading threshold 0.25 nm) showed intra- and inter-assay variability of <4 % and 1.5 %, respectively (Figure 9A, Table 3). TNFα (threshold 0.12 nm) exhibited intra-assay variability of <5 %CV and combined inter-assay variability of <3.5 %CV (Figure 9B, Table 3) and CD16a V176 (loading threshold 0.25 nm) showed intra- and inter-assay variability of <4 % and 3 %, respectively (Figure 9C, Table 3).

	Kinetic Screen Qualification - Repeatability	Intra-assay Biosimilars	Inter-assay (combined)
CD64	1.02	3.20	1.12
TNFα	3.48	4.34	3.20
CD16a V176	2.37	3.8	2.21

Table 3: Ligand Loading %CV During Kinetic Screen Biosimilar Assessment.

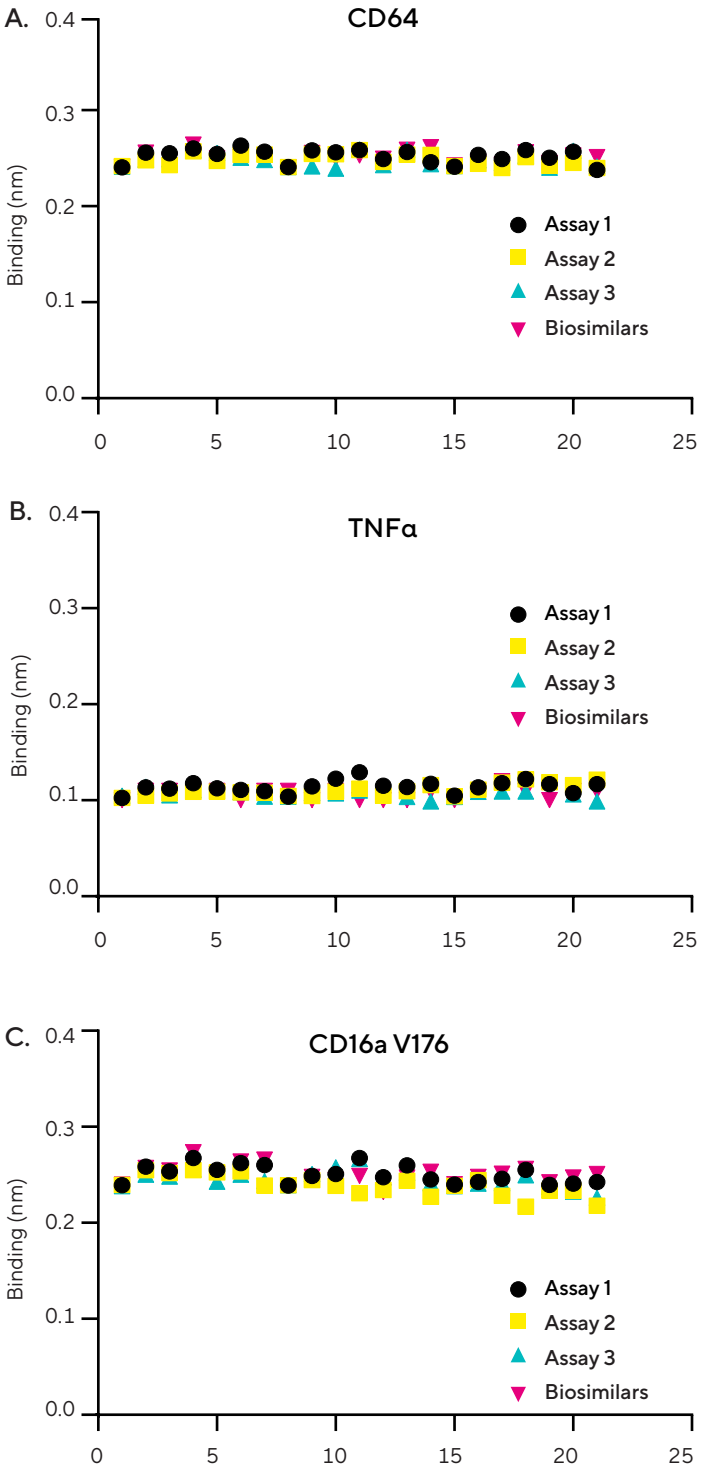

Figure 9: Ligand Loading Including Biosimilar Column Eluate Assessments. (A) CD64 (loading threshold 0.25 nm), (B) TNFα (loading threshold 0.12 nm) and (C) CD16a V176 (loading threshold 0.25 nm) show good precision across the kinetic screen qualification and biosimilar assessment.

Unlike the kinetic screen qualification where a single analyte was assessed repeatedly, assessment of biosimilar column eluates or any other samples, the analyte response is dependent upon the binding properties of the analyte and therefore, may either be higher or lower than the RMP. Kinetic screening of the biosimilar column eluates using Humira® showed an intra- and inter-assay CV of <3 % and inter-assay CV of <6 % when the biosimilar analyte combined responses were considered for CD64 (Figure 10A, Table 4). TNFα, which exhibited precise ligand loading levels also exhibited excellent intra- and combined inter-assay CV of < 5 %, respectively when assessing Humira® in kinetic screening (Figure 10B, Table 4). CD16a V176 intra-assay %CV of <5 % when assessing Humira® and kinetics screening of the biosimilar and a combined inter-assay analyte responses CV of <7 % (Figure 10C, Table 4).

	Kinetic Screen Qualification - Repeatability	Intra-assay Biosimilars	Inter-assay (combined)
CD64	1.81	2.57	5.72
TNFα	5.15	6.04	4.89
CD16a V176	4.65	4.48	6.46

Table 4: *Analyte Response %CV During Kinetic Screen Biosimilar Assessment.*

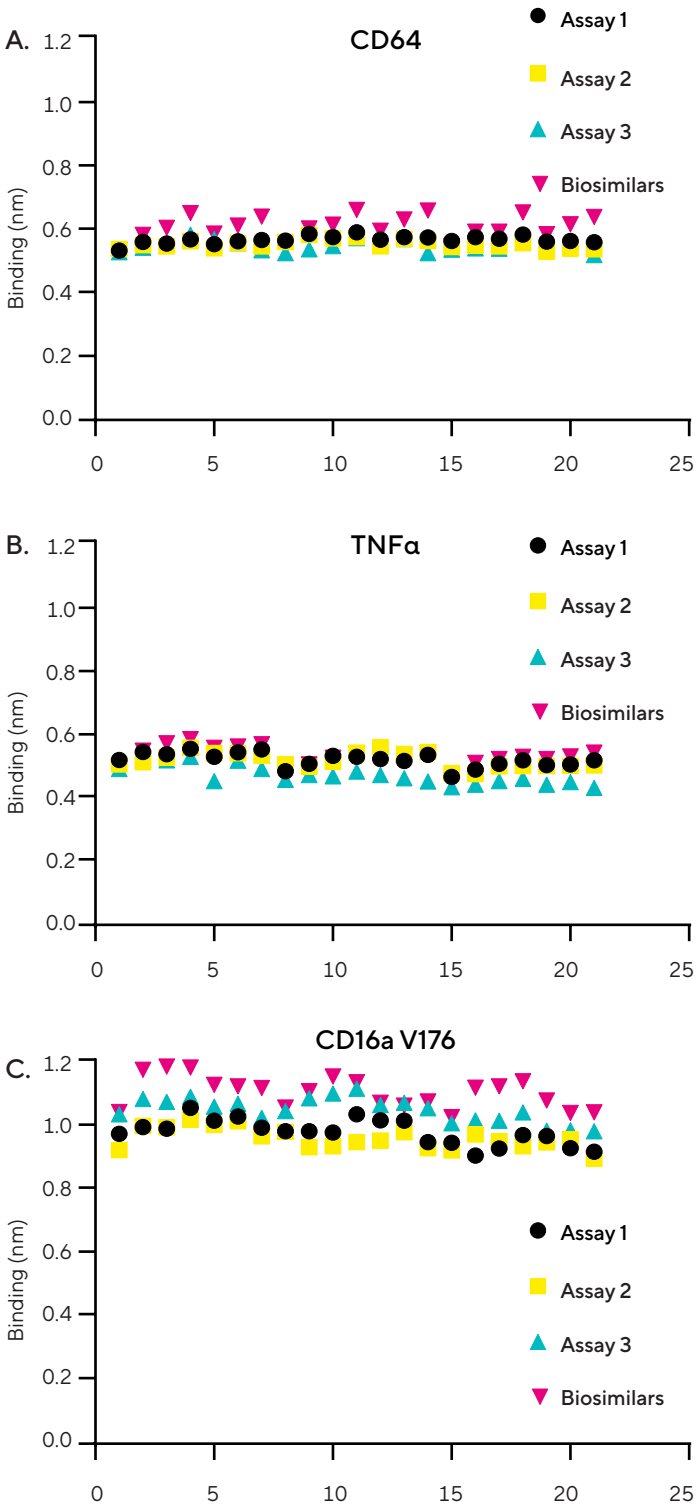

Figure 10: Analyte Response Including Biosimilar Column Eluate Assessments. *(A) TNFα (B) CD64 (C) CD16a V176 show good precision across the kinetic screen qualification and biosimilar assessment.*

CD64

As shown in Figure 11, both the k_a and k_d of the Humira® reference standard (RMP) used determined during screening of the column elution fractions of the biosimilar were within the range of parameters determined during the kinetic screen qualification for the Humira® RMP.

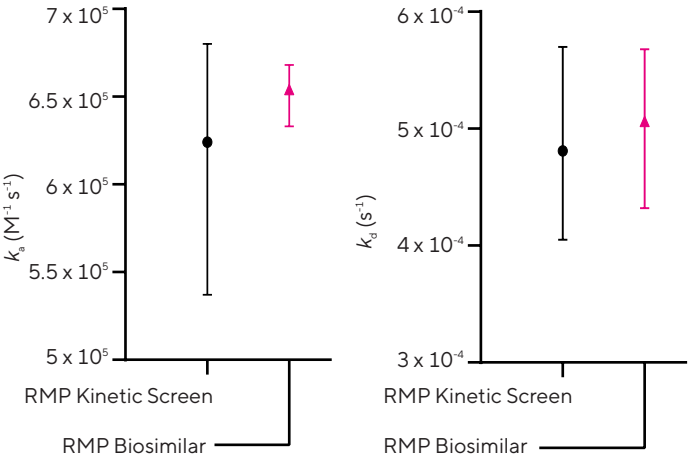

Figure 11: Kinetic Parameters are Within Pre-Determined Ranges for CD64. *Humira reference (A) k_a and (B) k_d from the biosimilars assessment were within range specified by the kinetic screen qualification.*

The k_a determined for most column elution conditions is close to those values determined for the Humira® RMP but as can be seen in Figure 12A, a clear deviation can be observed for the conditions pH 3.6 + L-Arginine and pH 3.8 + L-Arginine.

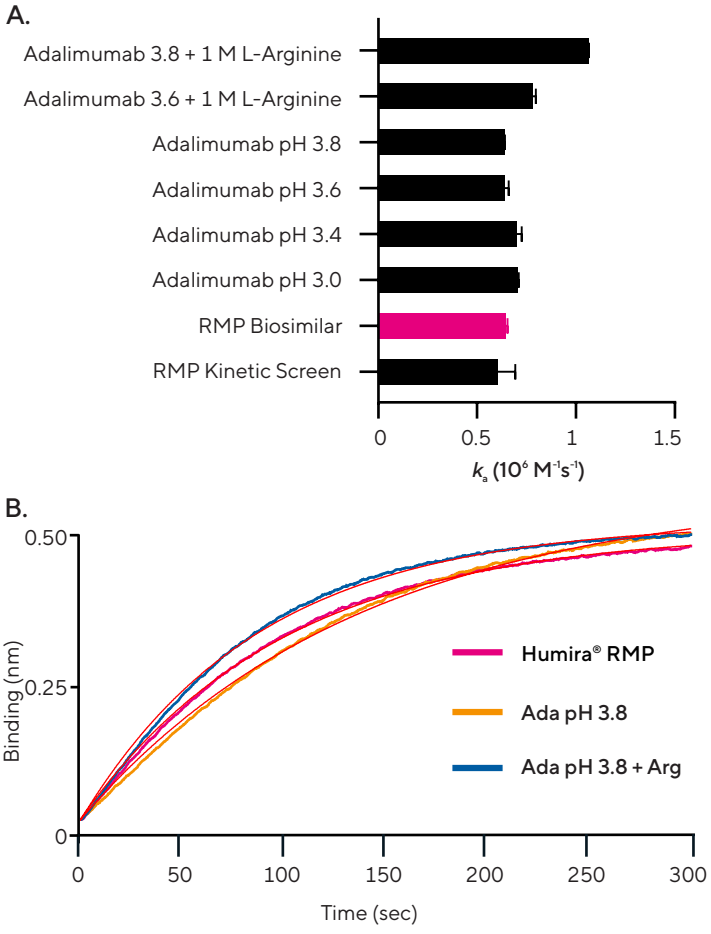

Figure 12: The Effect on L-Arginine upon Association Kinetics. *(A) k_a values for kinetic screen biosimilars show a deviation for those sample containing L-Arginine compared to those without. (B) Humira RMP (Magenta) exhibits different binding kinetics to adalimumab pH 3.8 (orange) and adalimumab pH 3.8 + 1M L-Arginine (blue).*

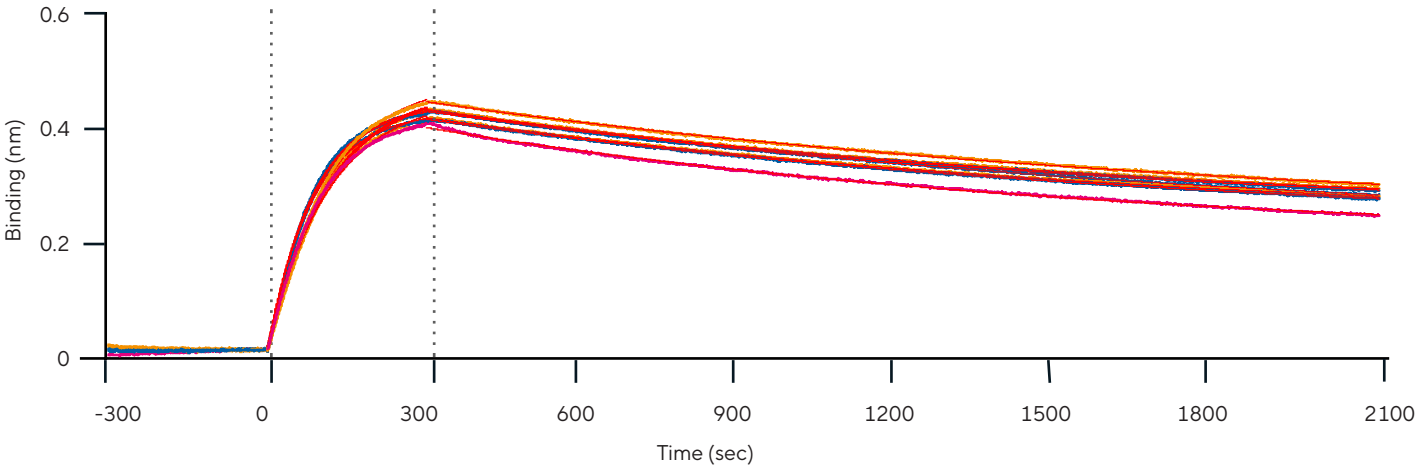

Figure 13: The Effect on L-Arginine upon Dissociation Kinetics *pH 3.6 + 1M L-Arginine (orange) and pH 3.8 + 1M L-Arginine (blue) column elution conditions show a clear deviation from the Humira® RMP (magenta)*

When displayed against the Humira® RMP, the dissociation traces for pH 3.6 + L-Arginine and pH 3.8 + L-Arginine show a clear deviation away from the Humira® RMP samples with an average k_d of 6.61*10^4 and 6.48*10^4, respectively (Figure 13).

Considering the precise CD64 ligand loading and analyte response, differences in the observed kinetics post column elution indicate that additional orthogonal studies are required to determine the reason for any differences against both the Humira® RMP and the column elution fractions at the same pH without the L-Arginine.

TNFα

As with CD64, both the k_a and k_d of the Humira® RMP determined during screening of the column elution fractions of the biosimilar were within the range of parameters determined during the kinetic screen qualification for the Humira® RMP (Figure 14).

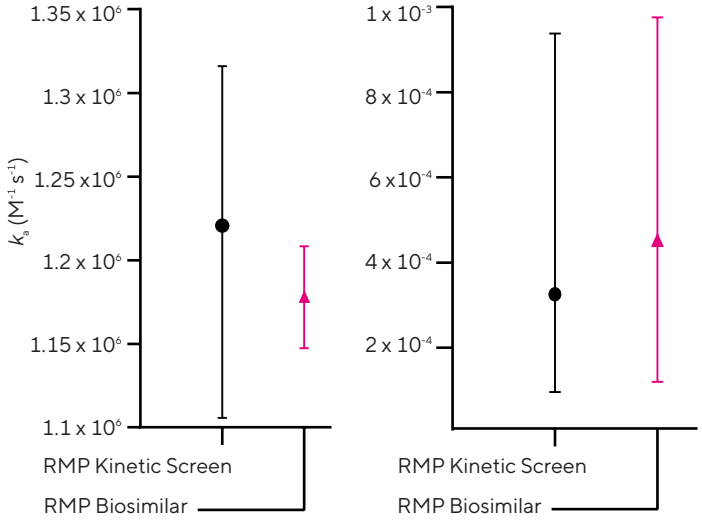

Figure 14: Kinetic Parameters are Within Pre-Determined Ranges for TNFα. *Humira® RMP binding to TNFα exhibited similar values during the kinetic screen qualification and during the column elution assessment.*

When compared to the Humira® RMP all column elution samples exhibited association kinetics that were outside the range determined during the qualification (Figure 15A). Column elution samples containing L-Arginine again, exhibited different binding properties to those eluted without L-Arginine and show a rapid association phase that saturated the biosensor and moved away from the RMP observed 1:1 kinetics (Figure 15B).

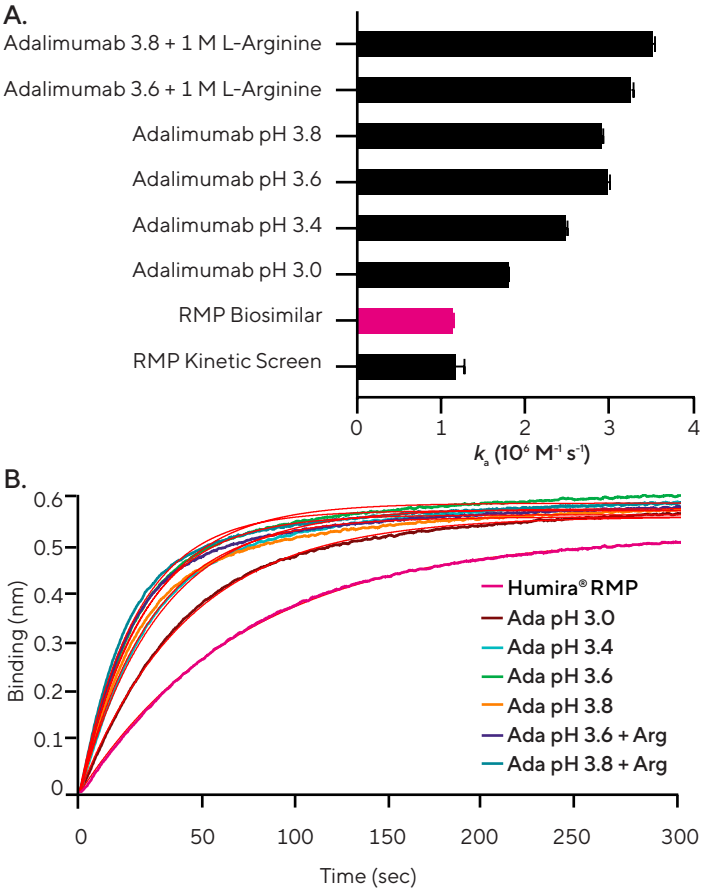

Figure 15: The Effect on L-Arginine upon Association Kinetics. *(A) All samples exhibited higher k_a values for the biosimilar column eluates show a deviation for those sample containing L-Arginine compared to those without (B) Biosimilar column eluate samples in general show a rapid association phase and those containing L-Arginine saturated the biosensor and moved away from the Humira® RMP observed 1:1 kinetics.*

CD16a V176

Although CD16a V176 was fitted using a heterogenous ligand model the kinetic screen qualification and biosimilars RMP association and dissociation kinetics showed good agreement (Figure 16). Although independent kinetic parameters are determined for k_a, k_{a2}, k_d and k_{d2} the observed binding response is the sum of these two independent reactions.

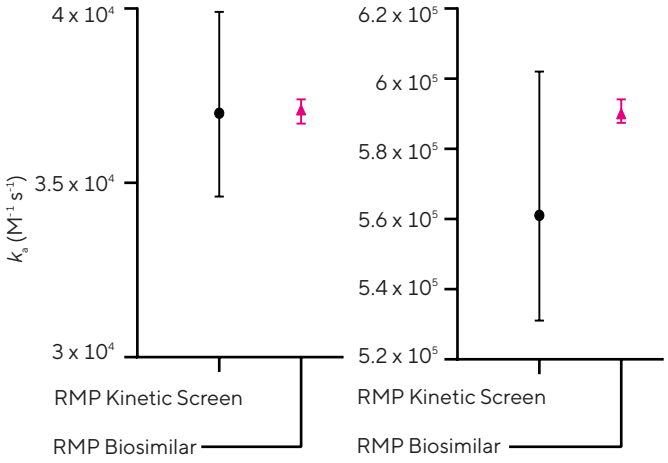

Figure 16: The Heterogenous Ligand Model Shows Good Precision Across Assays. *CD16 k_a and k_{a2} (A, B) show values for the Humira® RMP that are comparable to those determined for the Humira® RMP during the kinetic screen qualification – repeatability.*

Out of the three ligands assessed, binding of Humira® to CD16a V176 shows the clearest changes between the biosimilar column elution fractions and the Humira® RS. The association rate constant k_a is visually slower than the Humira® RMP (Figure 17A) and the same effect is seen for k_{a2} (Figure 17B). During the association phase in the Octet® BLI assay k_a and k_{a2} are observed as a single binding event as shown in Figure 17C, as with CD64 and TNFα, a clear gradation between the Humira® RMP, pH 3.6 + L-Arginine and pH 3.8 + L-Arginine samples which occupy the middle band and the pH eluates which cluster together in the top band.

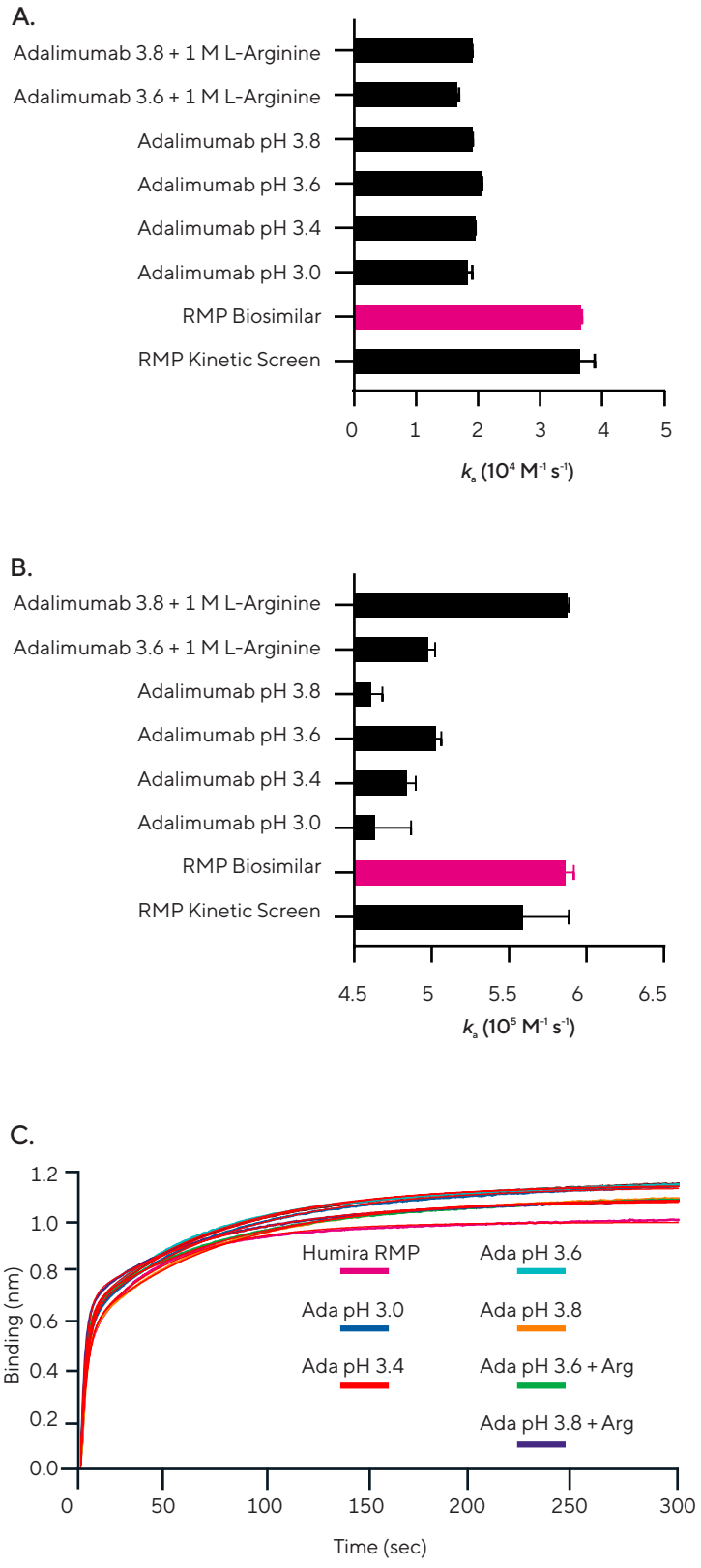

Figure 17: CD16A V176 Association Kinetics *(A) k_a (B) k_{a2} (C) binding response plot shows Humira® RMP binding to CD16a V176 in the lowest band and a clear grouping of the L-Arginine containing samples (pH 3.6 and pH 3.8), whilst the pH eluates cluster together as a separate top band.*

Dissociation kinetics for the biosimilar column elution fractions show a similar trend to association kinetics in that there is a large difference between themselves and the Humira® RMP. As shown in Figure 18A, k_d values for all samples are slower than the Humira® RMP but the secondary dissociation constant k_{d2} is faster than the Humira® RMP and as can be seen in Figure 18B, pH 3.6 + L-Arginine and pH 3.8 + L-Arginine exhibit a clear difference to the pH elution samples, which is also visible in the dissociation binding response (Figure 18C), where Humira® RMP is fastest and the + L-Arginine samples cluster together in the middle and the pH eluates show a clear clustering together.

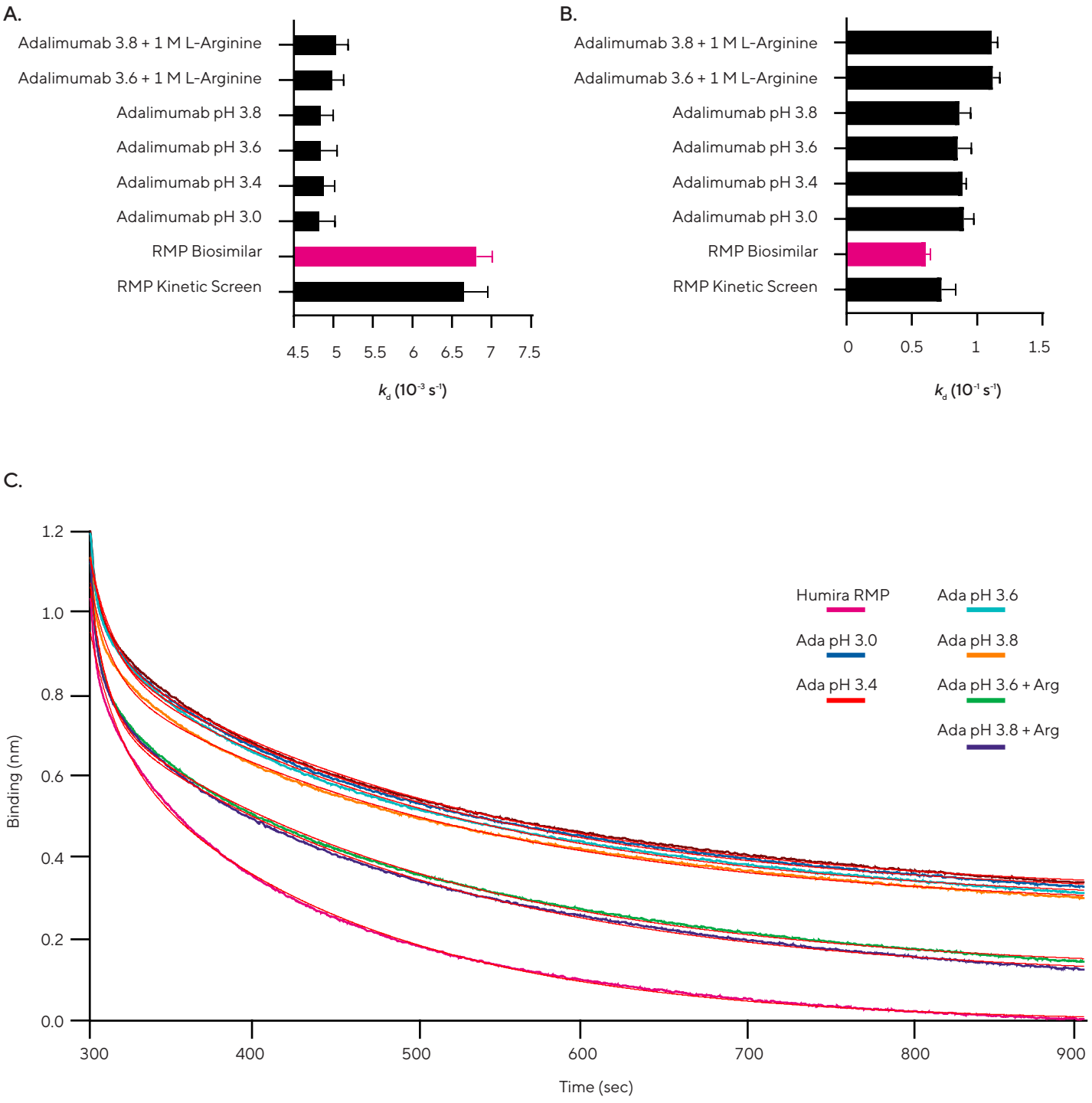

Figure 18: Dissociation Kinetics of CD16A *(A) k_d for the biosimilar column eluates is slower than the Humira® RMP and V176 and faster for k_{d2} with the L-Arginine containing samples showing a clear difference to the other pH eluted fractions (B). (C) The dissociation phase for the Humira® RMP and biosimilar column eluates show a clear separation between those eluted with or without L-Arginine.*

Conclusions

Unlike standard kinetic studies that require several analyte concentrations which require a large amount of space on a standard 96- or 384-well micro plate, a single concentration kinetic screen means a range of relevant screening parameters can be rapidly determined while maximizing microplate space.

As shown in this application note, a Humira® biosimilar (adalimumab) development process sample (Protein A antibody purification) was able to be rapidly tested against three key ligands that would be required in a full qualification to regulatory bodies. Although triplicates are shown above for biosimilar column eluates, single replicates were sufficient to highlight key trends such as seen by the addition of 1M L-Arginine to the elution buffer. Single replicate assays ranged from ~30 minutes (CD16a V176) to ~1 hour 45 minutes for the high affinity TNFα interaction, though as discussed in Results and Discussion the CD16a V176 assay duration could have been decreased by shortening the dissociation period.

After selection of a suitable analyte concentration and ligand load level, single concentration kinetic screens can comprehensively describe an analytes kinetic profile and allow ranking and selection of the optimal antibodies, proteins, or production processes according to their kinetic profiles (or where required, binding responses). This makes decision making simpler and the low analyte concentration requirements and facile assay setup allow better decisions to made earlier in the drug discovery cycle; saving time and helping to alleviate downstream issues by combining CQAs with production yields.

This simplified kinetic screen assay setup can rapidly generate a large amount of association, dissociation and affinity data that is critical to ensure that reselection of the optimal clone is not required further downstream. As it is possible to load multiple ligands, analytes, and biosensors into a single Octet® BLI assay it is simple to envision a single kinetic screen assay where multiple binding interactions between different ligand and analytes can be rapidly assessed with a day.

This approach can also accelerate the drug discovery cycle for complete libraries of antibodies as they can be tested against a de novo antigen rapidly and cost-effectively. Unlike the classical analytical techniques where hits are identified and subsequent optimization of antibodies is performed, which can be costly, time-consuming and have a high failure rate, the combination of kinetic screening with machine learning allows the creation of large libraries of antibodies that can be tested, selected, and filtered rapidly. This has the potential to decrease the discovery cycle from years to months or weeks due to a direct increase in usable data.

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