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Combining Advanced Flow Cytometry and BLI Label-Free Detection for Broad Characterization of Antibody Binding and Function

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Introduction

Monoclonal antibody (mAb) therapeutics comprise a fast-growing segment of the pharmaceutical market, with growth expected to continue due to a record-high number of mAb therapeutics currently under regulatory review.[1] There are now over 100 approved mAb therapeutics across a vast array of disease areas, ranging from cancer treatments that target checkpoint inhibitors such as PD-1, to interventions against infectious diseases, such as HIV. Many mAbs act to enhance the immune response against infected or cancerous cells, combining high target specificity via the variable region with the potential to induce a multitude of Fc receptor functions via the constant region.

For the mAb market to keep expanding and diversifying, it is crucial to have robust, high-throughput methods for screening and profiling mAb drug candidates. If optimal binders with desirable functional profiles can be identified early in the antibody discovery process, there is a higher likelihood of approval in later-stage clinical assessment. Technological advancements that have improved the quality of novel mAbs being fed into the clinical pipeline have enabled rapid response to crises such as the 2020 COVID-19 pandemic, which led to seven antibody drug products either receiving or requesting emergency use authorizations before the end of 2021.[1]

With the broad range of characteristics that can impact therapeutic efficacy, full profiling of mAb candidates during drug development often requires the measurement of many different parameters across several platforms. Conventional techniques for mAb characterization typically:

- Involve low-throughput acquisition (e.g., traditional flow cytometry)
- Require purified forms of the target protein
- Utilize laborious, time-consuming processes involving protocol optimization, fixation, and repetitive washes
- Necessitate the use of large volumes of precious sample and antibody
- Require complex and lengthy data analysis

Here, we present a streamlined approach for high-throughput profiling mAbs, using both iQue® Advanced Flow Cytometry and Octet® Bio-Layer Interferometry (BLI) Label-Free Detection. We have created a multi-phase workflow, which utilizes the two instruments in parallel to extensively characterize therapeutic antibodies. The iQue® assays employ live cell-based models to profile binding, competition, and Fc function of test antibodies, while the Octet® provides antibody characterization through the determination of antigen binding kinetics, relative antibody glycosylation content and Fc-gamma receptor (FcγR) binding. Together, the power of these instruments can be leveraged to provide comprehensive assessment of novel mAbs, to improve the identification and qualification of hits during screening and to advance drug discovery workflows.

Method

In this application note, we demonstrate the combined iQue® and Octet® approach for antibody assessment by profiling the binding and functional characteristics of three anti-HER2 antibodies (see workflow in Figure 1). We split the process up into multiple phases, beginning in phase 1 with extensive characterization of antigen binding. In phase 2, we used the iQue® to assess cross-competition between the antibodies, whilst on the Octet®, we analyzed post-translational modifications (PTMs). Finally, in phase 3 we explored antibody Fc function and FcγR interactions.

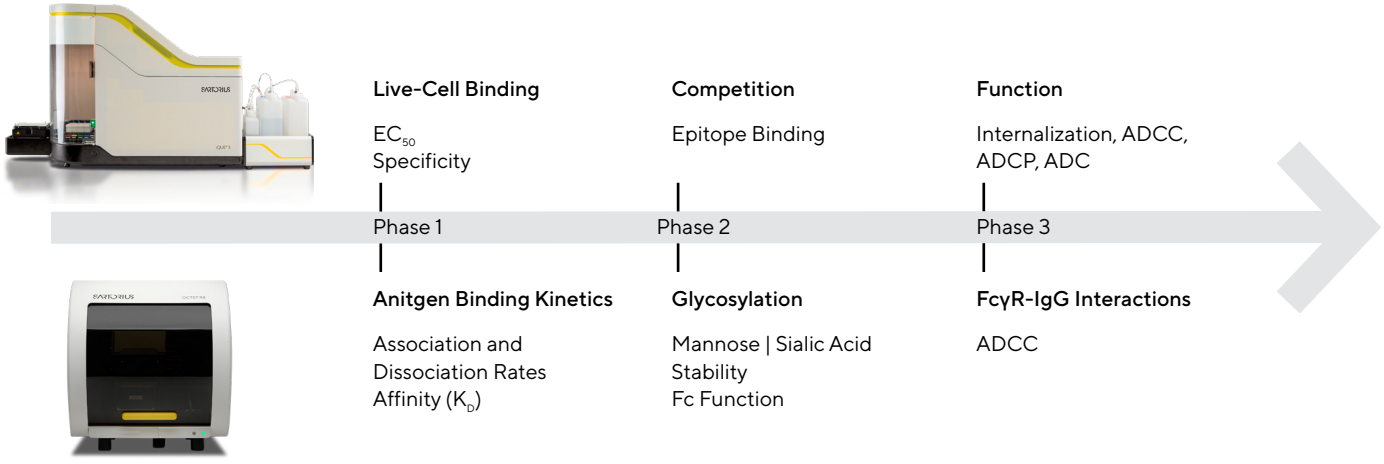


Figure 1

Overview of the Combined iQue® Advanced Flow Cytometry and Octet® BLI Label-Free Detection Workflow for mAb Characterization

Phase 1 utilizes the iQue® live-cell antibody binding assay to assess antibody binding to the native target on cells whilst kinetic characterization of antigen binding is performed using the Octet®. Phase 2 couples cross-competition assessment using the iQue® with PTM analysis in the form of ranking the glycosylation status of the antibodies using the Octet®. The focus of phase 3 was functional characterization of the antibodies, achieved by combining phenotype and function assessment using iQue® reagent kits with analysis of FcγR interactions using the Octet®.

Antibodies

Three anti-HER2-hIgG1 antibodies were characterized: a Trastuzumab biosimilar (Absolute Antibody); Kadcyla (Trastuzumab emtansine; therapeutic antibody-drug conjugate (ADC) based on Trastuzumab and the chemotherapy drug emtansine (also known as DM1)) and a Pertuzumab biosimilar (Proteogenix). An Anti-β-Gal-hIgG1 mAb from Invivogen was used as an isotype control. For the iQue® live-cell binding assay, the anti-HER2 antibodies were either analyzed in their native state, or after heating in a water bath at 69°C for 45 or 90 minutes.

iQue® Live-Cell Antibody Binding

Test antibodies (10 µL) were incubated with 20 µL of mixed HER2-positive AU565 and HER2-negative MDA-MB-468 target cells (10 K/cell type/well) in 96- or 384-well plates for 30 minutes (on ice, to inhibit internalization). MDA-MB-468 cells were pre-labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye (1:2000) and the AU565 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 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® Determination of Antigen Binding Kinetics

Anti-hIgG Fc Capture (AHC) biosensors (Sartorius) were immobilized with 0.25 µg/mL of each anti-HER2 antibody. Association and dissociation kinetics of antibody binding to a soluble tag-free recombinant HER2 protein (MW 70.1 kDa, ACROBiosystems, 50 nM to 3.1 nM, 1 in 2 dilution) were measured using the Octet® BLI Label-Free Detection System. Association was measured for 180 seconds, and dissociation was measured for 1800 seconds. Octet® Kinetics Buffer (diluted from 10X to 1X in PBS) was used for all sample dilutions and buffer steps. 10 mM glycine pH 1.7 was used to regenerate the biosensors between replicates. A shake speed of 1000 RPM and temperature of 30°C was used throughout. Resulting traces were fitted using a 1 to 1 global binding model.

iQue® Competition Assay

Anti-HER2 antibodies (4.5 µg/mL, 3X final assay concentration) were pre-labeled with fluorophore-conjugated secondary antibody (R-Phycoerythrin-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, Jackson ImmunoResearch) at a 1:1 concentration ratio. Labeled antibodies were added to AU565 target cells (HER2-positive breast cancer line, 20 K/well) alongside a concentration range of unlabeled anti-HER2 antibody and iQue® Cell Membrane Integrity (R/Red) Dye. Plates were incubated for 45 minutes on ice, washed, and run on the iQue®. A lower MFI than the IgG control for the secondary antibody fluorophore indicated that there had been competition between the labeled and unlabeled antibodies for binding to HER2 on cells.

Octet® Glycosylation Analysis

Antibodies were analyzed using the Octet® GlyM and GlyS Kits, which utilize lectin pre-immobilized biosensors specific for binding to mannose and sialic acid, respectively. These kits measure relative glycan content of test antibodies. Each kit was used as described in the assay protocol for one-step direct quantitation.[2,3] Following optimization to determine an optimal fixed titer for glycan analysis, test antibodies were diluted in glycan sample prep buffer (GSB) to a concentration of 10 µg/mL. GSB only was included as a reference for background subtraction. The endpoint binding rate equation was used to quantify binding from curves at 570 seconds for mannose and 1140 seconds for sialic acid.

iQue® Functional Assays

- Internalization
  Target cells (HER2-positive AU565 cells, 7.5 K/well) were seeded overnight in 96-well flat bottom plates (Corning® 3595). Antibodies were labeled using the pH-sensitive probe from the iQue® Antibody Internalization Kit, which fluoresces upon internalization into acidic lysosomal or endosomal pathways. The kit also includes an iQue® Cell Membrane Integrity (B/Green) Dye to measure cell viability and facilitate exclusion of dead cells from internalization analysis. After three hours, cells were lifted using Accutase (Corning®), transferred to a V-bottom plate (Corning® 3363) and run on the iQue®. Antibody internalization is quantified in the iQue® Forecyt software using the supplied template as the percentage of live cells positive for the fluorescent probe.
- Antibody-Dependent Cell Cytotoxicity (ADCC)
  AU565 cells (5 K/well) were labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye and seeded in 96-well flat bottom plates overnight. Test antibodies were added at a range of concentrations alongside unlabeled natural killer (NK) cells (Purchased frozen from StemCell Technologies, 25 K/well). After 24 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, CD16, 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.

- AntibodyDependent Cellular Phagocytosis (ADCP)
  Incucyte® Nuclight Green labeled AU565 cells (5 K/well) were incubated in 96-well plates for 15 minutes with test

antibodies at room temperature. Effector cells (PBMCs isolated from whole blood and incubated overnight with 10 ng/mL IL-2) were added at a 20:1 effector-to-target ratio. After 1 hour at room temperature, cells were labeled with Antibody Detection Cocktail and Cell Membrane Integrity (R/Red) Dye from the iQue® Human Antibody Dependent Cellular Phagocytosis (ADCP) Kit (30 minutes, room temperature). Plates were then centrifuged (300g, 5 minutes), resuspended and run on the iQue® using a 10 second sip time. ADCP was quantified as the percentage of CD14-positive monocytes that were also positive for the Nuclight green dye as this indicated co-localization between the targets and phagocytic immune cells.

- Antibody-Drug Conjugate (ADC) Activity
  AU565 cells (5 K/well) were seeded in 96-well flat bottom plates overnight before test antibodies were added at a range of concentrations. After 72 hours, cells were lifted using Accutase and transferred to V-bottom plates for labeling with iQue® Cell Membrane Integrity (R/Red) Dye. After 30 minutes plates were washed and run on the iQue®. ADC activity was quantified in iQue® Forecyt software as an increase in cell death.

Octet® Measurement of FcγR-IgG Interaction
Biotinylated Human FcγIIIA (CD16a, ACROBiosystems, 0.325 µg/mL) was immobilized on to Octet® high-precision streptavidin biosensors (SAX) for 75 seconds. Kinetics of binding to a range of concentrations of anti-HER2 antibodies (top concentration 0.4 µM, 1 in 2 dilution) was measured using the Octet® R-8. Rates of association were measured for 300 seconds and dissociation for 600 seconds. The plate was shaken at 1000 RPM throughout sampling and its temperature maintained at 25 °C. Octet® Kinetics Buffer (diluted from 10X to 1X in PBS) was used for all sample dilutions and buffer steps. Resulting traces were fitted using a 2 to 1 (heterogenous ligand) global binding model.

Results

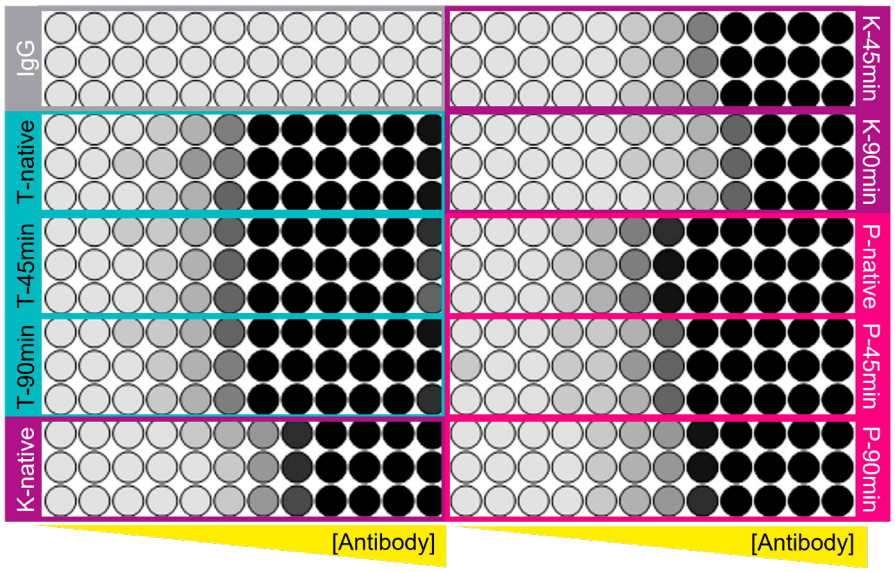
Comparing Live-Cell Binding and Heat Stability of Anti-HER2 Antibodies
Analysis of relative binding of antibodies to live cells using advanced flow cytometry provides a powerful tool for drug development processes, with the key advantage of delivering readouts for binding to the native form of the target protein as it is found on the surface of live cells. Moreover, the presence of other membrane bound proteins found on the cell provides an excellent first look at whether binding to off-target receptors may present an issue. These assays can be applied at the screening stage, to perform high-throughput screening of a library of novel antibody candidates, for example from a hybridoma or phage display library. They can also be used after screening to further qualify 'hits', for example by producing full concentration-response curves and generating EC_{50} values to compare antibody binding.

In this application note, we have used the iQue® Advanced Flow Cytometry Platform to assess binding of three anti-HER2-IgG1 antibodies: a Trastuzumab biosimilar, Kadcyla (an ADC based on Trastuzumab) and a Pertuzumab biosimilar. We coupled this measurement with a thermal stability evaluation, by testing how the binding of native antibodies compared to the binding of antibodies that had been heated at 69°C for 45 or 90 minutes. Unlabeled HER2-positive AU565 cells and iQue® Cell Proliferation and Encoding (V/Blue) dye-labeled HER2-negative MDA-MB-468 cells were mixed and incubated with the test antibodies, followed by an RPE-conjugated secondary antibody.

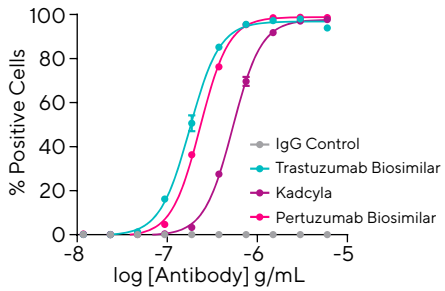
The data in figure 2 shows that the Trastuzumab biosimilar had both the highest level of binding to AU565 cells and the greatest resistance to heat denaturation, with the EC_{50} remaining constant across the native, 45- and 90-minute heated antibodies, ranging only between 160 and 180 ng/mL. EC_{50} values for binding of Kadcyla were highest and it was sensitive to heat denaturation, with a two-fold increase in EC_{50} from the native state (530 ng/mL) compared to after 90 minutes of heating (1250 ng/mL). This suggests that the modification of the Fc portion to include the ADC payload, compared to the native Trastuzumab, has resulted in a loss in both thermal and cell binding stability. The Pertuzumab biosimilar in its native state had similar binding to Trastuzumab, with an EC_{50} of 230 ng/mL, but it was also sensitive to heating and saw a two-fold loss in binding activity after 90 minutes of heating (EC_{50} 530 ng/mL).

Combining the antigen-positive and antigen-negative cells in the wells meant that we could get an indication of antibody binding specificity. At the top concentration of 6 µg/mL of antibody, binding to the HER2-negative cells was less than 1% for all three antibodies, indicating they are all highly selective, as is essential with therapeutic antibodies. Off-target binding of therapeutic antibodies can have a serious impact *in vivo* with risks of high toxicity and reduced efficacy.[4]

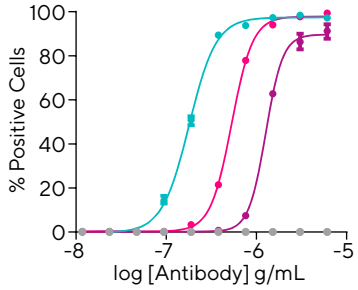
A. Secondary Antibody MFI on AU565s



B. Native



C. 90 Minute Heat



D. EC_{50} Values (ng/mL ≡ nM) for % Positive AU565

Antibody	Native	45 Min. Heat	90 Min. Heat
Trastuzumab Biosimilar	180 ≡ 1.2	160 ≡ 1.1	180 ≡ 1.2
Kadcyla	530 ≡ 3.6	840 ≡ 5.7	1250 ≡ 8.4
Pertuzumab Biosimilar	230 ≡ 1.6	340 ≡ 2.3	530 ≡ 3.6

E. Specificity

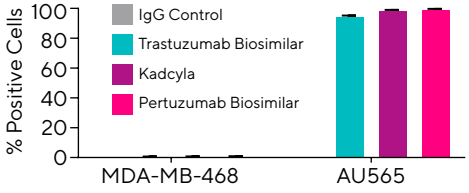


Figure 2

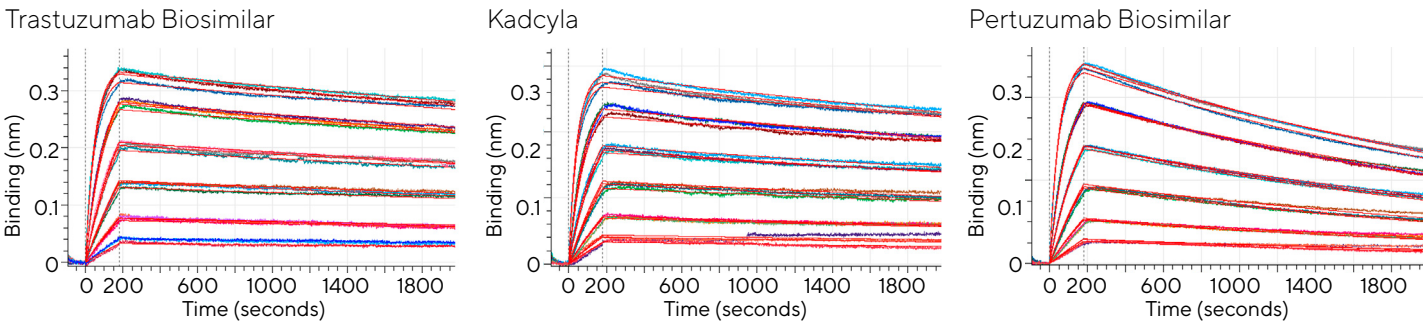
Live-Cell Antibody Binding Assessment Revealed Differences in Binding and Heat Stability of Antibodies

Unlabeled HER2-positive AU565 cells and iQue® Cell Proliferation and Encoding (V/Blue) dye labeled HER2-negative MDA-MB-468 cells were mixed and incubated with varying concentrations of test antibodies (T= Trastuzumab biosimilar, K= Kadcyla and P= Pertuzumab biosimilar) or an IgG control (n=3). Test antibodies were tested in their native state, or after heating at 69°C for 45 or 90 mins. (A) Heat map showing MFI for the secondary antibody on AU565 cells. Darker grey color indicates higher MFI. (B) and (C) concentration response curves plotting the percent positive cells over a defined MFI threshold for the native and 90 min-heated antibodies. (D) EC_{50} values for percent positive cells for binding with the native and heated antibodies. (E) Binding to antigen-negative compared to antigen-positive cells with 6 µg/ml of each antibody to show specificity (± SEM).

Determination of Kinetic Parameters to Characterize Antibody-Protein Interactions

The iQue® assay provides only a snapshot of the binding profile, with an endpoint measurement of the amount of antibody bound to cells, giving little insight into the binding mechanism. Complementing flow cytometry with a technique such as BLI can bridge this limitation. BLI measures the kinetics of the antibody-antigen interaction, with readouts for association and dissociation rates facilitating calculation of K_D (affinity) values.

To assess the binding kinetic parameters of the interaction between the three anti-HER2 antibodies to HER2 protein, we used the Octet® BLI Label-Free Detection System with AHC biosensors (Figure 3). The biosensors come pre-immobilized with an Fc capture antibody, onto which we immobilized a pre-optimized concentration (0.25 µg/ml) of the test mAbs. Following a baseline step, ligand immobilized sensors were dipped into wells containing a range of concentrations of soluble, recombinant HER2 protein to measure association (180 seconds), then moved back to buffer to measure dissociation (1800 seconds) of the anti-HER2 antibody – HER2 protein complex.



Comparison of Binding Affinity and Kinetics

Antibody	K_D (pM)	K_D (%CV)	k_a ($M^{-1}s^{-1}$) ($x10^5$)	k_a (%CV)	k_d (s^{-1}) ($x10^{-5}$)	k_d (%CV)	X^2 (mean)	R^2 (mean)
Trastuzumab Biosimilar	248	6.8	3.9	2.3	9.6	6.8	0.4	0.999
Kadcyla	243	10.6	4.6	7.9	11.3	7.9	0.6	0.998
Pertuzumab Biosimilar	808	9.6	4.0	6.3	32.2	4.0	0.9	0.999

Figure 3

Binding Kinetics Parameters Characterizing the Interaction Between Immobilized Anti-HER2 Antibodies and HER2 Protein Using the Octet® BLI System

AHC biosensors were loaded with 0.25 µg/ml of each of the anti-HER2 antibodies (Trastuzumab biosimilar, Kadcyla and Pertuzumab biosimilar). The kinetics of association and dissociation of a soluble, tag-free, recombinant HER2 protein (Mw 70.1 kDa, 50 nM to 3.1 nM, 1 in 2 dilution) were measured using the Octet® BLI Label-Free Detection System (n=3). Octet® kinetics buffer (1X) was used for sample dilution, baseline and dissociation steps. Mean X^2 and R^2 values were used to evaluate the quality of the fit and were within the defined limits ($X^2 < 3$ and $R^2 > 0.99$).

Traces (sensorgrams) were fitted using a 1:1 global model to calculate parameters as highlighted in Figure 3D. Like the iQue® assay (Figure 2), the Trastuzumab biosimilar displayed relatively high affinity to HER2, with a K_D of 248 pM. The K_D for Kadcyla was very similar to Trastuzumab (243 pM), which was expected as they are both Trastuzumab-based antibodies and have the same variable region for antibody-antigen interaction. This contrasted the relative level of binding as given by the EC_{50} in the iQue® assay, in which the EC_{50} for Kadcyla was 3-fold higher than Trastuzumab. Providing further evidence that the ADC linkers on the Kadcyla Fc region may influence its binding to live cells.

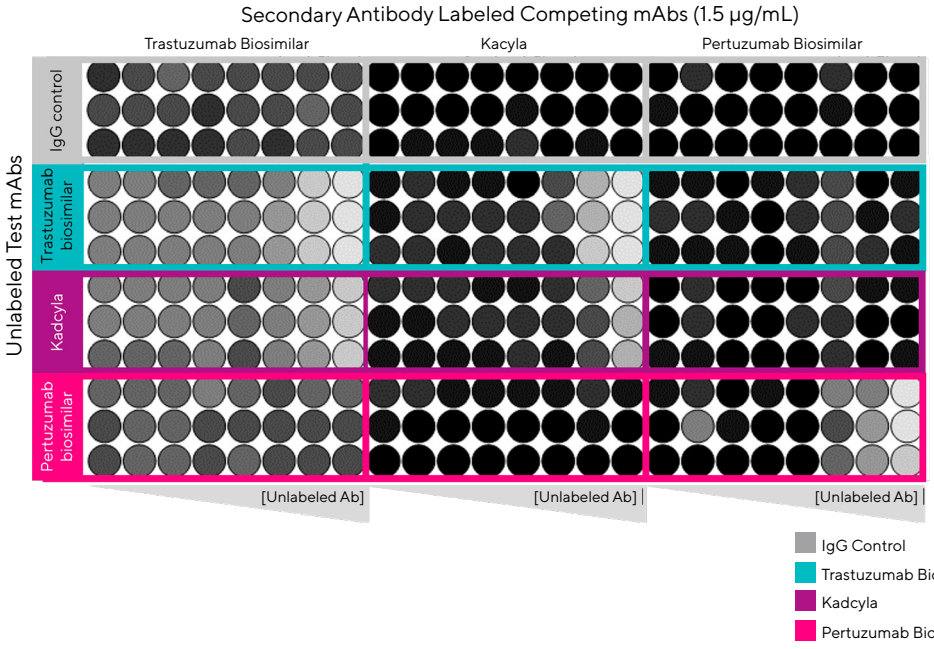
The measured K_D for the Pertuzumab interaction with HER2 was 3-fold higher than Trastuzumab and Kadcyla (808 pM). Both the traces and kinetic data in Figure 3 clearly show that this difference in K_D is due to faster dissociation between HER2 and Pertuzumab, with a dissociation rate of 32.2×10^{-5} 1/s compared to 9.6×10^{-5} 1/s and 11.3×10^{-5} 1/s for Trastuzumab and Kadcyla, respectively. These kinetic differences in the antibody binding profile were not elucidated using the iQue® assay, and further display the benefit of measuring antibody binding on both the advanced flow cytometry and BLI platforms. The iQue® assesses specificity and binding to the native protein as it is found on the surface of live cells, whilst the Octet® necessitates use of a solubilized form of the HER2 protein, meaning a truncated form of transmembrane protein had to be tested. The Octet® reveals kinetic information, such as k_a and k_d measurements, which could critically impact mechanism of action *in vivo*. The two data sets combine to provide a much fuller picture of antibody binding to antigen compared to the stand-alone measurements.

Determining Binding Competition Between Antibodies

During antibody discovery, it is important to determine which epitope a novel drug candidate binds to on the target antigen. In some cases, this can be done by assessing whether the antibody competes for binding with antibodies for which the epitope binding site is already known. This is exemplified by the data in Figure 4, which shows the results from an iQue® competition assay used to characterize the epitope binding of anti-HER2 antibodies. In instances where antibody libraries are being screened without antibodies with known epitope binding already available, an Octet® epitope binning assay could be used instead to elucidate which antibodies have similar binding characteristics and may exert the same desirable biological activity.

For the iQue® assay, a single concentration of each antibody was labeled with fluorophore-conjugated secondary antibody, and the presence or absence of competition with a range of concentrations of unlabeled antibody was assessed, in a pairwise manner. Trastuzumab binds HER2 at the C-terminal portion of domain IV, meaning Kadycla, with the same variable region should also bind domain IV5.

A. Secondary Antibody MFI on AU565 Cells



B. Table 1. Antibodies That Compete for the Same Epitope (Y/N)

Antibody	Trast. Biosim.	Kadcyla	Pert. Biosim.
IgG Control	N	N	N
Trast. Biosim.	Y	Y	N
Kadcyla	Y	Y	N
Pert. Biosim.	N	N	Y

C. Competition Analysis

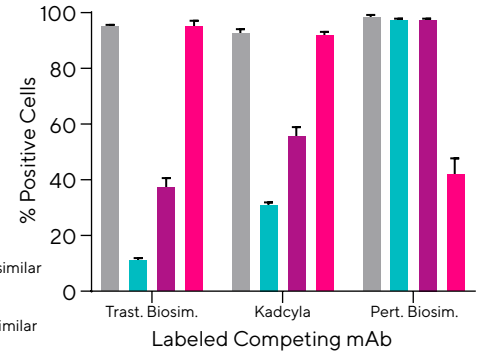


Figure 4

A Cross Competition Assay Confirmed a Lack of Competition Between Trastuzumab and Pertuzumab for Binding to HER2

Three anti-HER2 antibodies (a Trastuzumab biosimilar, Kadcyla and a Pertuzumab biosimilar) were labeled with RPE-conjugated secondary Ab (1:1 labeling ratio) and added to a 384-well plate alongside iQue® Cell Membrane Integrity (R/Red) Dye and a range of concentrations of unlabeled anti-HER2 antibodies. High HER2-expressing AU565 cells were added, and the plate was incubated on ice for 45 minutes before data was collected using the iQue®. (A) Heat map shows median fluorescence intensity (MFI) for the secondary antibody on live cells. Lighter grey indicates lower MFI and shows that competition has occurred between the labeled and unlabeled antibodies. (B) Table summarizing the results in A, highlighting antibodies that competed for binding to the same epitope. (C) Bar chart showing the percent of cells above a defined MFI threshold at the top concentration (20 µg/ml) of unlabeled antibody.

Pertuzumab binds HER2 in a central region of domain II, which is thought to inhibit HER2 dimerization.[5] The data in Figure 4 support the expected epitope binding, with competition observed between Trastuzumab and Kadcyla, as indicated by the drop in MFI and percent positive cells when these two antibodies were combined. There was no competition between the Trastuzumab-based antibodies and Pertuzumab as they bind different epitopes. This data suggests that these antibodies can be given as a combination therapy, which has been shown to increase median overall survival in treatment of HER2-positive breast cancers.[6]

Ranking Antibody Glycosylation as a Predictor of the Quality and Relative Stability of Antibodies

During drug development, it is useful to profile the levels of PTMs on novel drug candidates as they can be linked to a range of functional and physical characteristics of drug activity. Examples of the PTMs that can be of interest in antibody-based therapeutics include glycosylation, methylation, and phosphorylation. Protein glycosylation can affect factors such as shelf-life, stability, and pharmacokinetics. In these experiments, we used the Octet® BLI platform to assess the relative glycosylation status of the three anti-HER2 antibodies.

Antibodies were analyzed for their mannose and sialic acid content using the Octet® GlyM and GlyS kits, respectively. The kits compare relative levels of glycosylation of each antibody sample. The data in Figure 5 shows how the three anti-HER2 antibodies ranked in terms of their mannose and sialic acid content. For both PTMs, Pertuzumab ranked highest and Kadcyla ranked lowest. It is unsurprising that Kadcyla displayed the lowest level of glycosylation as the inclusion of the ADC linker and payload in its Fc region likely leads to the removal of glycosylation sites.

Elevated levels of sialic acid have been linked to improved thermal stability of proteins.[7,8] Sialic acid groups can stabilize the protein structure by aiding folding and reducing aggregation.[9] The results in Figure 2 that compared antibody binding with heated and unheated antibodies suggested that the Trastuzumab biosimilar had higher resistance to heating than Kadcyla. Combining these results with the results in Figure 5 supports published data,

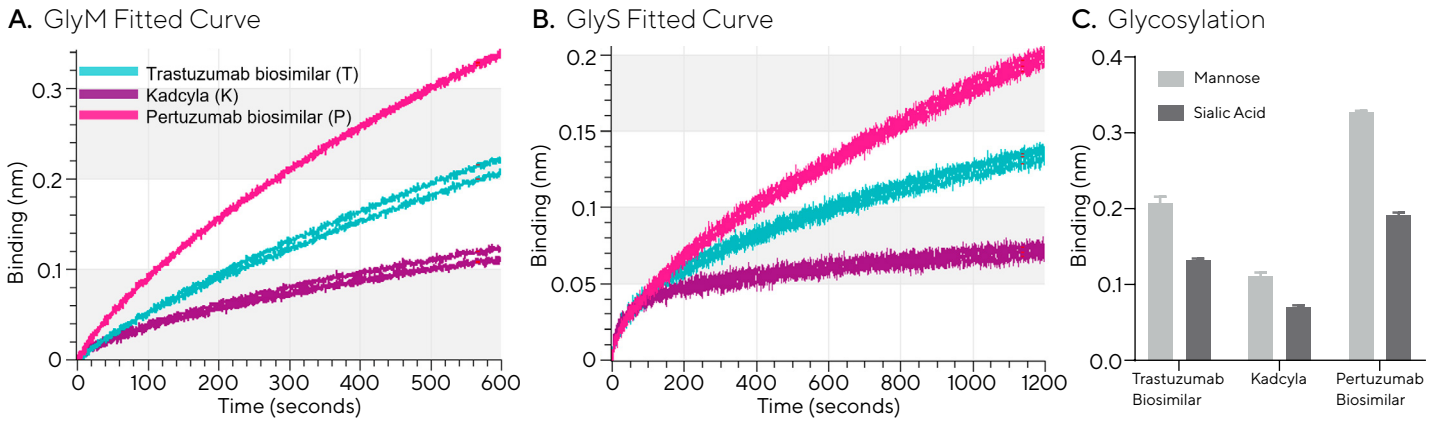


Figure 5

Prediction of Relative Stability Through Analysis of Antibody Glycosylation

Octet® GlyM and GlyS kits were used to rank antibodies based on relative mannose and sialic acid content. Each antibody was diluted to a concentration of 10 µg/mL in Glycan Sample Prep Buffer (GSB). GSB only reference control has been subtracted. Full traces (A) mannose [GlyM] and (B) sialic acid [GlyS] binding (n=3). (C) Mean binding (nm) signal for mannose and sialic acid (± SEM).

suggesting that the removal of glycosylation sites to produce Kadcyla (i.e., Trastuzumab emtansine) from Trastuzumab has led to a reduction in the thermal stability of the antibody.

In contrast to these findings, we saw that the Pertuzumab biosimilar had the highest glycosylation of the three anti-HER2 antibodies (Figure 5), but it was as sensitive as the Kadcyla to denaturation by heating (i.e., they both saw a two-fold increase in EC_{50} for live-cell binding after 90 minutes of heating). This highlights that there must be other factors at play in determining the stability of an antibody and that the two factors do not always correlate, hence the large variability that can exist between molecules.[7] Further work will need to be done, with a larger sample size, to elucidate whether there is a link between glycosylation level and the binding and function of the antibodies.

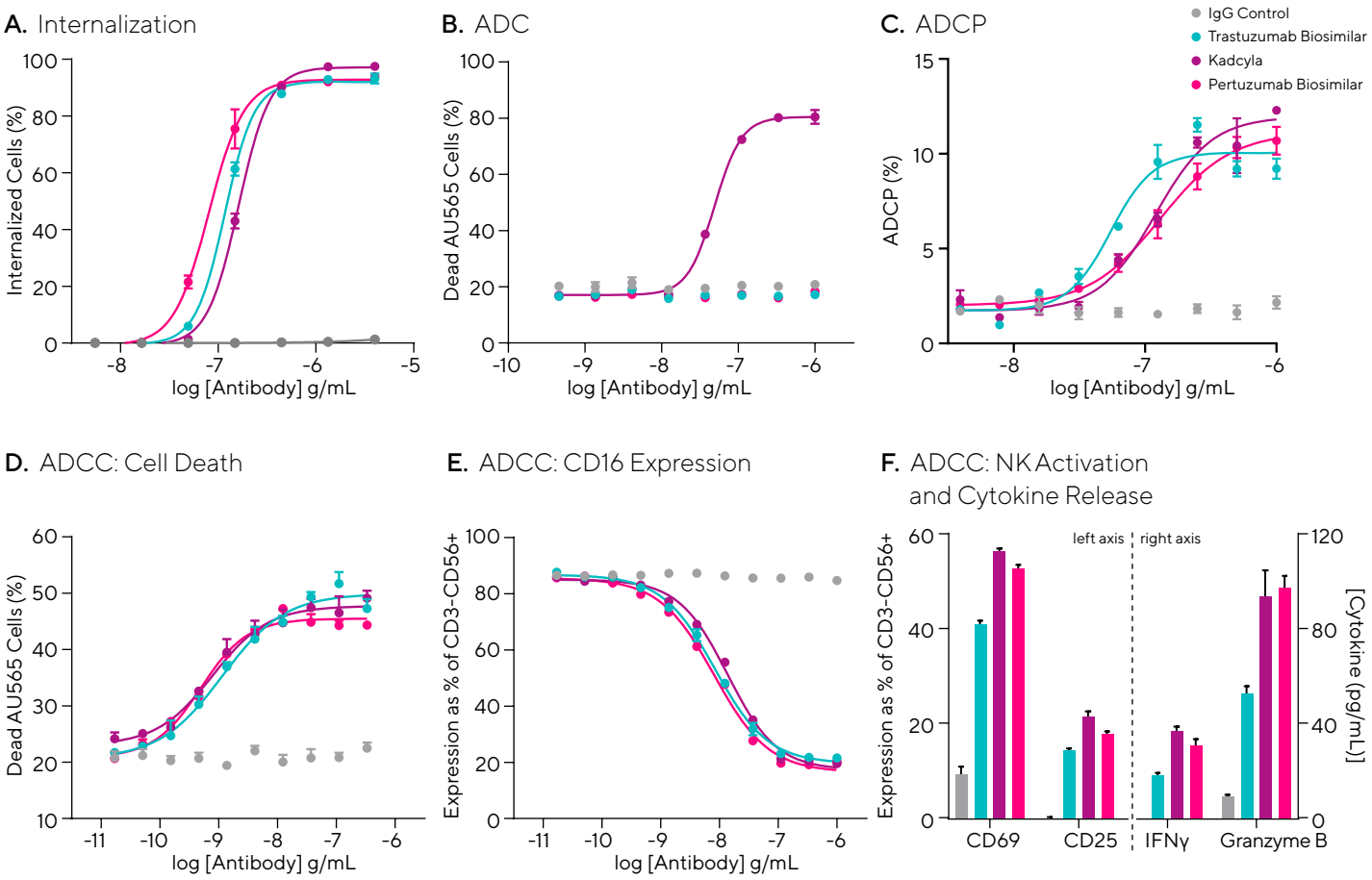
Functional Profiling to Assess Antibody Mechanism of Action (MoA) Against Tumor Cells

There are multiple MoAs through which targeted immunotherapies such as mAbs and ADCs can act to enhance the immune response against cancer. Often, *in vivo* tumor cell killing is due to a combination of MoAs, acting on several immune cell types. Advanced flow cytometry provides an effective technique for exploration of these mechanisms using live cell-based assays. Here we used the iQue® Advanced Flow Cytometry Platform, with several of the associated reagents and kits, to probe the MoA of three anti-HER2 antibodies.

Figure 6A shows the percentage of cells above a defined threshold for internalization of a pH-sensitive probe provided with the iQue® Antibody Internalization Kit. The probe is used to label the antibodies and a fluorescence signal is produced upon internalization into acidic lysosomal or endosomal pathways. The concentration-response curves for internalization after 3 hours were similar for the three antibodies, although the EC_{50} value (Figure 6G) for Pertuzumab was 2-fold lower than Kadcyla, indicating greater internalization (EC_{50} for Kadcyla = 161 ng/mL, Pertuzumab = 80 ng/mL). The antibody internalization capability is essential for delivery of the ADC payload on Kadcyla into target cells. The ADC activity is exemplified in Figure 6B in which, in the absence of immune cells, only Kadcyla induced target cell death after 72 hours.

Two of the Fc effector functions were quantified using co-culture assays. The first was the iQue® ADCP assay, which measured colocalization between CD14-positive monocytes and live, green-labeled target cells. Trastuzumab induced the highest ADCP activity, with an EC_{50} of 55 ng/mL, compared to 121 ng/mL and 130 ng/mL for Kadcyla and Pertuzumab, respectively. The second assay was the iQue® ADCC assay, in which target cells were co-cultured with NK cells, prior to analysis of cytokine concentrations and cell marker expression using the iQue® NK Killing Kit. Induction of ADCC activity by Trastuzumab was lowest with more than two-fold increase in EC_{50} relative to Pertuzumab, although it must be noted that we are measuring relatively small shifts in the nanomolar range, and it is unclear whether these differences are large enough to produce a significant effect *in vivo*. Reduction in CD16 expression is an indicator of ADCC activity, and here was highly comparable between the three antibodies.

In the presence of all three antibodies, levels of activation



G. Summary of EC_{50} Values (ng/mL)

Antibody	Internalization	ADC	ADCP	ADCC
Trastuzumab Biosimilar	117	EC_{50} > max	50	1.1
Kadcyla	161	49	120	0.8
Pertuzumab Biosimilar	80	EC_{50} > max	130	0.5

Figure 6

iQue® Phenotype and Function Kits Probed Differences in Anti-tumor Activity of the Anti-HER2 Antibodies

iQue® immunophenotyping kits were used to measure anti-tumor functions of anti-HER2 antibodies. Assays included HER2-positive AU565 target cells, cultured either alone or with immune cells. (A) Percent of target cells positive for antibody internalization after 3 hours using the iQue® Antibody Internalization Kit. (B) Cell death (%) after 72 hours with test antibodies indicated ADC activity. (C) ADCP (%) is defined as the percent of live, CD14-positive phagocytic effector cells that are positive for the Incucyte® Nuclight Green-labeled target cells as determined using the iQue® Human ADCP Kit. (D) AU565s were co-cultured with NK cells and ADCC activity was measured across a range of anti-HER2 concentrations. (E) CD16 expression indicates ADCC as measured using the iQue® Human NK Cell Killing Kit. (F) Activation marker expression and cytokine release during ADCC. Data shown for top concentration of anti-HER2 (3 µg/mL) (± SEM). (G) Table summarizing EC_{50} values

marker (CD69 and CD25) expression on NK cells, and release of cytokines IFNγ and Granzyme B (indicative of NK cell activation and killing) increased in a concentration-dependent manner. Notably, Trastuzumab induced the lowest levels of expression of all activatory markers and cytokines, which may explain its reduced potency of ADCC induction. A further *in vitro* study on the temporal killing profile of each antibody could be used, for example, to reveal the temporal nature of ADCC and NK activation, or to explore the impact of Kadcyla ADC compared to ADCC activity. Moreover, further investigation may reveal whether the reduced activation by Trastuzumab results in reduced risk of over-activation or inflammatory side effects, as could be explored by quantifying the release of inflammatory cytokines.

Assessment of FcγR:Fc Interactions

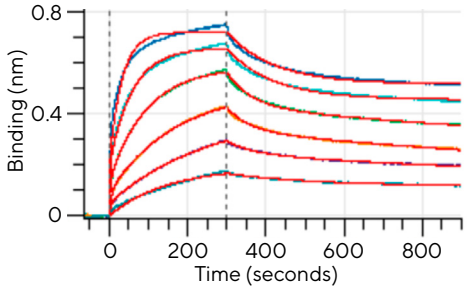
The key to the Fc receptor-mediated functions of antibody therapeutics, such as ADCC and ADCP, is the ability for the Fc region of the antibody to bind to Fc receptors on immune cells. There are multiple classes of Fc receptors, each typically associated with specific Fc

functions. For example, FcγRI (CD64) controls processes such as ADCP, while FcγRIIIa (CD16a) activation is linked to ADCC activity. To explore the interaction between the Fc portion of anti-HER2 antibodies and CD16a, biotinylated CD16a protein was immobilized onto Octet® SAX biosensors and the binding kinetics monitored using the Octet®. These data were fit using a 2:1 (heterogenous ligand) global binding model, meaning two K_D values were generated to describe affinities of antibody Fc binding to each site on the CD16 molecule (Figure 7).

The Trastuzumab biosimilar displayed the highest affinity binding to both CD16a binding sites, with a K_{D1} value of 1.1 pM compared to 6.6 pM for Kadcyla and 3.3 pM for Pertuzumab. This was primarily influenced by the slower rate of dissociation for Trastuzumab, as is evidenced in the k_d values, which were two-fold higher for Pertuzumab and 6-fold higher for Kadcyla. The K_{D2} for Trastuzumab was 5.3 pM, compared to 12.5 pM and 19.6 pM with Kadcyla and Pertuzumab, respectively. Both a faster rate of association and a slower rate of dissociation for Trastuzumab led to the lower relative K_{D2}.

In these assays, increased binding to CD16a as seen with Trastuzumab, was not linked to increased ADCC activity (Figure 6D-F). As seen with other data sets discussed in this application note, the Fcγ receptor binding data has highlighted the benefit of combining both BLI kinetic analysis and advanced flow cytometry data to build a full picture of an antibody's functional profile, especially when measuring small differences between molecules that have similar function *in vitro*. As complexity is added (as in the cell-based assay), several factors and MoAs interact to induce overall cell death. Future work will compare Fcγ receptor binding and ADCC activity between the heated antibodies used in Figure 2, to explore whether, when all other antibody characteristics are kept constant (i.e., an antibody is being compared to a denatured version of itself), a correlation between ADCC and CD16a binding is revealed.

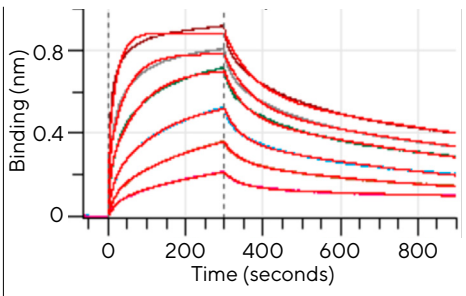
A. Trastuzumab Biosimilar



	K_{D1} (nM)	k_{a1} ($M^{-1}s^{-1}$) ($x10^5$)	k_{d1} (s^{-1}) ($x10^5$)	K_{D1} (%CV)
Value	1.1	0.89	10.0	75
CV (%)	6.9	5.7	1.2	

	K_{D2} (nM)	k_{a2} ($M^{-1}s^{-1}$) ($x10^5$)	k_{d2} (s^{-1}) ($x10^5$)	K_{D2} (%CV)
Value	5.3	18.0	960	25
CV (%)	10.8	2.0	12.8	

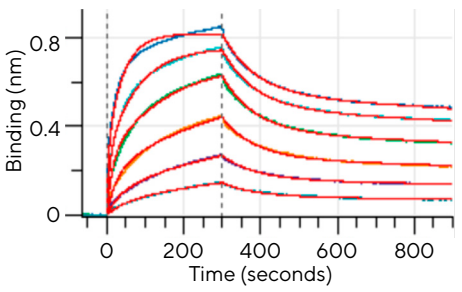
B. Kadcyla



	K_{D1} (nM)	k_{a1} ($M^{-1}s^{-1}$) ($x10^5$)	k_{d1} (s^{-1}) ($x10^5$)	K_{D1} (%CV)
Value	6.6	0.95	62.8	72
CV (%)	11.3	1.8	9.5	

	K_{D2} (nM)	k_{a2} ($M^{-1}s^{-1}$) ($x10^5$)	k_{d2} (s^{-1}) ($x10^5$)	K_{D2} (%CV)
Value	12.5	10.8	1340	28
CV (%)	7.1	9.7	2.6	

C. Pertuzumab Biosimilar



	K_{D1} (nM)	k_{a1} ($M^{-1}s^{-1}$) ($x10^5$)	k_{d1} (s^{-1}) ($x10^5$)	K_{D1} (%CV)
Value	3.3	0.63	20.9	62
CV (%)	8.2	2.9	5.4	

	K_{D2} (nM)	k_{a2} ($M^{-1}s^{-1}$) ($x10^5$)	k_{d2} (s^{-1}) ($x10^5$)	K_{D2} (%CV)
Value	19.6	5.90	1157	38
CV (%)	2.6	0.6	1.9	

Figure 7

Analysis of FcγRIIIa (CD16a) to Fc Region Binding Kinetics Using Octet® Revealed Differences Between Anti-HER2 Antibodies

Biotinylated CD16a (0.325 µg/mL) was loaded on to Octet® SAX Biosensors. Binding to a range of concentrations of anti-HER2 antibodies (top concentration 0.4 µM, 1 in 2 dilution) was measured using the Octet®. Association was measured for 300 seconds and dissociation for 600 seconds. Octet® Kinetics Buffer (diluted from 10X to 1X in PBS) was used for all sample dilutions and buffer steps. Resulting traces were fit using a 2:1 (heterogenous ligand) global binding model, resulting in generation of two K_D values per antibody. Traces and binding kinetic parameters shown for: (A) Trastuzumab biosimilar, (B) Kadcyla and (C) Pertuzumab biosimilar (n=2).

Conclusions

We used the iQue® Advanced Flow Cytometry Platform and Octet® BLI Label-Free Analysis System in parallel to perform broad antibody characterization. Live-cell binding assays on the iQue® analyzed antibody binding to native protein and provided functional assessment of immune cell-killing activity. Measurements using the Octet® platform allowed comparison of the kinetics of both antigen and Fc receptor binding, alongside PTM analysis. The experiments in this application note have displayed the advantages of using these instruments in a combined workflow, including:

- Comprehensive assessment of antibody binding parameters, including specificity, live-cell binding, and kinetics. Using the two systems in parallel reveals subtle differences in the binding characteristics of each antibody, which when applied *in vivo* could have major effects on efficacy.
- Simple, pre-optimized workflows allow for data generation across multiple platforms with ease, facilitating broader profiling of many different antibody characteristics.
- High-throughput instrumentation enables large numbers of antibodies to be profiled in minimal time and allows for enhanced replication leading to improved robustness of data.
- Low volume requirements for the iQue® allows for conservation of precious antibody samples.
- Both systems come with inbuilt data analysis software, streamlining data processing, and speeding up the time to actionable results.

Together, these advantages create a powerful workflow for antibody characterization that provides unique insights into binding mechanisms and comparative analysis of parameters that may impact *in vivo* efficacy. Both instruments have vast potential to accelerate and simplify antibody discovery processes.

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