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Application Note

Keywords or phrases:
Antibody discovery, live-cell antibody binding, monoclonal antibodies, antibody:antigen interactions, iQue®, advanced flow cytometry, iQue Forecyt® software

Utilizing Advanced High-Throughput Flow Cytometry to Quantify Direct and Competitive Live Cell Antibody Binding

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Introduction

Monoclonal antibody (mAb) therapeutics comprise one of the most rapidly growing drug classes, with the milestone for the 100th FDA approval of a mAb product reached in 2021[1,2]. Amongst these approved products are treatments against a huge range of diseases, namely, a large number of cancer therapeutics, comprising about 45% of approved mAbs, and interventions against immune disorders, comprising about 27% of products[3]. Within these categories are mAbs raised against a wide variety of target antigens, for example anti-HER2 (human epidermal growth factor receptor 2) mAbs to target breast cancers and anti-TNF (tumor necrosis factor) antibodies to treat symptoms of inflammatory disease[3].

The success of mAbs as a class of therapeutics is largely due to their high specificity and affinity for the target antigen. Evaluation of mAb target binding is a critical part of the antibody development process and robust, high-throughput techniques are needed to facilitate rapid screening of antibody libraries. Utilizing such techniques as early as possible in the drug discovery pathway can enhance the quality of 'hits' generated for mAbs with desirable characteristics, providing increased likelihood of candidates being progressed through the clinical pipeline.

Specific binding of antibody to the target antigen is one of many key attributes that must be evaluated during development, alongside characteristics such as Fc function, stability and post-translational modifications. Conventional techniques for evaluating antibody binding can often:

- Have low-throughput and long sample acquisition times
- Measure binding to purified, truncated or tagged recombinant forms of the target protein
- Be laborious and time-consuming, requiring steps such as protocol optimization, fixation and repetitive washes
- Necessitate the use of large volumes of precious sample and antibody.

Here we present two simple assays that utilize the iQue® Advanced Flow Cytometry Platform and validated reagents to measure binding of unlabeled therapeutic mAbs to your target on live cells. The first is a direct antibody binding assay which ranks mAbs based on binding to target cells, with the ability to analyze binding to multiple cell types in a single well. The second is a competitive binding assay which can reveal mAbs that target different epitopes.

Method and Workflow

Direct binding assay

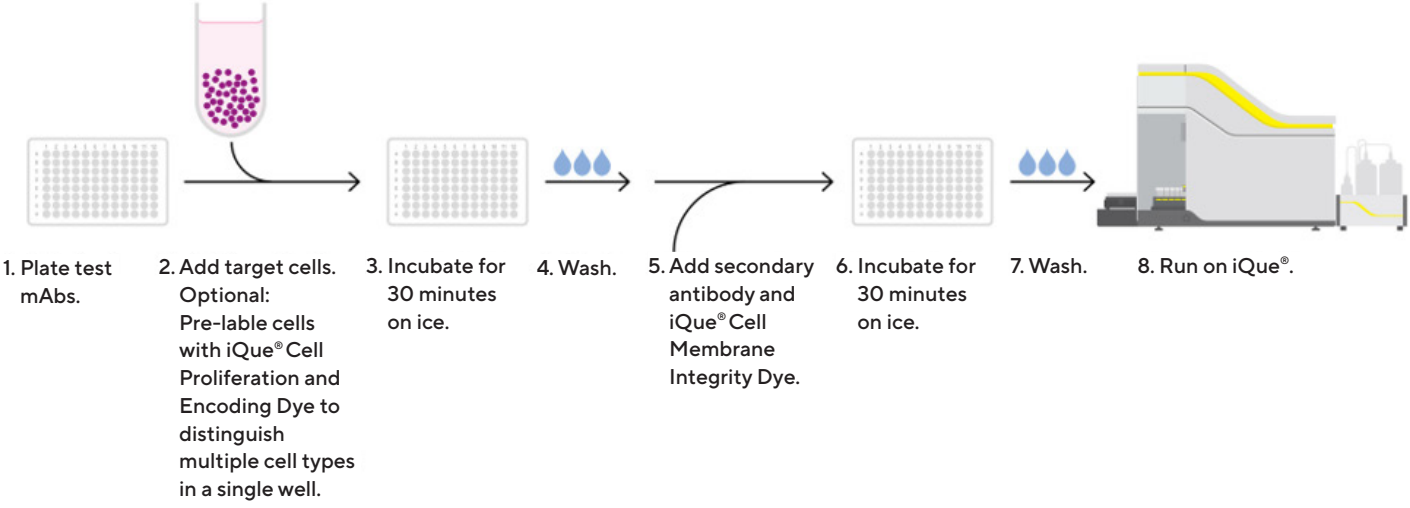

Figure 1: *Direct Antibody Binding iQue® Assay*

Note: Simple workflow to assess binding of test mAbs to live cells using the iQue® Advanced Flow Cytometry Platform. Target cells are optionally labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye and incubated with test mAbs for 30 minutes on ice. Plates are then washed and labeled with iQue® Cell Membrane Integrity (R/Red) Dye. After 30 minutes incubation, plates are washed and run on the iQue®.

1. Dilute test mAbs to desired concentration in cell culture media at 3× final assay concentration (FAC). Add 10 µL/well to a 96- or 384-well V-bottom plate (e.g., Corning 3363 or 3656).
2. Optional: If adding multiple cell types per well, distinguish cells by labeling with iQue® Cell Proliferation and Encoding (V/Blue) Dye:
   I. Prepare a working stock of encoder dye by diluting in PBS (dilution factor e.g., 1 in 1000 for bright staining).
      - Note: If using more than two cell types per well, the bright dye can be diluted (e.g., 1 in 10) to create dimmer populations.
   II. Collect cells in a conical tube, wash in PBS and resuspend at 2×10^6 cells/mL.
   III. Add an equal volume of the prepared dye solution (1:1) and incubate (37 °C, 15 minutes).
   IV. Wash cells with at least 2× volume of cell culture media (including 10% serum), spin (500 g, 5 minutes).
   V. Repeat wash step twice more.

3. Prepare target cells to an appropriate density in cell culture media and add 20 µL/well to the plate. Incubate for 30 minutes on ice.
   - Note: Recommend a starting cell density of approximately 5–10 K cells/well for each cell type.
4. Add 100 µL of wash buffer (e.g., PBS + 2% FBS) and centrifuge (300 g, 5 minutes).
5. Remove media and shake (2000 RPM, 1 minute) using the iQue® plate shaker to resuspend cells.
6. Combine fluorophore conjugated secondary antibody (e.g., R-Phycoerythrin-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, Jackson ImmunoResearch) with iQue® Cell Membrane Integrity Dye (choice of V/Blue (if not using V/Blue encoder dye), B/Green, B/Red, R/Red) at the concentration recommended in the dye protocol. Add 10 µL/well of the mixture to the plate and incubate for 30 minutes on ice.
7. Wash, spin and resuspend as in steps 3–5. Add 20 µL/well wash buffer.
8. Run on the iQue® using a 4 and 5 second sip time and analyze median fluorescence intensity (MFI) using iQue® Forecyt® software.

Competitive binding assay

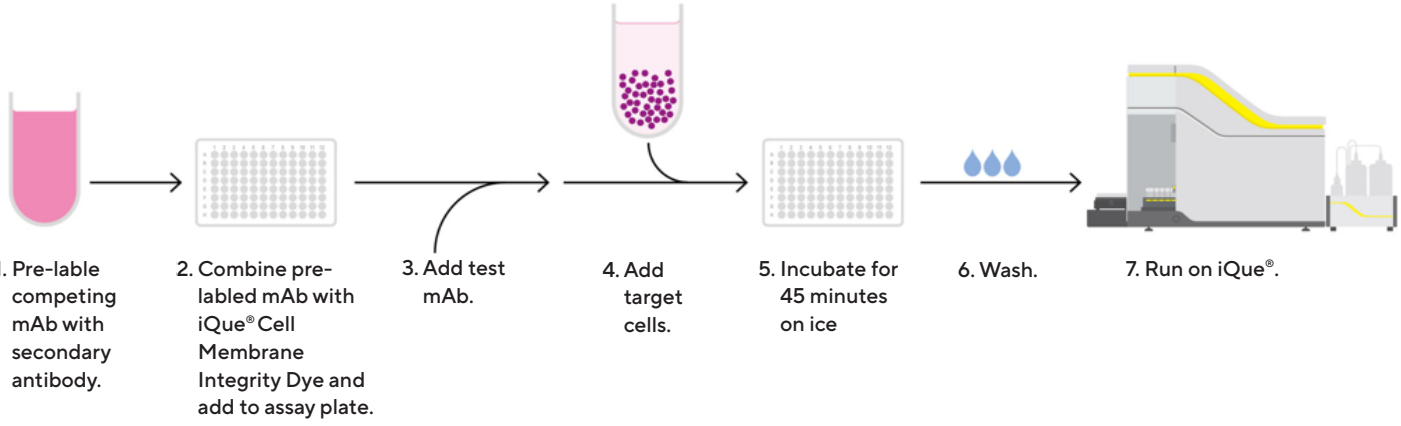

Figure 2: *iQue® Competition Binding Assay Workflow*

Note: A competing antibody is pre-labeled in a tube with a fluorophore-conjugated secondary antibody, prior to combination with the unlabeled test antibody of interest in the assay plate. The target cells are then added to this antibody mixture and the labeled competing mAb and the test mAb will compete for binding to the target antigen, provided they both target the same epitope. Therefore, the greater the concentration of the test antibody, the less of the labeled competing antibody will bind, resulting in a lower MFI value for the secondary antibody fluorophore. If the two antibodies don't compete for the same epitope, the MFI should be unaffected by test mAb concentration.

1. Pre-label test mAb (3×FAC) in a tube with fluorophore conjugated secondary antibody at a 1:1 concentration ratio for 15 minutes at room temperature.
2. Combine with iQue® Cell Membrane Integrity Dye and add 10 µL/well of the mixture to a 96- or 384-well plate.
3. Dilute unlabeled test competing mAbs to desired concentration and add to the assay plate (10 µL/well).
4. Prepare target cells to an appropriate density in cell culture media and add 10 µL/well to the plate. Incubate for 45 minutes on ice.
   - Note: Recommend a starting cell density of approximately 10–20 K/well for each cell type.
5. Add 100 µL of wash buffer (e.g., PBS +2% FBS) and centrifuge (300 g, 5 minutes).
6. Remove media and shake (2000 RPM, 1 minute) using the iQue® plate shaker to resuspend cells.
7. Add 20 µL/well wash buffer.
8. Run on the iQue® using a 3–5 second sip time and analyze MFI using iQue Forecyt® software.

Results

Assessment of mAb binding and specificity in both adherent and suspension cell models

Historically, many flow cytometry assays focused more on analyzing cells grown in suspension culture and less on adherent cell types, largely due to issues with lifting and handling the cells. This presents a huge limitation in the field of oncology, since around 90% of adult cancers are solid tumors formed from adherent cell types[4,5]. To expand the use of the iQue® antibody binding assay, we validated it for use with both suspension and adherent cell types.

For the suspension cell model, we compared binding of anti-CD20 antibody Rituximab to CD20-antigen positive Ramos cells, from a B lymphocyte cell line, and CD20-negative Jurkat cells, from a T lymphocyte line. For the adherent cell model, binding of anti-HER2 antibody Trastuzumab was quantified on HER2-positive AU565 cells and HER2-negative MDA-MB-468 cells, both from breast cancer cell lines. In both models, the antigen negative cell type was labeled using the iQue® Cell Proliferation and Encoder (V/Blue) Dye to distinguish them from the antigen positive cells, as displayed in the dot plots in Figure 3.

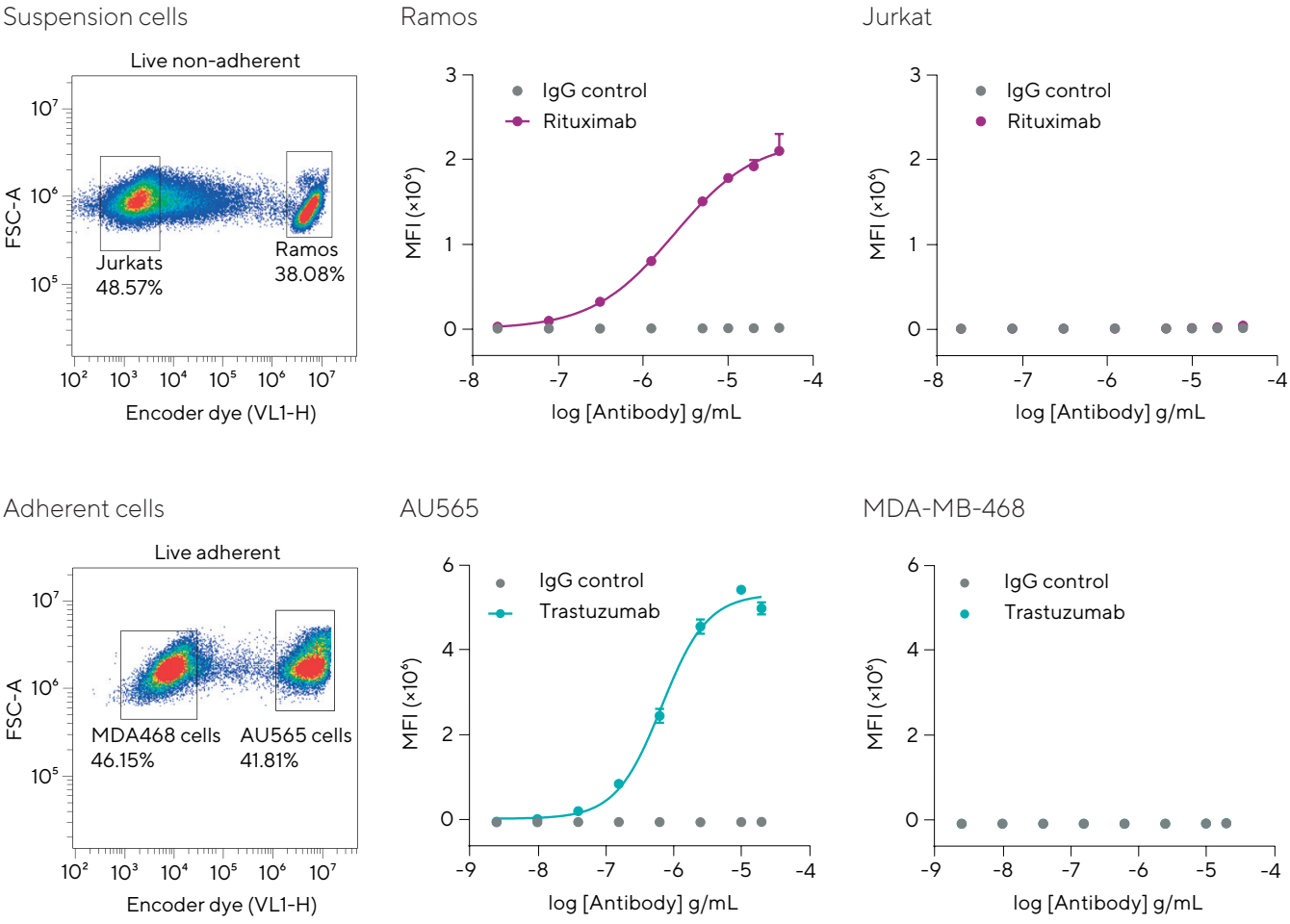

Figure 3: *Assess Specificity of Antibody Binding to Target Antigens on Both Suspension and Adherent Cells*

Note: Binding and specificity of two therapeutic antibodies, Rituximab and a Trastuzumab biosimilar, was assessed using a suspension and an adherent cancer cell line model. CD20-positive Ramos cells and HER2-positive AU565 cells were labeled with iQue® Cell Proliferation and Encoder (V/Blue) Dye to distinguish them from antigen negative Jurkat and MDA-MB-468 cells. Cells were incubated with a range of concentrations of unlabeled test mAb or an IgG control followed by a single concentration of RPE-conjugated secondary antibody (5 µg/mL).

In both the suspension and adherent models, there was an antigen positive cell type-specific increase in binding with increasing antibody concentration. There was no binding observed with the antigen negative cell types, or with the IgG control antibody.

It is crucial to assess whether there is any off-target binding of a novel antibody candidate in the early stages of drug discovery using *in vitro* assays, as this can present issues such as reduced efficacy and increased toxicity *in vivo*[6].

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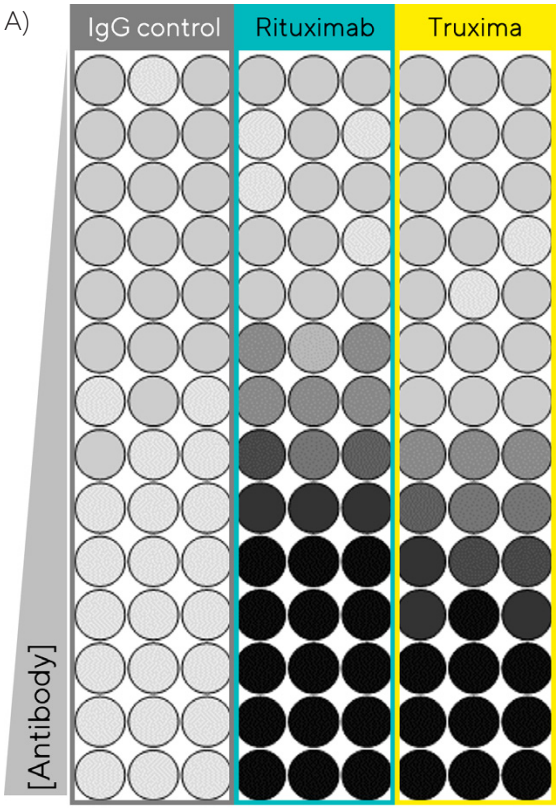
Comparing biosimilar binding to live target cells

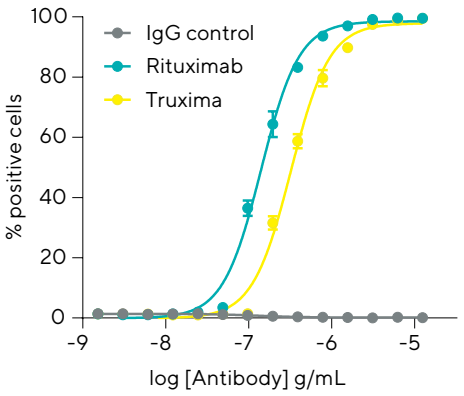
mAb biosimilars have been a relatively recent emergence on the bio-therapeutic market and have the potential to encourage lower cost treatments and to increase access for patients. For a novel mAb biosimilar to be approved for use, characteristics such as antigen binding, Fc receptor interactions and Fc function must be rigorously tested to show that they are comparable to the reference product. Here we used the iQue® direct binding assay to compare the binding of anti-CD20-IgG1 antibody Rituximab to one of its biosimilars, Truxima (Figure 4). Although there was a concentration dependent increase in binding of both antibodies to the Raji target cells, there was a clear shift to indicate decreased potency binding of the Truxima compared to Rituximab, with EC_{50} values of 0.14 ± 0.01 µg/mL and 0.32 ± 0.03 µg/mL for Rituximab and Truxima, respectively.

This difference between Rituximab biosimilars has been seen before, for example, our previous work showed there was a difference in the level of ADCP induced by Rituximab and Truxima. Other studies have shown differences in ADCC and CDC activity between Rituximab and its biosimilars[7,8]. There are several possible reasons for these differences in Fc function between biosimilars, for example, their levels of post-translational modifications could differ, which depends on factors such as the expression system, or culture conditions used[8,9].

A)



B)



C)

Compound	Top	EC_{50} ± 95% CI (µg/mL)	Slope	R^2
Rituximab	98.6	0.14 ± 0.01	1.8	0.995
Truxima	98.0	0.32 ± 0.03	1.9	0.994

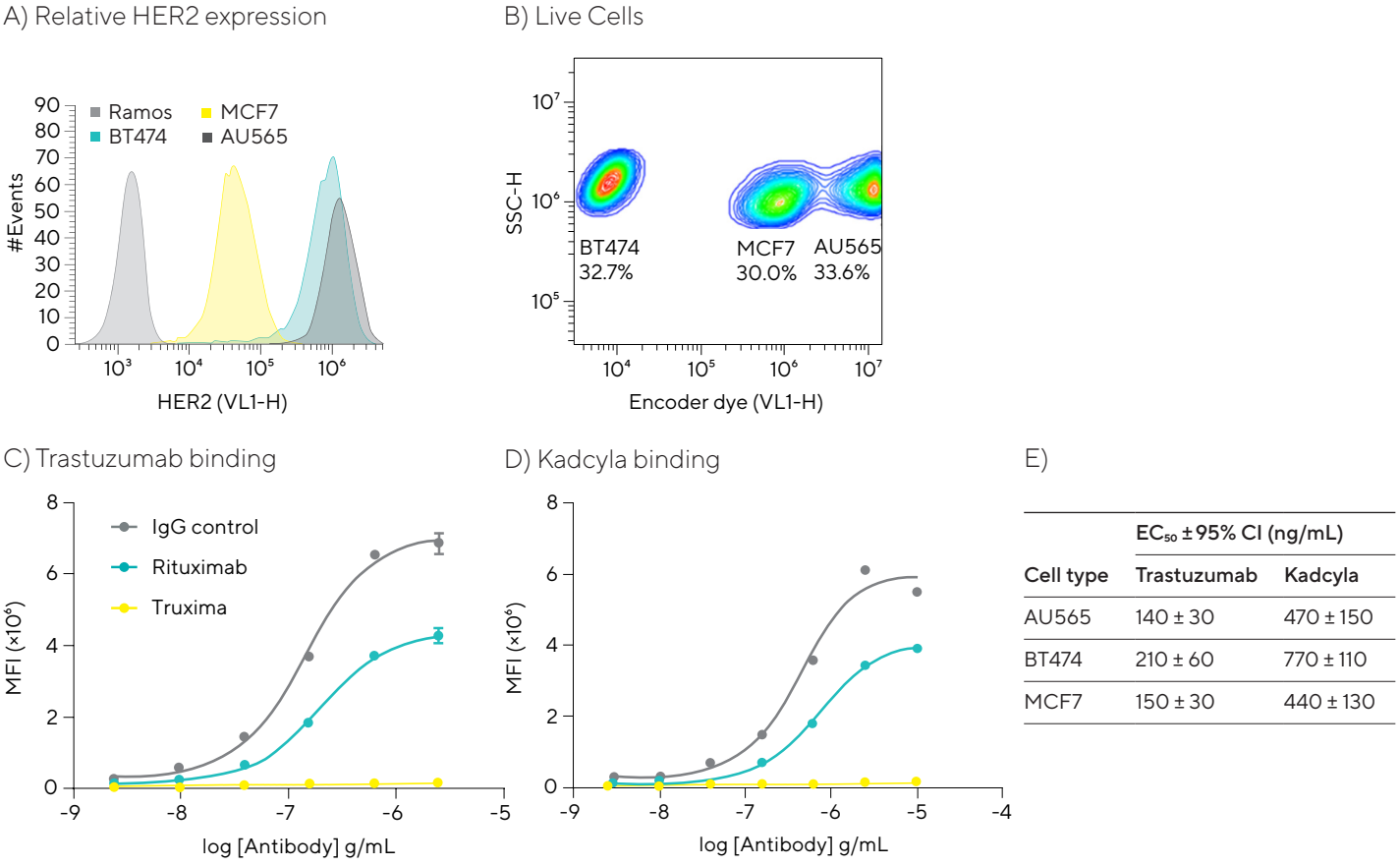
Figure 4: *Comparison of Live Cell Binding by Anti-CD20 Biosimilars*

Note: A direct mAb binding assay was used to measure the binding of anti-CD20 mAb Rituximab and one of its biosimilars, Truxima, to high CD20 expressing Raji cells. (A) Heat map of the percent of live cells that are positive for binding the antibody over a gated threshold. (B) Concentration-response curves show the % of cells positive for mAb binding over the gated threshold. (C) Table of values to describe curves in (B).

Measuring antibody binding to a range of target cells in a single well

A key factor influencing the efficacy of targeted immunotherapies, such as mAbs, for treatment of cancer, is the prevalence of the target antigen on the specific patient's cancer. To this end, expression of biomarkers such as HER2 on breast cancer cells and EGFR (epidermal growth factor receptor) on lung cancers may be tested to indicate a patient's suitability for a particular treatment[10].

It can therefore be useful to measure the binding and function of a novel therapeutic against a range of cell types, with different levels of expression of the target antigen, to give an indication of how specific patients may be affected by the treatment. To explore this using the iQue® direct antibody binding assay, we compared binding of two anti-HER2-IgG1 antibodies, a Trastuzumab biosimilar and Kadcyla (an antibody drug conjugate (ADC) based on Trastuzumab) to three adherent breast cancer cell types: AU565s, BT474s and MCF7s.



E)

	EC_{50} ± 95% CI (ng/mL)	
Cell type	Trastuzumab	Kadcyla
AU565	140 ± 30	470 ± 150
BT474	210 ± 60	770 ± 110
MCF7	150 ± 30	440 ± 130

Figure 5: *Analyze Antibody Binding to a Range of Antigen Expressing Cell Types in a Single Well*

Note: A) HER2 expression on three adherent cell lines (AU565, MCF7 and BT474) was analyzed using a conjugated anti-HER2 antibody and compared to expression on HER2-negative Ramos cells. (B) To distinguish cells in the binding assay, AU565 cells were labeled with a high concentration of iQue® Cell Proliferation and Encoder (V/Blue) Dye, MCF7 cells were labeled with a lower concentration and BT474 cells were left unlabeled. Curves plot MFI for binding of (C) a Trastuzumab biosimilar and (D) Kadcyla to the three cell lines. (D) EC_{50} values for Trastuzumab and Kadcyla binding (±95% confidence interval).

To begin with, a fluorophore conjugated HER2 antibody was used to profile the relative HER2 expression of each adherent cell type compared to Ramos cells, which were included as a HER2-negative control line (Figure 5A). The MFIs revealed highest HER2 expression on AU565 cells (MFI ($\times 10^5$) of 12.9 ± 0.08), then next highest expression on BT474 cells (MFI ($\times 10^5$) = 8.2 ± 0.2) followed by lowest expression on MCF7 cells (MFI ($\times 10^5$) = 0.4 ± 0.009). Background expression on the negative control line was negligible (MFI ($\times 10^5$) of 0.015 ± 0.003). For measurement of Trastuzumab and Kadcyla binding, like in Figure 3, the iQue® Cell Proliferation and Encoder (V/Blue) Dye was used to label cells, so that the three adherent cell types could be distinguished in a single well (Figure 5B). This facilitated simultaneous quantification of binding to each cell type.

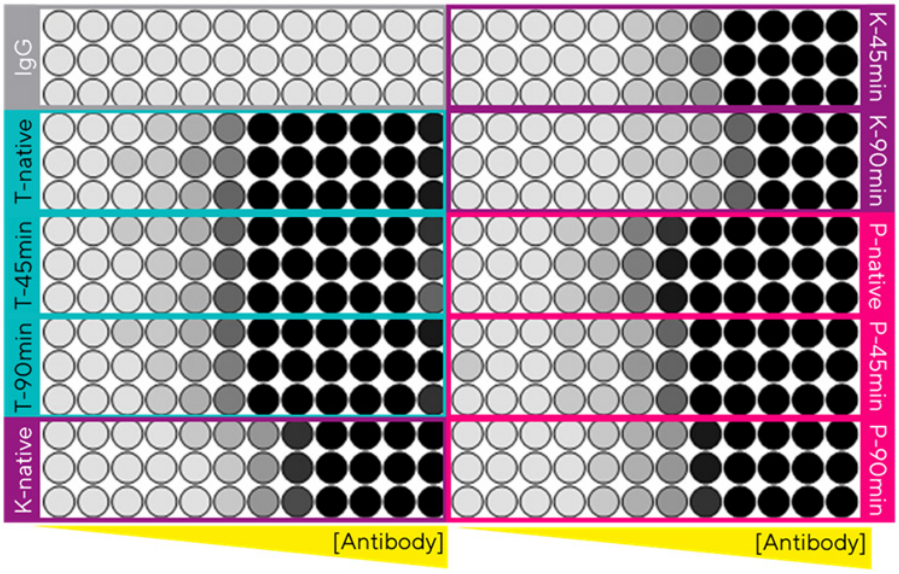
Figures 5C to E show that binding of both Trastuzumab and Kadcyla correlated with expression of HER2 on cells, with greatest binding to AU565 cells, mid-level binding to BT474s, then much lower-level binding to MCF7s.

The EC_{50} values for binding of each antibody to cells was similar, showing greater potency of binding by Trastuzumab, with EC_{50} values ranging from 140 to 210 ng/mL, compared to Kadcyla with EC_{50} values in the 440 to 770 ng/mL range.

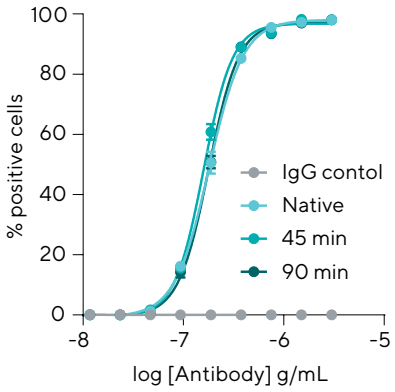
High-throughput screening of binding and thermal stability in 384-well format

High-throughput techniques are beneficial throughout the drug discovery process. They can be applied both in the early stages, where large numbers of antibodies, for example in a phage display or hybridoma library, can be screened in minimal time, and in later stages, once promising hits have been identified, to generate a full profile of their relative EC_{50} values using multiple replicates. In Figure 6, we have demonstrated the latter use of this high-throughput capability to profile binding of 10 antibodies, across 12 concentrations, with 3 replicates of each, within a single 384-well plate.

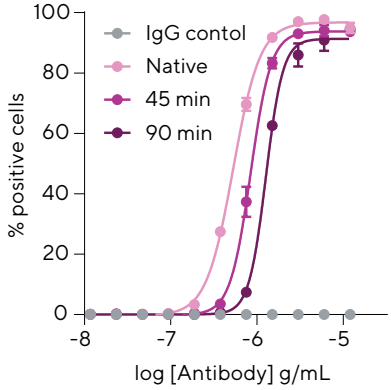
A) Secondary antibody MFI on AU565s



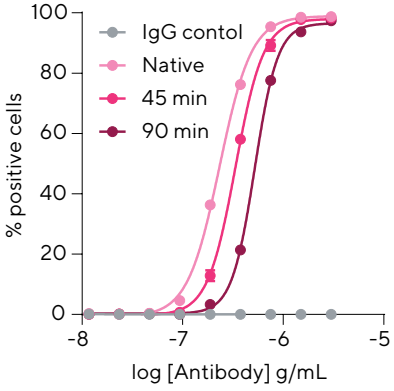
B) Trastuzumab



C) Kadcyla



D) Pertuzumab



E)

Antibody	Native	45 min heat	90 min heat
Trastuzumab biosimilar	180 ± 8	160 ± 6	180 ± 7
Kadcyla	530 ± 16	840 ± 36	1270 ± 53
Pertuzumab biosimilar	230 ± 8	340 ± 10	520 ± 12

Figure 6: *Screening Binding and Thermal Stability of Anti-HER2 Antibodies in a 384 Well Format*

Note: Unlabeled HER2-positive AU565 cells were incubated with varying concentrations of test antibodies (T= Trastuzumab biosimilar, K= Kadcyla and P= Pertuzumab biosimilar) or an IgG control (n=3). Test mAbs 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. Concentration response curves show the % positive cells over a defined MFI threshold for the (B) native (C) 45 minute heated and (D) 90 minute heated antibodies. (E) EC_{50} values for % positive cells for binding with the native and heated antibodies.

The antibodies tested included the Trastuzumab biosimilar and Kadcyla, alongside another anti-HER2-IgG1 antibody, a Pertuzumab biosimilar. The antibodies were tested in their native state, and after 45 or 90 minutes of heating at 69 °C, with the aim of measuring their thermal stability. Binding was assessed on HER2-positive AU565 cells and compared to binding of an anti-βgal-IgG1 control.

The data in Figure 6 shows that the Trastuzumab biosimilar had both the highest level of binding and the greatest resistance to heat denaturation, with the EC_{50} remaining constant across the native, 45- and 90-minute heated antibodies, ranging between 160 and 180 ng/mL. Kadcyla displayed the lowest potency of binding to cells and was sensitive to heat denaturation, with a two-fold increase in EC_{50} from the native state (530 ng/mL) after 90 minutes of heating (1270 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 had similar binding to Trastuzumab in its native state, with an EC_{50} of 230 ng/mL but it was sensitive to heating and saw a two-fold loss in binding activity after 90 minutes of heating (EC_{50} 520 ng/mL).

Competition binding assay reveals antibodies that bind to the same epitope

During antibody discovery, it is important to determine which epitope a novel drug candidate binds to on the target antigen. If it is a well characterized target for which antibodies are available with known epitope binding, this can be achieved by evaluating whether the novel antibody competes with those known binders.

This is exemplified by the data in Figure 7 which shows the results from an iQue® competition assay used to assess competition between the anti-HER2 antibodies that were profiled in Figure 6. In these experiments, a single concentration of each antibody was labeled with fluorophore conjugated secondary antibody, and the presence or absence of competition with 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 IV[11]. Pertuzumab binds HER2 in a central region of domain II, which is thought to inhibit HER2 dimerization[11]. The data in Figure 7 support the expected epitope binding, with competition observed between Trastuzumab and Kadcyla, as indicated by the drop in MFI (left shift of the histogram relative to the IgG control) when these two antibodies were combined. The Trastuzumab biosimilar induced the greatest reduction in MFI when competing both with itself, and with the Kadcyla, which fits with the data seen in Figure 6 to suggest it is the strongest binder of the two antibodies.

There was no competition between the Trastuzumab-based antibodies and Pertuzumab as they bind different epitopes. This illustrates why 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[12].

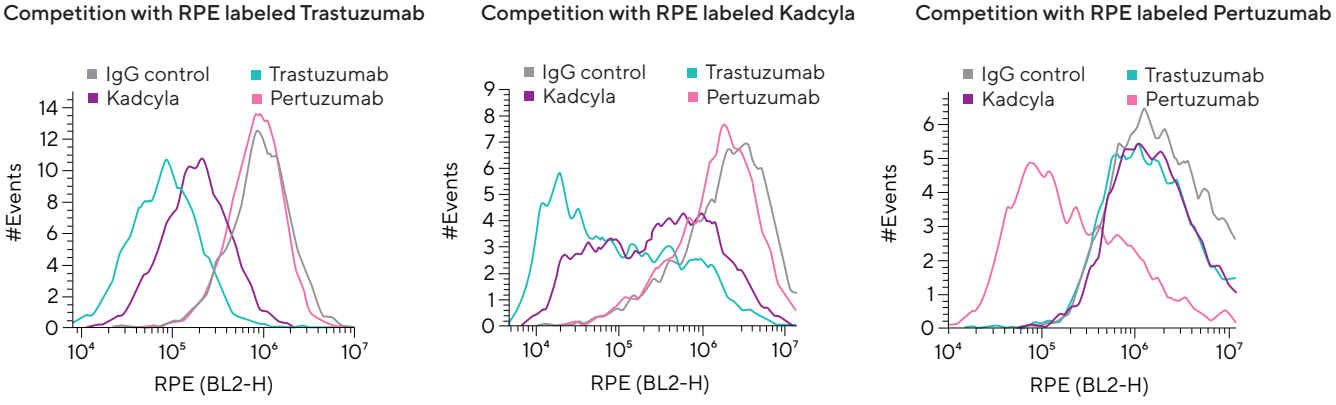


Figure 7: *Competition Binding Assay Reveals Antibodies which Bind the Same Epitope on the Target Antigen*

Note: A single concentration (1.5 µg/mL) of three mAbs (a Trastuzumab biosimilar, Kadcyla [an ADC based on Trastuzumab] and a Pertuzumab biosimilar) were labeled with RPE-secondary antibody. Labeled mAbs were paired with unlabeled mAbs (a βGal-IgG1 control, Trastuzumab, Kadcyla and Pertuzumab, 20 µg/mL) and added to HER2-positive AU565 cells. A left shift in the histogram relative to the IgG control shows a decrease in intensity of RPE, indicating competition for binding to HER2.

Conclusion

The iQue® Advanced Flow Cytometer with optimized assays enables high-throughput measurement of antibody binding activity. The direct binding workflow provides robust quantification of binding to native protein on live cells, with simple generation of pharmacological readouts, such as EC_{50} values using the iQue Forecyt® software. The competition binding assay provides a streamlined workflow to assess antibodies that bind the same epitope on the target antigen. The experiments shown in this application note have exemplified the advantages of these workflows, including:

- Easy-to-follow protocols for measuring antibody binding to target antigens on both suspension and adherent cells facilitates assessment of both solid and blood tumor targets.
- 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. A full 96-well plate can be read in less than 15 minutes and a 384-well plate in 40 minutes.
- Low volume requirements for the iQue® allows for conservation of precious antibody samples.
- Inbuilt data analysis software streamlines data processing and speeds up the time to actionable results.
- The broad range of detection channels gives the flexibility to mix and match dyes, such as encoder or membrane integrity dyes, in a panel with your chosen secondary antibody fluorophore.

Together, these advantages create a powerful tool for measurement of antibody binding, with the potential to enhance the speed and quality of hits generated during antibody discovery.

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Status 01 | 2023