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Evaluation of Complement-Dependent Cytotoxicity (CDC) Using a Streamlined, Miniaturized Advanced Flow Cytometry Assay

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Introduction

Research and development of monoclonal antibody (mAb) therapeutics is a vast and expanding area of drug discovery, largely due to mAb potential for high specificity and affinity towards target molecules. The past decade has seen a stark increase in the number of approvals for mAb therapeutics, with 11 molecules granted first approval in the US or EU in 2021.¹ Approved mAb-based therapeutics span a wide range of disease areas, although most are indicated for treatment of cancers (45%), such as anti-CD20 treatment of B-cell cancers, or for immune-mediated disorders (27%), such as anti-TNF treatment of inflammatory diseases.^{1,2}

There are several mechanisms of action (MoAs) through which mAbs are cytotoxic towards target cells, such as cancer cells. These MoAs often harness the power of the body's own immune system to exert anti-tumor effects. Examples of this include the three key Fc-mediated functions: antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC). ADCC and ADCP rely on a mAb simultaneously engaging with Fc receptors on immune cells and the antigen on target cells. This brings the target cells into proximity with cytotoxic cells (e.g., natural killer cells) or phagocytic cells (e.g., monocytes or macrophages), leading to

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enhanced immune clearance. Contrastingly, in CDC, the antigen-bound mAb recruits proteins present in the blood to induce lysis. This process begins with binding of the C1q protein to the mAb Fc region, which triggers activation of the complement pathway. A complex molecular cascade follows, which eventuates in the formation of the membrane attack complex (MAC).³ The MAC creates a pore in the target cell membrane, resulting in cell death.

During early stage mAb development, *in vitro* assays are often used to profile activity towards several MoAs, including CDC, to ensure mAbs with desirable characteristics are selected for clinical evaluation. Conventional techniques for measuring CDC activity often:

- Require large volumes of precious antibody and serum samples
- Use instrumentation with low-throughput acquisition (e.g., traditional flow cytometry)
- Are laborious and time-consuming, requiring steps such as protocol optimization, fixation, and multiple washes

In this application note, we present a streamlined, miniaturized workflow for quantifying mAb-induction of CDC activity using the iQue® Advanced Flow Cytometry Platform. Combining the high-throughput and low sample volume requirements of the iQue® with rapid data analysis using the integrated iQue Forecyt® software has the potential to simplify and speed up antibody characterization and drug discovery processes.

Methods

Assay Concept

Target cells of interest are seeded into 96- or 384-well V-bottom plates. The iQue® Cell Proliferation and Encoding (V/Blue) Dye can be used to label the target cells so that multiple cell types can be distinguished in a single well (for example a bright, mid-bright, dim, and unstained population). This assay is appropriate for use with both suspension and adherent target cells. Test mAbs are added to cells before human serum is added to induce CDC. Cells are then washed and labeled using the iQue® Cell Membrane Integrity (R/Red) Dye to enable

quantification of cell death. Fluorophore-conjugated antibodies may be included at this stage if cell surface marker expression is desired. Following a final wash step, cells are resuspended and run on the iQue® Advanced Flow Cytometry Platform. In the integrated iQue Forecyt® software, a simple gating strategy is applied to determine the proportion of dead cells per well.

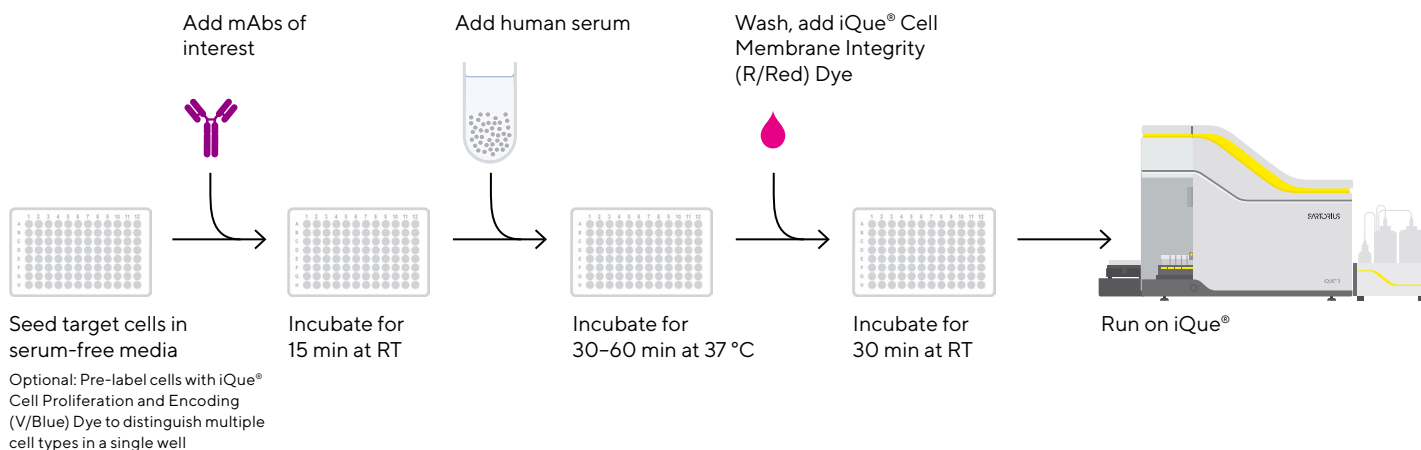


Figure 1

Workflow Schematic for the CDC Assay Using High-Throughput Flow Cytometry

Combine target cells (optionally labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye to distinguish multiple cell types in a single well) and mAbs of interest in a 96- or 384-well plate. Incubate for 15 min at room temperature (RT) to promote antibody binding to targets. Add human serum and incubate for a further 30–60 min (37 °C). Wash then label with iQue® Cell Membrane Integrity Dye (choice of V/Blue, B/Green, B/Red, R/Red) for 30 min (RT) and collect data using the iQue® Advanced Flow Cytometer.

Assay Workflow

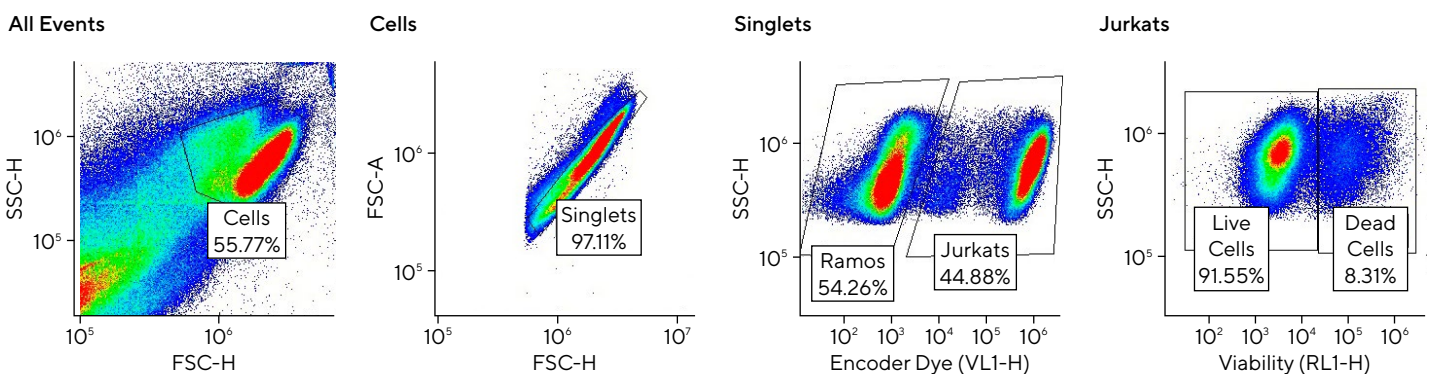
- Optional: If adding multiple cell types per well, distinguish cells by labeling with iQue® Cell Proliferation and Encoding (V/Blue) Dye:
 - Prepare a working stock of encoder dye by diluting in phosphate-buffered saline (PBS) (e.g., dilution factor, 1 in 1000 for bright staining).

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.
 - Collect cells in a conical tube, wash in PBS, and resuspend at 2×10^6 cells/mL.
 - Add an equal volume of the prepared dye solution (1:1) and incubate (37 °C, 15 min).
 - Wash cells with at least 2X volume of cell culture media (including 10% serum) and spin (500 g, 5 min).
 - Repeat wash step twice more.
- Prepare target cells to an appropriate density in serum-free cell culture media and seed 10 μ L/well in a V-bottom 96- or 384-well plate (e.g., Corning 3363 or 3656).

Note: Recommend a starting cell density of approximately 5-10 K/well for each cell type.
- Add 10 μ L of test mAbs (prepared in serum-free media) and incubate for 15 min at room temperature.
- Add 10 μ L of human serum prepared at 3X final assay concentration (FAC) and diluted in serum-free media (e.g., 45% to give a FAC of 15%), and incubate for 30–60 min at 37 °C.

Note: Recommended controls include serum-free media alone (i.e., no serum) and/or 15% heat inactivated serum (56 °C, 30 min).
- Add 100 μ L of wash buffer (e.g., PBS + 2% FBS) and centrifuge (300 g, 5 min).
- Remove media and shake (2000 RPM, 1 min) using the iQue® plate shaker to resuspend cells.
- Add 10 μ L iQue® Cell Membrane Integrity Dye (choice of V/Blue, B/Green, B/Red, R/Red) using concentration recommended in dye protocol and incubate for 30 min at room temperature.

Optional: Fluorophore-conjugated antibodies can be added at this stage to simultaneously measure marker expression.
- Wash, spin, and resuspend as in steps 5 and 6. Add 20 μ L/well wash buffer.
- Run on the iQue® using a 3–5 seconds sip time and analyze data using iQue Forecyt® software, applying a simple gating template (Figure 2).



Gating Strategy for CDC Assay

Figure 2

Gating Strategy for Quantifying Cell Death in the iQue® CDC Assay

First, cells and single cells are gated. If multiple cell types were included in the well, they can then be separated based on fluorescence of the iQue® Cell Proliferation and Encoding (V/Blue) Dye. Cell viability can then be determined based on iQue® Cell Membrane Integrity (R/Red) Dye staining.

Results

Serum Donor and Concentration May Impact Levels of CDC

We have previously shown that the donor from which immune cells are acquired for an ADCC or ADCP assay can have a large impact on the level of the induced antibody-mediated killing mechanism.⁴⁵ To explore whether this effect also applied to CDC, we looked at the level of CDC in the presence of a range of concentrations of Rituximab (anti-CD20-IgG1) with four different donors as the source of human serum (Figure 3A and B). High-CD20-expressing Ramos cells from a B-lymphocyte line were used as target cells. Overall, the levels of CDC induced by Rituximab in

the presence of each of the four donors was similar, particularly for donors 1, 2, and 3, between which EC_{50} values for the CDC response ranged from 0.29–0.36 $\mu\text{g}/\text{mL}$. The EC_{50} for CDC in the presence of donor 4 serum was slightly outside of these values at 0.53 $\mu\text{g}/\text{mL}$. These data highlight that if performing antibody screening for CDC activity, it may be worth testing activity with several different donors to ensure similar trends are observed. However, based on our observations, fluctuations due to the serum donor are likely minimal and perhaps less critical than in an ADCC or ADCP assay (data not shown).

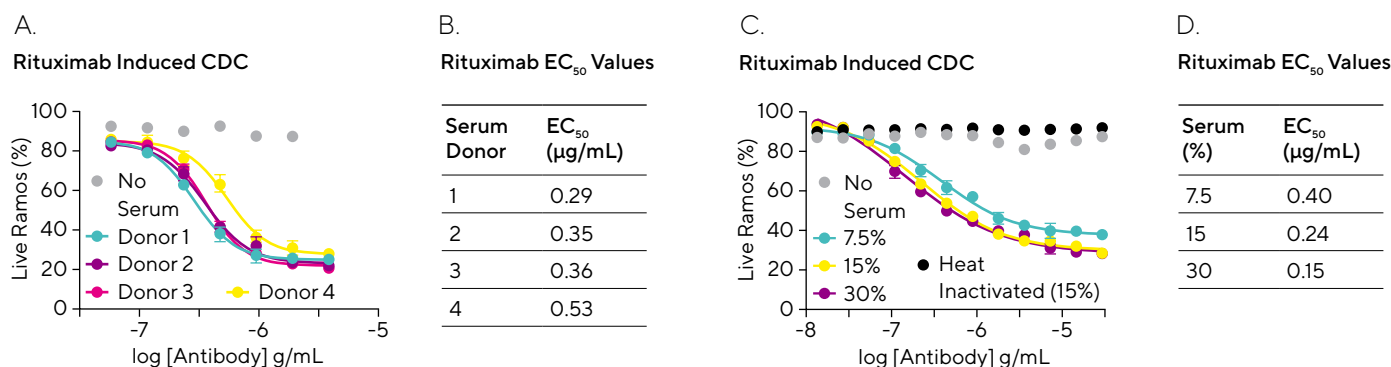


Figure 3

Minimal Impact of Serum Donor and Concentration on CDC by Rituximab

(A) Ramos cells were seeded in 96-well plates and incubated with Rituximab (anti-CD20-IgG1) and human serum from four different donors or with no serum. (B) Summary of EC_{50} values from the data in (A). (C) Ramos cells were seeded in a 384-well plate alongside Rituximab and a range of concentrations of serum from a single donor. No serum or heat-inactivated serum were included as controls. (D) EC_{50} values from curves in (C). CDC was quantified by measuring the decrease in percentage of live Ramos cells through labelling with the iQue[®] Cell Membrane Integrity (R/Red) Dye.

The assay optimization process included measurement of how the level of CDC differed with the concentration of human serum added. Figure 3C shows the CDC activity of Rituximab, measured with three different concentrations of serum from the same donor. The EC_{50} values in Figure 3D show a general increase in activity with increasing serum concentration. To get the balance between maximizing CDC response while minimizing usage of both serum and antibody samples we concluded to move ahead with a serum concentration of 15% for subsequent assays.

Levels of CD20 Expression on Target Cells Correlated with CDC Activity

Several studies have shown links between CD20 expression on cancerous target cells and the level of CDC activity exerted on them by anti-CD20 antibodies.⁶⁷ Subsequent experiments aimed to explore differences in CDC between suspension cell types using the iQue[®] assay. The histogram

in Figure 4A shows the relative cell surface CD20 expression on three cancer cell lines: Ramos, Rajis (another B-lymphocyte cell line) and Jurkats (a T-lymphocyte cell line). These data show that CD20 expression is highest on Ramos cells, with a median fluorescence intensity (MFI) $\times 10^6$ of 3.08 ± 0.1 , compared to 1.51 ± 0.03 on Raji cells. Expression of CD20 on Jurkat cells was similar to the IgG background control, with an MFI $\times 10^6$ of 0.06 ± 0.0005 .

To conserve time and reagents, iQue[®] Cell Proliferation and Encoding (V/Blue) Dye was used to differentially label cells to enable them to be distinguished within a single well, allowing comparison of CDC across multiple antibodies, concentrations, and cell types using a single 384-well plate. The histogram in Figure 4B shows how the cell types were separated, with the Rajis brightly labeled, Ramos more dimly labeled, and Jurkats unlabeled.

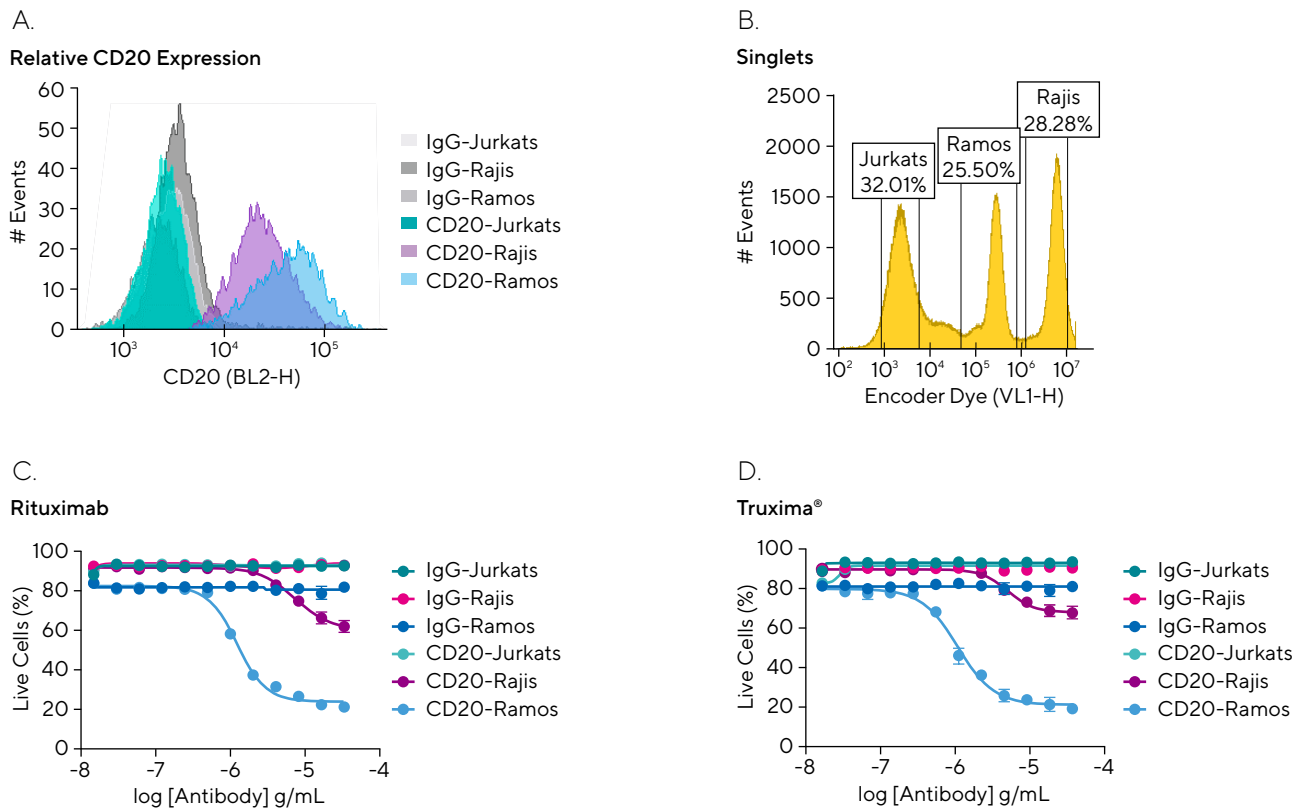


Figure 4
Higher CD20 Expression Elicits Greater CDC Induction by Anti-CD20 mAbs

(A) Relative CD20 expression on Jurkat, Raji, and Ramos cells compared to an IgG background control. (B) 5K/well of each cell type were seeded in a 384-well plate for a CDC assay and separated using differential labeling with iQue® Cell Proliferation and Encoding (V/Blue) Dye. CDC activity was measured with a range of concentrations of (C) Rituximab and (D) Truxima® (a Rituximab biosimilar).

Figures 4C and 4D reveal how CDC induction by both Rituximab and Truxima® differed depending on the target cell type. The high-CD20-expressing Ramos cells saw the greatest percentage cell death at the highest concentrations of both mAbs, with a 68 and 70% reduction in live cells compared to the IgG

control for Rituximab and Truxima®, respectively. The mid-CD20-expressing Raji cells saw considerably less CD20 mAb induction of CDC compared to the Ramos, at 34% (Rituximab) and 24% (Truxima®). As expected, no CDC was induced in the presence of CD20-negative Jurkat cells.

Multiplexed Measurement of Complement Protein Binding and CDC Activity

To delve deeper into the CDC mechanism, it can be useful to combine quantification of CDC activity with measurement of expression of cell surface markers or binding of proteins, for example those involved in the formation of the complement complex. The data in Figure 5 show the results from a CDC assay in which a FITC-labeled antibody was included during the labeling step to look at the binding of complement proteins C4c and C4b. C4b binds cells during the complement cascade and is processed to C4c, which remains bound to the cell surface.⁸ The cell surface location and lack of occlusion of these complement proteins makes them an ideal target for binding

antibodies. This method did not work for measuring binding of the C1q protein (data not shown). This is likely due to recruitment of other proteins to C1q in the complex obstructing antibody binding. Instead, its binding should be measured using purified C1q protein, a method which has been utilized in other studies.⁹

The results in Figure 5 show that both CDC and C4c and C4b binding increased in response to increasing Rituximab concentrations on the high-CD20-expressing Ramos cells. The EC₅₀ value for CDC activity was 0.17 µg/mL while the EC₅₀ for C4c and C4b binding was 0.64 µg/mL. No CDC or C4c and C4b binding was observed with either the IgG control antibody or with the antigen-negative Jurkat cell line.

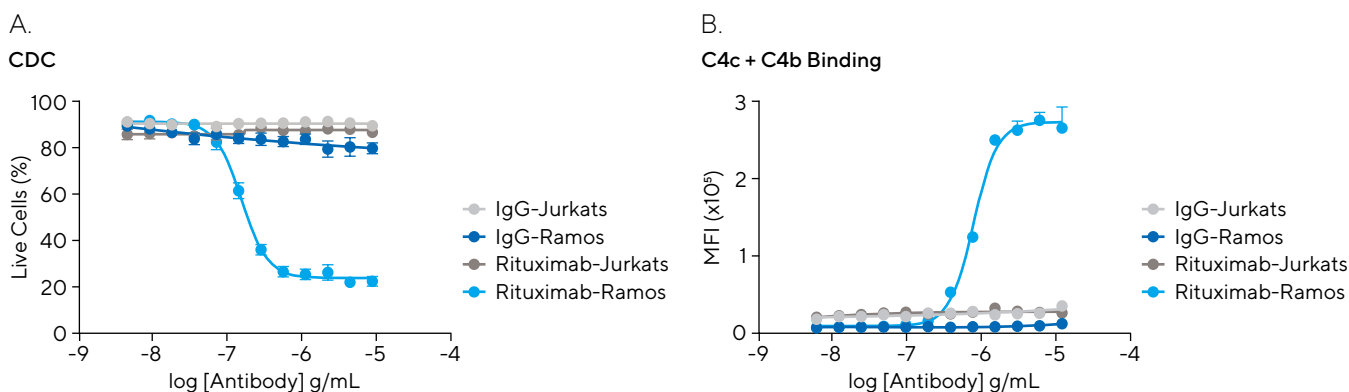


Figure 5

Binding of Complement Proteins C4c and C4b to the Cell Surface Increased with CDC

10K/well each of Ramos and Jurkat cells were seeded with a range of concentrations of Rituximab or an IgG control antibody. Human serum was added for 30 min to induce CDC. (A) Quantification of the percentage of live cells to give an indication of cell death due to CDC activity. (B) MFI on cells for binding of an anti-C4c + C4b antibody (part of the complement protein cascade).

Induction of Fc Receptor Mechanisms Is Dependent on Antibody Isotype

Many recent drug development efforts have been aimed at producing the next generation of antibody therapeutics with modifications to improve characteristics such as efficacy and stability compared to native antibodies.¹⁰ Examples of these next generation therapeutics include bispecific antibodies, ADCs, nanobodies, and engineered antibodies. Antibodies can be engineered via mutations to the Fc region which can improve function by changing the binding affinity to molecules such as Fc receptors and/or C1q proteins—both of which impact Fc mediated function.¹⁰

Here we used the iQue[®] to compare Fc functions of an anti-CD20-IgG1 Rituximab-based antibody with two Fc mutants: an IgG1fut (non-fucosylated) and IgG1NQ (non-glycosylated) (Figure 6). The CDC assay was used for this comparison alongside an ADCP assay, which utilized the

iQue[®] Human ADCP Kit. This kit measures the level of colocalization between phagocytic immune cells, such as monocytes and macrophages, and target cells to give a read-out for the level of ADCP induced by a test mAb.

The IgG1NQ mutant showed similar CDC activity to the IgG1 antibody, with a lower maximal death response of 50% ± 2% compared to 66% ± 4% with the native antibody. Contrastingly, its ADCP activity was completely abolished, with a response similar to the IgG control. This is likely because antibody glycosylation is essential for Fc-receptor mediated effector functions.¹¹ The primary function of the mutation to the IgG-Fc to remove the fucose residue is to enhance ADCC activity and aside from this, its effector function should be similar to the non-mutated IgG1.¹¹ This is again in line with what we measured in Figure 6 with the IgG1fut mutant inducing comparable, but slightly reduced, CDC and ADCP activity compared to the native antibody.

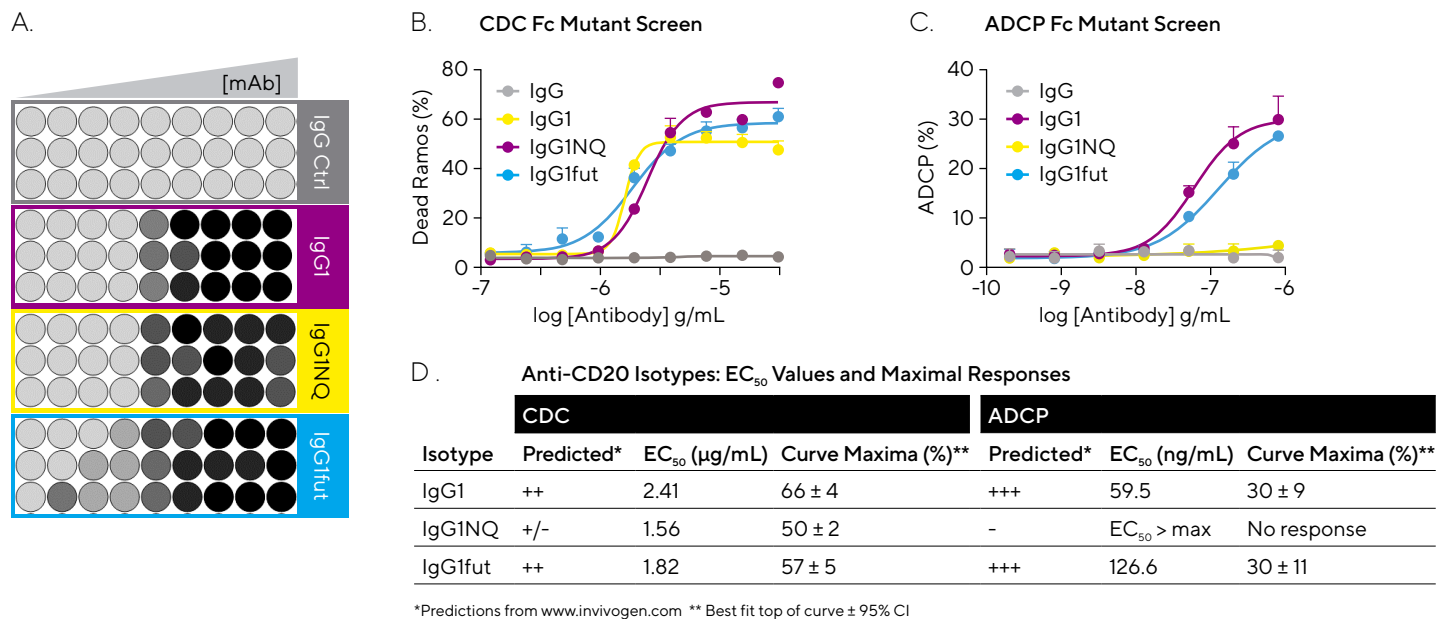


Figure 6
CDC and ADCP Responses Differ between Anti-CD20 mAb Isotype Fc Mutants

(A) Heat map from a CDC assay in which Ramos cells were seeded at 5K/well alongside several anti-CD20-IgG1 antibodies, including IgG1 and two IgG1 isotype mutants: IgG1NQ (non-glycosylated) and IgG1fut (non-fucosylated). βGal-IgG1 antibody was included as a control. CDC was induced by addition of human serum. Darker grey indicates more cell death. (B) Data from (A) shown as concentration response curves for percentage of dead Ramos cells due to CDC. (C) The IgG1 isotypes were also tested for their ADCP activity using the iQue® Human ADCP Kit which measures co-localization between phagocytic immune cells and target cells to indicate ADCP. Ramos cells (2.5K/well) labeled with iQue® Cell Proliferation and Encoding (B/Green) Dye were seeded with PBMCs (20:1 E:T) in a 384-well plate alongside antibodies prior to labeling with the components of the ADCP kit. Analysis was performed using the iQue®. (D) Data from (B) and (C) summarized as a table.

Adherent Cells are Highly Resistant to CDC

It has been estimated that approximately 90% of adult cancers are solid tumors formed from adherent cell types.^{12,13} It is therefore crucial when we are developing *in vitro* models that we aim to make them suitable for assessment of antibody anti-tumor activity against both suspension and adherent cells. To this end, as well as using the iQue® CDC assay to measure anti-CD20 mAb-induced CDC of B cells, we also wanted to measure anti-HER2 antibody activity against cells from solid tumors, specifically breast cancer cell lines.

Figure 7D shows there was no impact on the percentage of live cells when two adherent cell types (high-HER2-expressing AU565 cells and HER2-negative MDA-MB-468 cells) were incubated with a single, high concentration (30 µg/mL) of anti-HER2 antibodies and human serum. This exact same assay setup induced high levels of death of the antigen-positive cell type in the suspension cell model with all three anti-CD20 antibodies, as is evidenced in Figure 7B, with a 53, 57, and 59% reduction in viability compared to the IgG control.

The lack of CDC activity exerted by the anti-HER2 antibodies to the adherent cells is in line with literature sources, of which a number have shown little or no CDC activity by Trastuzumab.^{14,15} These studies suggest that resistance may be conveyed due to overexpression of complement regulatory proteins on the surface of cancer cells. Complement regulatory proteins CD46, CD55, and CD59 are expressed on normal tissues to inhibit complement activity, but their overexpression on the surface of some tumors is linked to inhibition of CDC activity.¹⁶

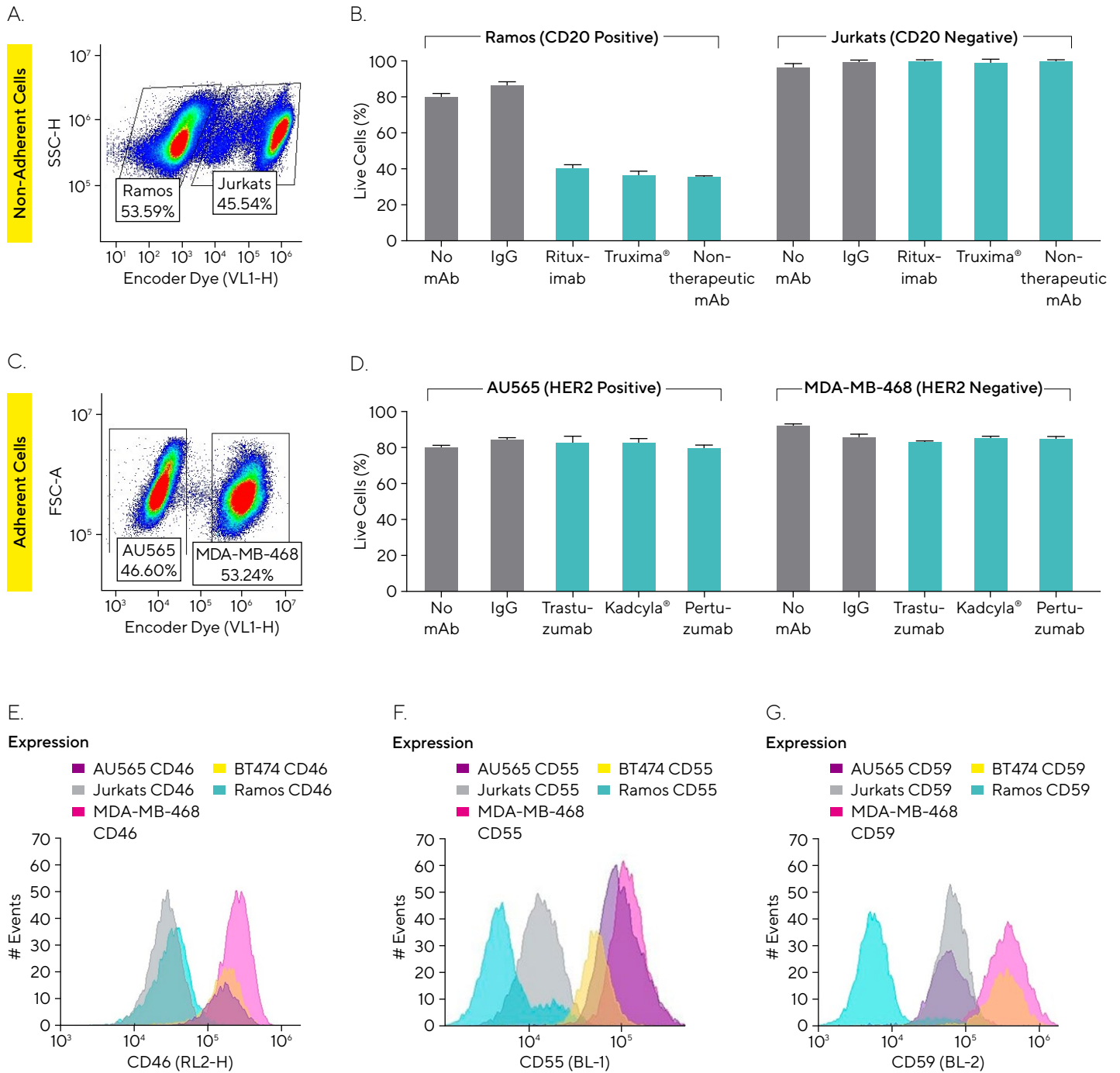


Figure 7

Adherent Cells Express Higher Levels of Complement Regulators and Are Resistant to CDC by Anti-HER2 mAbs

(A) Dot plot showing separation of antigen-negative Jurkat cells (labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye) and antigen-positive Ramos cells (unlabeled) enabling assessment of CDC of both cell types simultaneously in each assay well. (B) Cells were incubated with 30 µg/mL of three different anti-CD20-IgG1 antibodies: Rituximab, Truxima® and a non-therapeutic mAb based on Rituximab. CDC was induced by addition of human serum. (C) Plot showing separation of HER2-antigen-negative MDA-MB-468 cells labeled with encoder dye and HER2-antigen-positive AU565 cells (unlabeled). (D) Adherent cells were incubated with human serum and three different anti-HER2-IgG1 antibodies: Trastuzumab, Kadcylla® (an antibody-drug conjugate based on Trastuzumab) and Pertuzumab. A range of suspension and adherent cell types were profiled for their expression of complement regulatory proteins: (E) CD46, (F) CD55, and (G) CD59.

Figures 7E–G show the results from profiling experiments we carried out to compare the relative expression of complement regulatory markers on five cell lines: two suspension cell types (Ramos and Jurkats) and three adherent cell types (AU565s, BT474s, and MDA-MB-468s). As expected, we saw a general trend towards higher expression of the complement regulatory proteins on adherent cells with lower expression on suspension cells, supporting the hypothesis that this may be the reason for adherent cell resistance to CDC.

Following confirmation that adherent cells express higher levels of complement regulatory proteins, we attempted to inhibit these proteins to enhance CDC (data not shown). Wang *et al.* (2017) reported enhanced Trastuzumab CDC activity towards BT474 cells when complement regulators were inhibited with blocking mAbs or sheared by phosphatidylinositol-specific phospholipase C (PI-PLC), however we were unable to recreate these results.¹⁷ Several studies reported enhanced CDC activity following knockdown of complement regulatory proteins using siRNAs (short interfering RNA or silencing RNAs), although we have not yet tested this approach.^{14,18} It has also been suggested that combining multiple anti-HER2 antibodies that bind different epitopes, for example Trastuzumab and Pertuzumab, may work to enhance CDC activity.¹⁴

Conclusions

In this application note, we have exemplified a simple workflow for quantifying CDC activity using the iQue[®] Advanced Flow Cytometry Platform. Fast sample acquisition by the iQue[®] combined with streamlined data analysis using the iQue Forecyt[®] software enables rapid comparisons between antibody function to be drawn. The experiments in this note have highlighted the advantages of this workflow including:

- Low volume requirements for the iQue[®] allow conservation of precious antibody and serum samples and lead to a reduction in reagent costs.
- Ease of multiplexing the CDC readout with other markers and dyes allows much more information to be gathered from a single assay and negates the need to combine data from multiple sources.
- Pharmacological readouts, such as EC₅₀ values, generated using the iQue Forecyt[®] software, can be used to rank antibodies based on their CDC-inducing activity.
- High-throughput data acquisition by the iQue[®] means that a 96-well immune assay can be read in 15 min or a 384-well in 40 min. This facilitates rapid screening of large antibody libraries along with the capacity to increase replication to enhance robustness of data.
- Combining CDC data with Fc function analysis using the iQue[®] Human ADCP Kit and iQue[®] Natural Killer Cell Killing Kit can provide full profiling of the three key Fc receptor mediated functions of antibodies using a single instrument.

Together these advantages demonstrate the power of the iQue[®] to measure antibody Fc functions, such as CDC, and show the potential for this workflow to improve antibody drug discovery processes, both through enhanced speed and quality of hits generated.

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