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Quantification of Antibody-Dependent Cellular Phagocytosis via a Streamlined Advanced Flow Cytometry Workflow

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Introduction

Antibody-dependent cellular phagocytosis (ADCP) is the immunological process of antibody-stimulated engulfment of tumor cells by phagocytic immune cells, such as monocytes and macrophages. This results in specific internalization and degradation of tumor cells by the immune cells. Monoclonal antibodies (mAbs) are used extensively as cancer therapeutics due to their ability to induce selective killing of tumor cells. Such therapeutics have been designed to target both solid tumors and blood cancers, for example Trastuzumab (an anti-HER2 mAb) is used as a treatment of HER2 positive breast cancers and Rituximab (an anti-CD20 mAb) is given in combination with chemotherapy to treat non-Hodgkin's lymphoma and chronic lymphocytic leukemia.[1-3]
ADCP, alongside antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), is one of three key mechanisms of action (MoAs) adopted by mAbs to induce clearance of cancerous cells from the body. An important stage in the development of mAbs for therapeutic use is the characterization of their effect on each of these MoAs. In ADCP, mAbs engage Fc receptors on immune cells via their constant region. Simultaneously, their variable region binds specifically to receptors over-expressed on tumor cells; targeting them for engulfment. This means that the ADCP activity of a potential therapeutic can be enhanced by modifying either the constant or variable regions of the mAb.[4] Powerful, high-throughput techniques are therefore crucial to facilitate discovery of novel therapeutics by screening large libraries of antibodies in minimal time.

Conventional techniques for measuring ADCP, such as traditional flow cytometry and confocal microscopy are limited due to the following reasons:

- Assay workflows are complex and time-consuming resulting in low throughput.
- They require lengthy fixation protocols, repetitive wash steps and complicated data analysis.
- These methods often involve multiple rounds of protocol optimization and necessitate large volumes of precious sample.
- Other techniques such as reporter assays are reliant on engineered reporter cell lines.
- True ADCP is challenging to quantify, meaning traditional assays may either provide only qualitative readouts or are heavily prone to measuring artifacts.

To address the challenges, we describe a high throughput, multiplex assay that measures ADCP via co-localization of encoding dye-labeled target cells with CD14 positive effector cells using a simplified, streamlined workflow. Data acquisition is performed using the iQue® platform for advanced flow cytometry, which combines high throughput sampling, flow cytometry detection, and plate level analytics, delivering rich content in both 96- and 384-well formats.

Assay Concept

Validated mix and read reagents from the iQue® Human Antibody Dependent Cellular Phagocytosis Kit were added to co-cultures of adherent or non-adherent target cells alongside primary effector cells. These data were produced using PBMC effectors, however the flexible assay format means isolated CD14+ effector cell preparations, such as monocytes or macrophages can also be used. The iQue® platform facilitated rapid sampling from 96- and 384-well plates allowing multiple mAbs to be characterized for their effects on ADCP in a short time. The pre-set gating template provided with the iQue® Human Antibody Dependent Cellular Phagocytosis Kit combined with automated data analysis by iQue Forecyt® software provided instant identification of co-localized live, encoded target cells with CD14+ effectors and generation of pharmacological readouts, such as EC_{50} values.

Assay Workflow

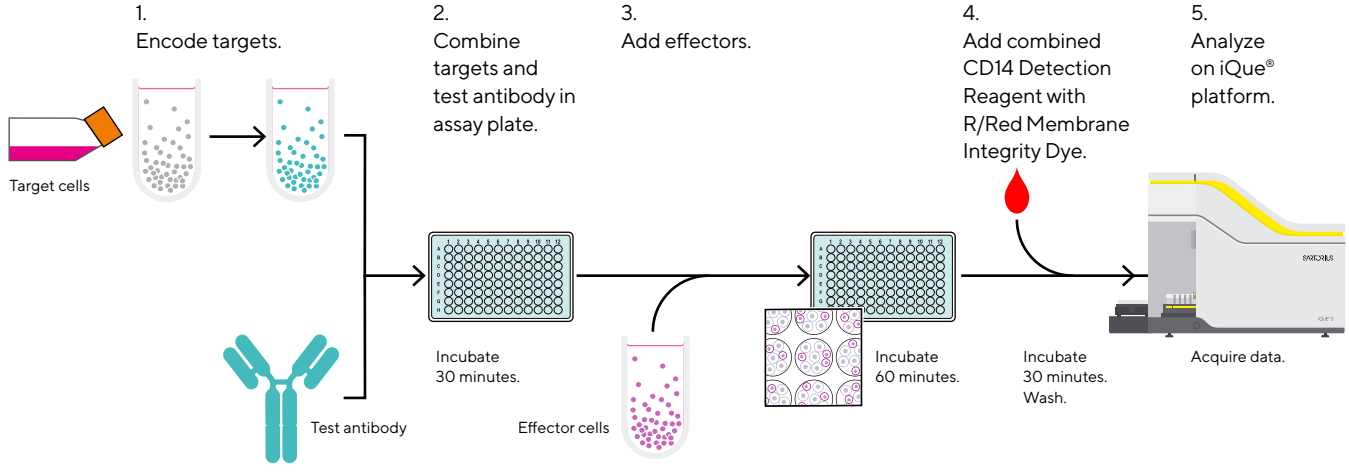


Figure 1. Schematic of the iQue® Human Antibody Dependent Cellular Phagocytosis Kit protocol

1. Label target cells using the iQue® Proliferation and Encoding (B/Green) Dye and protocol provided with the iQue® Human Antibody Dependent Cellular Phagocytosis Kit.
2. Prepare desired concentrations of test antibody(s) in cell culture media and add to either a 96 – or 384- well plate.
3. Resuspend B/Green labelled target cells at an appropriate density and add to plate. Incubate for 30 minutes.
4. Add an appropriate density of effector cells (rested overnight) and incubate for 1 hour.
   Note: We recommend effector-to-target ratios of 20:1 and 5:1 when adding PBMCs and monocytes, respectively.

5. Label cells using the Human ADCP Antibody Detection Reagent (containing the CD14 antibody) and iQue® Cell Membrane Integrity (R/Red) Dye for 30 mins.
6. Resuspend labelled cells in wash buffer and acquire samples using the iQue® platform (BR or VBR configuration).
7. Import the iQue® Human Antibody Dependent Cellular Phagocytosis Kit gating template (Figure 2) into the iQue Forecyt® software for automated analysis of ADCP.

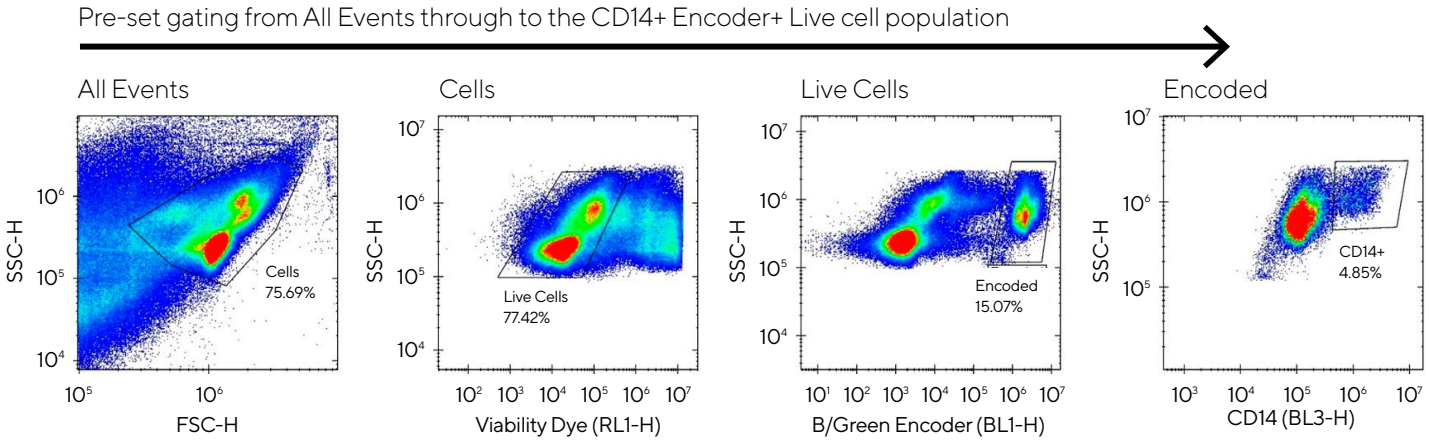


Figure 2. Gating strategy used to select for ADCP events. Pre-set gating template provided with the iQue® Human Antibody Dependent Cellular Phagocytosis Kit is imported into iQue Forecyt®. The singlet gate is omitted to allow quantification of co-localized B/Green encoded target cells with CD14+ monocytes.

Quantification of ADCP in a 384-Well Co-Culture Model

Initial experiments aimed to confirm that the iQue® Human Antibody Dependent Cellular Phagocytosis Kit workflow could be used to measure ADCP response to clinical mAbs. This was achieved using Truxima (anti-CD20-IgG1), a Rituximab biosimilar approved treatment for CD20 positive B cell cancers.[5] Ramos cells labelled with encoder dye were incubated with Truxima in a 384 well plate prior to addition of PBMCs from 2 different donors at various effector-to-target ratios. After 1 hour, all cells were labelled with viability dye and the Human ADCP Antibody Detection Reagent so that co-localization between live encoded targets and CD14+ monocytes could be quantified using the iQue® platform.

In the presence of Truxima (0.05-200 ng/mL), there was a clear concentration-dependent increase in ADCP (defined as the % CD14 and B/Green Encoder+ cells in the live population) of target cells mediated by the effector cells (Figure 3). This increase was observed at 5:1, 10:1 and 20:1 effector-to-target ratios, while, as expected, in the absence of PBMCs (0:1) Truxima had no effect on the proportion of CD14+ Green cells in the population. At the highest E:T (20:1), maximal ADCP (%) was very similar between donor 1 and donor 2, at 41 ± 5% and 43 ± 2% respectively. However, as the E:T was reduced, the difference in response between the two donors became more pronounced, with a maximal response at 5:1 E:T of 28 ± 8% with donor 1 compared to 19 ± 6 % with donor 2. Donor-to-donor variability in the level of mAb stimulated clearance of tumour cells may relate to: differences in Fc receptor density on the immune cells' surface; polymorphisms in the Fc receptor affecting the degree of binding by the IgG or could be due to differences in the number of phagocytic immune cells within each PBMC population.[6,7]

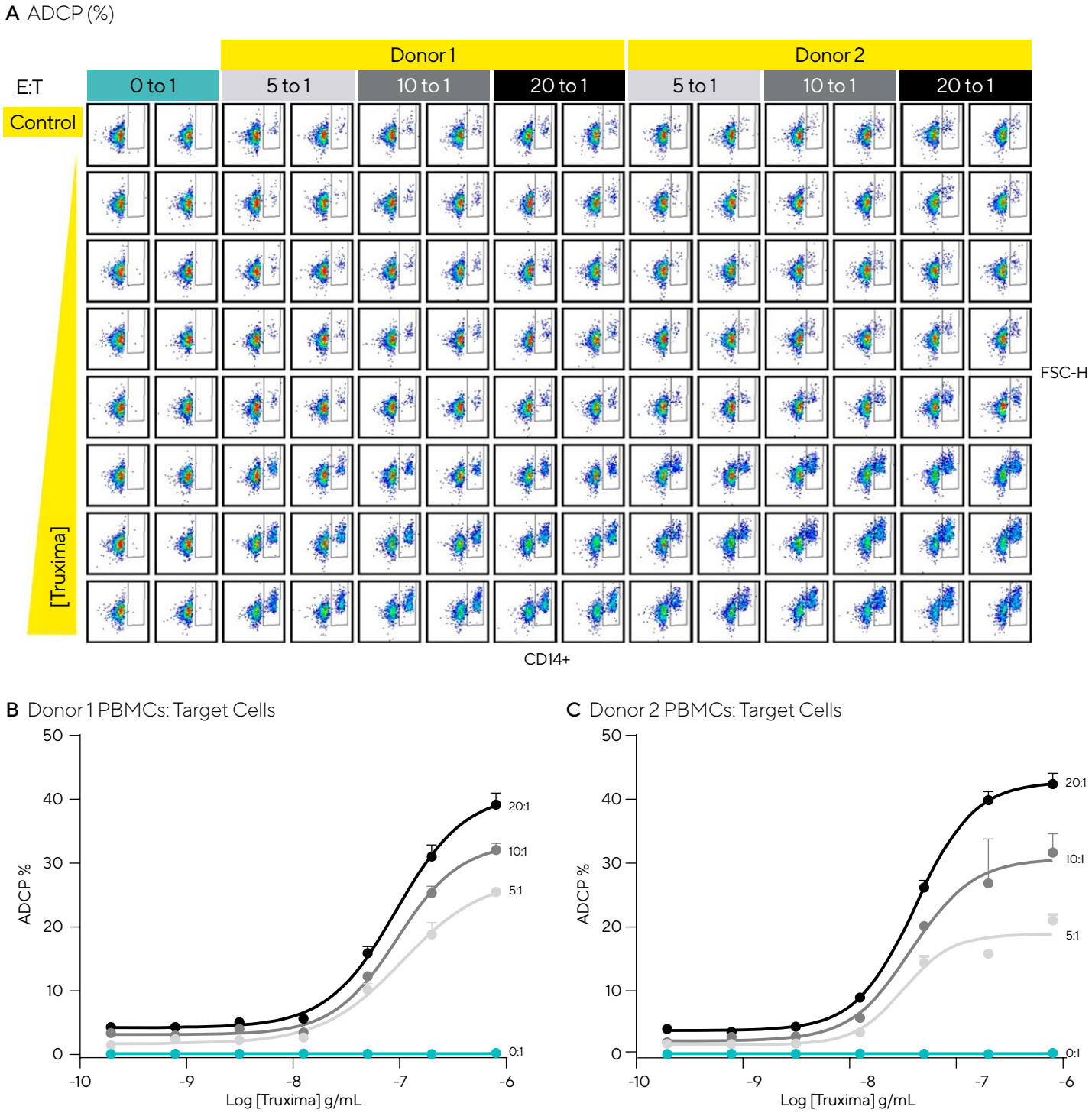


Figure 3. ADCP response to Truxima increases with concentration and effector-to-target ratio Ramos cells (2.5K/well) were incubated with varying concentrations of Truxima (anti-CD20-IgG1 mAb; Rituximab biosimilar; 0.05 ng/mL to 200 ng/mL). PBMCs were added at 5:1, 10:1 and 20:1 effector-to-target ratios. Control wells contained no antibody. ADCP was analyzed using the iQue® Human Antibody Dependent Cellular Phagocytosis Kit and the iQue® plaform. ADCP (%) is defined as the % CD14 and B/Green Encoder+ cells in the live population. (A) Plate view with gate for CD14+ cells within the B/Green encoded live population in each well. (B) and (C) Concentration-response curves with ADCP (%) by donor 1 and 2 PBMCs, respectively.

Pharmacological Analysis of mAb Fc Mutants and Isotypes

Development of novel therapeutic mAbs is reliant on the ability to characterize their effect on key MoAs using *in vitro* assays. Here we demonstrate the use of the iQue® Human Antibody Dependent Cellular Phagocytosis Kit and the iQue® platform to quantify the effect of a range of anti-CD20 mAbs on ADCP by PBMCs on CD20 positive Ramos target cells. The mAbs tested included three mutants of the mouse/human chimeric mAb, Rituximab: IgG1 (clinical mAb), IgG1fut (non-fucosylated) and IgG1NQ (non-glycosylated). Antibody glycosylation is essential for Fc-receptor mediated effector functions, meaning the mutation to remove the asparagine glycosylation sites from the IgG1 mAb to produce the IgG1NQ mutant should result in a loss of ADCP activity.[8] This is supported by the lack of ADCP response to the IgG1NQ mutant in Figure 4, which measured ADCP activity at a similar level to the IgG1 negative control antibody.

As highlighted in Table 1, both the IgG1 and IgG1fut isotypes are expected to exert a stimulatory effect on ADCP. The primary function of the mutation to the IgG-Fc to remove the fucose residue is to enhance ADCC activity.[8] Aside from this, its effector function is similar to the non-mutated IgG1. This was shown in Figure 4, with maximal ADCP of 30% for both isotypes. The potency of effect for the non-mutated IgG1 mAb was slightly greater with an EC_{50} value for ADCP induction of 60 ng/mL compared to 127 ng/mL for the non-fucosylated mutant. However, a difference of only 2.1-fold in the ng/mL range could be considered minimal and overall the pharmacological profiles of the mutants in terms of ADCP induction are similar.

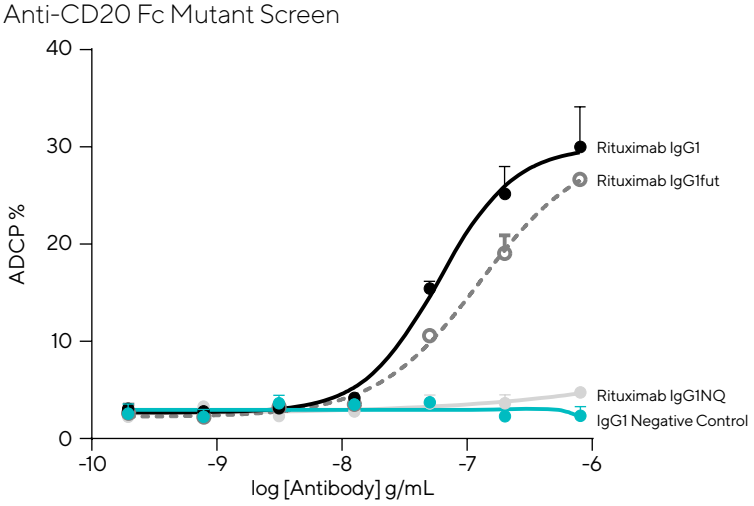


Table 1. Rituximab Fc mutants with EC_{50} values and maximal ADCP responses

Isotope	ADCP[8]	EC_{50} (ng/mL)	Maximal ADCP (%)*
IgG1	+	59.5	30 ± 9
IgG1fut	+	126.6	30 ± 11
IgG1NQ	-	No response	No response

* Best fit top of curve

Figure 4. Screening anti- CD20 mAb Fc mutants revealed differences in the pharmacology of ADCP induction between isotypes. Ramos cells (2.5K/well) labelled with B/Green encoder dye were seeded with PBMCs (20:1 E:T) in a 384-well plate. ADCP was stimulated with a range of isotypes of anti-CD20 mAb Rituximab (0.2 ng/mL to 800 ng/mL), including: IgG1 (clinical mAb isotype), IgG1fut (non-fucosylated) and IgG1NQ (non-glycosylated). Cells were labelled using the iQue® Antibody Dependent Cellular Phagocytosis Kit and ADCP was analysed using the iQue® platform. EC_{50} values were exported directly from iQue Forecyt®.

ADCP Quantification Using Adherent Cell Types

Having demonstrated that the iQue® Human Antibody Dependent Cellular Phagocytosis Kit workflow could be used to quantify ADCP of CD20 positive blood cancers, we also wanted to verify that ADCP of adherent tumour cells could be measured. Adherent HER2 positive AU565 breast cancer cells were incubated with HER2 mAbs and PBMCs (20:1 E:T); following the protocol previously described. ADCP was induced by three HER2 mAbs isotypes: anti-HER2-IgG1, (Trastuzumab), a native isotype and clinically available treatment for HER2 positive breast cancers; anti-HER2-IgA2, another native isotype with reduced ADCP activity relative to the IgG1 isotype and anti-HER2-IgG4 (S228P), an engineered isotype that also has reduced ADCP activity relative to IgG1 (Table 2).[8]

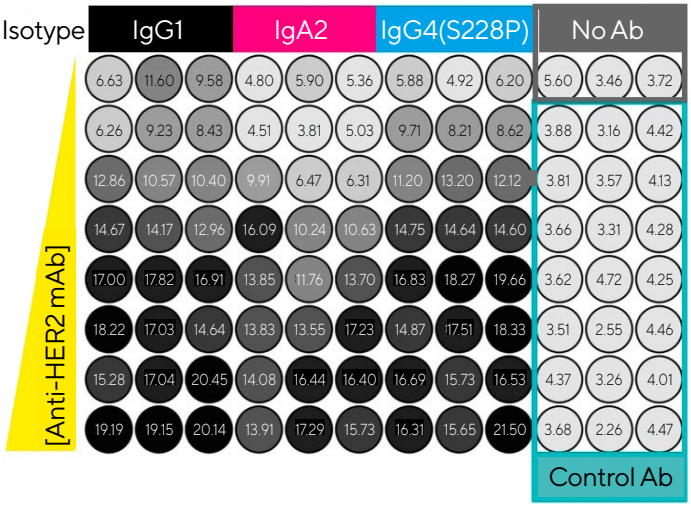
As shown in Figure 5, all three isotypes induced a concentration dependent increase in ADCP activity relative to the anti-β-galactosidase-IgG1 control antibody. As expected (Table 2), the highest maximal ADCP response (19 ± 4 %) and most potent response (EC_{50} = 6.6 ng/mL) was measured with the IgG1 isotype. The EC_{50} for the other native isotype (IgA2) was more than 5-fold greater than the IgG1 at 35.4 ng/mL while the engineered IgG4 (S228P) displayed only a marginal decrease in potency of effect on ADCP relative to the IgG1 at 18.2 ng/mL (2.8-fold increase). These data show that this assay is suitable for use with adherent target cells to model mAb induction of ADCP activity on solid tumors. They also highlight that the difference in effect on ADCP between mAb isotypes can be quantified.

Table 2. Anti-HER2 mAB Isotypes with EC_{50} Values and Maximal ADCP

Isotype	ADCP[8]	EC_{50} (ng/mL)	Maximal ADCP (%)*
IgG1	+++	6.6	19 ± 4
IgA2	+	35.4	15 ± 2
IgG4(S228P)	+	18.2	17 ± 1

* Best fit top of curve

A ADCP %



B ADCP Response to Anti-HER2 mAb Isotypes

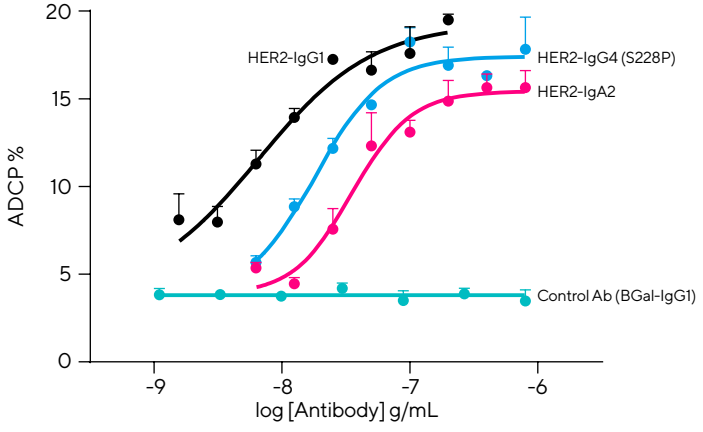


Figure 5. Pharmacological differences in ADCP induction can be quantified with anti-HER2 mAbs and adherent target cells. Adherent AU565 cells (10K/well) were seeded in a 96-well plate with PBMCs (20:1 E:T) and ADCP was stimulated with various isotypes of anti-HER2 mAb (IgG1, IgA2 and IgG4 (S228P). ADCP was quantified using the iQue® Human Antibody Dependent Cellular Phagocytosis Kit and the iQue® platform. (A) Heat map with % ADCP for each HER2 mAB isotype. No antibody or a Bgal-IgG1 mAb were included as negative controls. (B) Concentration response curves for ADCP response to anti-HER2 mAbs.

Investigating the Correlation Between Target Marker Expression and Induction of ADCP

To exert their MoA, targeted immunotherapies, such as mAbs, rely on the overexpression of specific surface markers on cancer cells compared to normal body cells. Therefore, the marker expression profile of an individuals' tumor is key in determining the course of treatment. For example, only breast cancer patients with amplified HER2 expression (an estimated 20% of tumors[9]) will be treated with Trastuzumab (anti-HER2-IgG1) because the selective anti-tumor effects of the drug will only be exerted in those patients.

To examine this effect in vitro we utilized the iQue® platform and a range of adherent breast cancer cell lines: MDA-MB-231, HCC38, MCF-7, BT474 and SK-BR-3 cells. HER2 on each cell type was labelled with an anti-HER2 fluorophore conjugated antibody and the level of HER2 expression quantified using the iQue® platform. The histogram in Figure 6A shows the relative HER2 expression on each cell type, with the associated Mean Fluorescence Intensity (MFI) values highlighted in Figure 6B. HER2 expression on MDA-MB-231 cells was lowest of the five cell types with an MFI of $2.0 \pm 0.2 \times 10^4$. Expression on HCC38 and MCF-7 cells was 2.8-fold greater than on MDA-MB-231 cells. This expression level is relatively low when compared to the highest expressing cell type, SK-BR-3s, with a 50-fold higher MFI than the MDA-MB-231 cells of $1.1 \pm 2.1 \times 10^6$. BT474 cells also expressed high levels of HER2 with an MFI of $9.0 \pm 2.4 \times 10^5$.

An ADCP assay was set up in a 384-well assay format to measure the ADCP response by PBMCs to a range of concentrations of Trastuzumab, in the presence of each of the five breast cancer cell types. The iQue® Human Antibody Dependent Cellular Phagocytosis Kit was used to label cells prior to quantification of the ADCP response (%) using iQue Forecyt®. In Figure 6C, the ADCP response (%) at a single Trastuzumab concentration (70 ng/mL) has been plotted for each cell type. These data show a very similar pattern to the HER2 expression data in 6A and 6B, with high ADCP on SK-BR-3 and BT474 cells and low ADCP on MDA-MB-231, HCC38 and MCF-7 cells. These data support the results from other studies that show that tumor cell clearing MoAs of targeted mAbs such as Trastuzumab are greatly enhanced when the cancerous cell over-expresses the target antigen.[9]

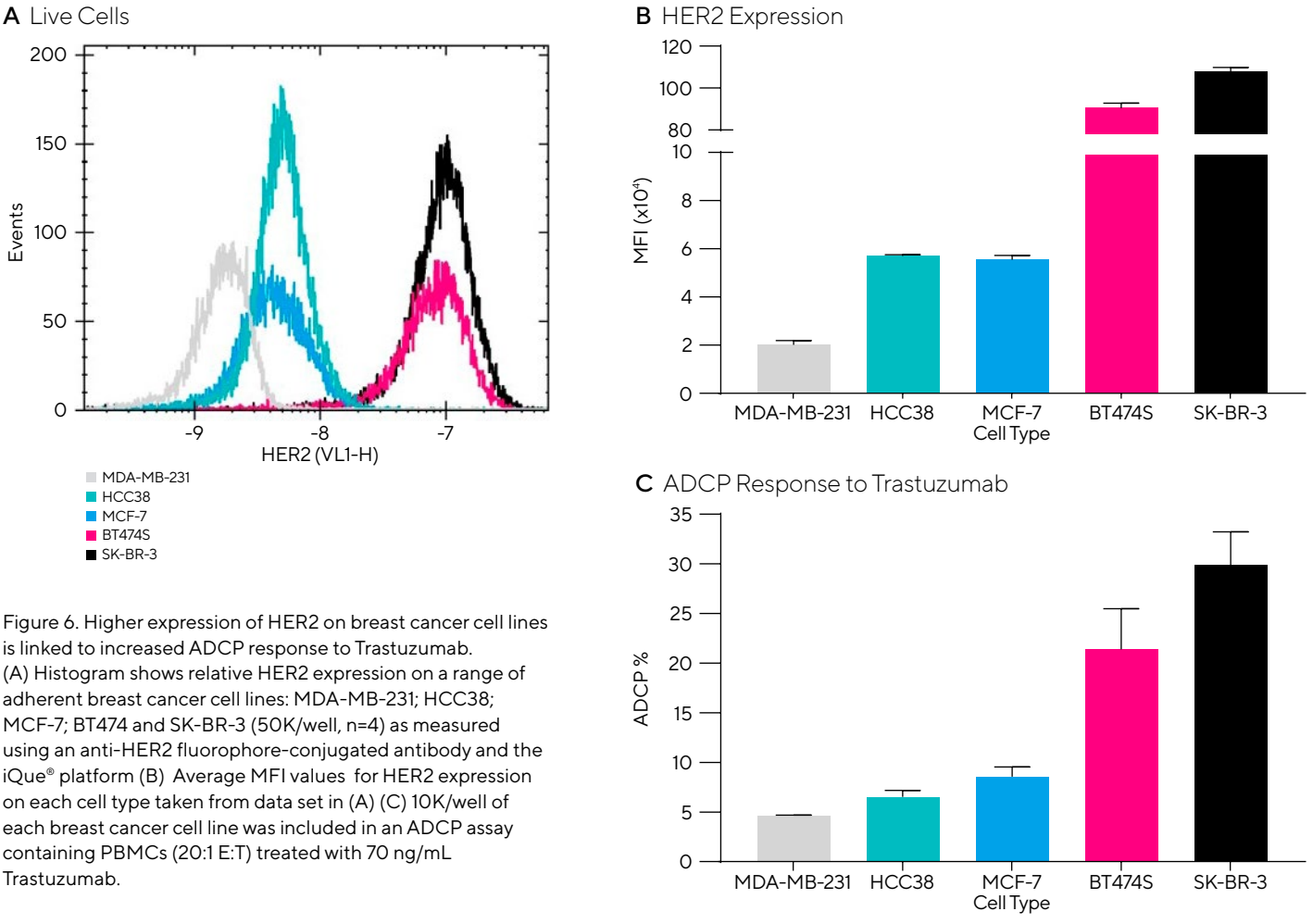


Figure 6. Higher expression of HER2 on breast cancer cell lines is linked to increased ADCP response to Trastuzumab. (A) Histogram shows relative HER2 expression on a range of adherent breast cancer cell lines: MDA-MB-231; HCC38; MCF-7; BT474 and SK-BR-3 (50K/well, n=4) as measured using an anti-HER2 fluorophore-conjugated antibody and the iQue® platform (B) Average MFI values for HER2 expression on each cell type taken from data set in (A) (C) 10K/well of each breast cancer cell line was included in an ADCP assay containing PBMCs (20:1 E:T) treated with 70 ng/mL Trastuzumab.

Conclusions

In this application note, we have demonstrated the value of using the iQue® Human Antibody Dependent Cellular Phagocytosis Kit in conjunction with the iQue® platform to provide a simple, robust assay for measuring ADCP. The pre-set gating template provided with the kit enabled instantaneous quantification of co-localized targets and effectors which were used to generate pharmacological readouts for ADCP response to mAbs. The experiments in this note have highlighted that:

- The iQue® Human Antibody Dependent Cellular Phagocytosis Kit workflow can be used to measure ADCP in response to mAbs, such as anti-CD20 mAb Rituximab. The level of response differed between effector cell donors and with increasing effector-to-target cell ratios.
- Pharmacological differences in the ADCP response to a range of anti-CD20 mAb Fc mutants with suspension target cells can be measured. This shows that this assay can be used to rank mAb therapeutics based on their effect on ADCP.
- This assay can be used with adherent target cells and pharmacological readouts for ADCP response to anti-HER2 mAbs, such as Trastuzumab, can be quantified. The flexibility to use either adherent or suspension cells in this assay means it is suitable for modelling mAb effects on both blood cancers and solid tumors.
- Differences in ADCP response due to expression levels of an mAbs target antigen on tumor cells can be measured. This mimics the clinical scenario with anti-tumour effects of mAb treatments only proving effective on target antigen over-expressing cancers.

The high-throughput power of the iQue® platform combined with inbuilt, automated data analysis by iQue Forecyt® can profile libraries of mAbs for their effects on ADCP in minimal time. This technique is a powerful tool with potential to reduce the time for MoA characterization in drug discovery applications.

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