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Exploring Antibody Internalization: Case Studies Using the iQue® HTS Platform

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Abstract

Antibody Internalization (ABI) is a pivotal process in drug development, particularly for Antibody-Drug Conjugates (ADCs), which combine immunotherapy and chemotherapy for targeted cancer treatment. ADCs, with their ability to deliver cytotoxic payloads specifically to tumor cells, have gained therapeutic traction with several FDA-approved options available. The iQue® High-Throughput Screening (HTS) by Cytometry Platform facilitates efficient ABI measurement using a pH-sensitive fluorescent probe and a streamlined workflow. This study explores the use of the iQue® Platform in various case studies, including the characterization of ADCs and the differentiation of monocytes into dendritic cells. The platform's enhanced features such as optimized optical alignment and improved data analysis capabilities support complex workflows and reagent compatibility.

Introduction

Antibodies targeting cell surface antigens can trigger endocytosis leading to their internalization, a process known as Antibody Internalization (ABI). Monitoring ABI is vital in ADC design as factors like the target antigen's epitope, ADC-antigen affinity, and intracellular trafficking affect internalization.

ADCs represent one of several modifications made to mAbs to enhance their anti-tumor activity. They have gained therapeutic traction, with twelve FDA-approved anti-cancer ADCs available. [1] Five of these target solid tumors, including Trastuzumab emtansine for HER2-positive breast cancers and Sacituzumab govitecan for triple-negative breast cancers. The remaining seven target antigens such as CD22 or CD19 on B cells, treating hematological malignancies.

ADCs combine immunotherapy and chemotherapy for targeted cancer treatment, using mAbs to deliver cytotoxic drugs.[2] These mAbs are modified with a linker for attaching toxic payloads, relying on ADC internalization into tumor cells to activate cytotoxicity, preventing release into the bloodstream or healthy tissues. Payloads are typically chemotherapeutic drugs disrupting microtubule polymerization or inducing DNA damage [3], targeting rapidly proliferating cancer cells.[4,5] ADCs also retain mAb Fc-mediated immune functions including antibody-dependent cellular cytotoxicity (ADCC).

The iQue® High-Throughput Screening (HTS) Cytometry Platform enables efficient ABI measurement in both suspension and adherent cells. It uses a pH-sensitive fluorescent probe and a one-step, no-wash protocol to label antibodies. Once internalized and processed through acidic pathways, the labeled antibody emits a quantifiable fluorescent signal. The iQue® Platform offers streamlined workflows with easy data analysis and visualization via iQue Forecyt® software. The latest iQue® 5 Platform features four laser options for enhanced multiplexing, individual channel gain control, reduced clogging, and user-friendly design.

Here we present several case studies demonstrating the use of HTS by cytometry to assess ABI, both as a standalone assay and within broader workflows incorporating live-cell antibody binding and function.

Materials and Methods

Cell Culture

A variety of human immortalized cells were used during these studies: NALM6 and WIL2-NS B lymphocyte, Jurkat T lymphoblast, THP-1 monocyte, AU565 and MDA-MB-468 breast cancer lines. Media formulations and materials used for cell culture and assays are described below (Tables 1, 2, and 3). For some studies, AU565 and MDA-MB-468 cells were transduced with Incucyte® Nuclight Green Lentivirus to produce stable cell lines expressing a nuclear restricted green fluorescent protein.

Cell Culture	Base medium	FBS	Penicillin-Streptomycin	Supplements
NALM6, WIL2-NS, Jurkat, THP-1, AU565	RPMI	10%	1%	_
iDC cells	RPMI	10%	1%	▪ GM-CSF 100 ng/mL ▪ IL-4 100 ng/mL
mDC cells	RPMI	-	1%	▪ GM-CSF 100 ng/mL ▪ IL-4 200 ng/mL ▪ TNF- α 20 ng/mL ▪ Ionomycin 200 ng/mL
MDA-MB-468	DMEM	10%	1%	_

Table 1. Media formulations used for cell culture.

Materials	Supplier	Cat. No.
RPMI 1640	Gibco	21875-034
DMEM	Gibco	41965-039
FBS	Cytiva HyClone™	SH30071.03
Penicillin-Streptomycin	Gibco	15140122
Ionomycin	Sigma	10634
Poly-L-ornithine Solution (PLO)	Sigma	P4957
96-well flat bottom plate	Corning™	3595
96-well V bottom plate	Corning™	3363
6-well flat bottom plate	Corning™	3516
TripleE	Gibco	12605-10
Recombinant Human Granulocyte-macrophage colony stimulating factor (GM-CSF)	Sartorius	CYK-0100-1019
Interleukin-4 (IL-4)	Sartorius	CYK-0100-1006
Recombinant Human Tumor Necrosis Factor-alpha (TNF-α)	Sartorius	CYK-0100-1004
Incucyte® Nuclight Green Lentivirus	Sartorius	BA-04888
iQue® Human Multiplex Antibody Internalization Kit	Sartorius	97048
iQue® Cell Membrane Integrity Dye (R/Red)	Sartorius	90350
iQue® Cell Membrane Integrity Dye (B/Green)	Sartorius	90342
iQue® Cell Proliferation and Encoding Dye V/Blue (Tag-it Violet™)	Sartorius	97054
Incucyte® Mouse IgG1 Fabfluor-pH orange Antibody Labelling Dye for Antibody Internalization	Sartorius	BA-04885

Table 2. Materials used for cell culture and assays.

Antibody	Supplier	Cat. No.
Anti-CD20-Ga-hIgG1	InvivoGen	Hcd20ga-mab1
Anti-CD20-hIgG4S228P	InvivoGen	Hcd20-mab14
Anti-CD20-hIgA2	InvivoGen	Hcd20-mab7
Anti-β-Gal hIgG1	InvivoGen	Bgal-mab1
Anti-human CD3	Biolegend	344801
Anti-human CD19	Biolegend	302214
Anti-human CD20	Biolegend	302302
Anti-human CD11b	Biolegend	301312
Anti-human CD54 IgG1	Biolegend	353102
Anti-human CD83 IgG1	Biolegend	305302
Anti-human CD71 IgG1	Sigma	SAB4700520
Anti-human IgG1 Isotype Control	Biolegend	400165
R-Phycoerythrin-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Human IgG	Jackson ImmunoResearch	109-116-097
Trastuzumab	Absolute Antibody	Ab00103-10.17
Kadcyla®	Midwinter Solutions	-
Enhertu®	Midwinter Solutions	-

Table 3. Antibodies used in studies.

Basic Antibody internalization assay

A pH-sensitive dye was provided as a pre-conjugated Fab fragment targeting the Fc gamma region of human IgG or mouse IgG1. During conjugation, the Fab fragment, sourced from either the iQue® kit or Incucyte® reagent (as detailed in Table 2), was combined with an isotype-matched test antibody at a molar ratio of three-to-one in cell media. This mixture was incubated at 37°C for 15 minutes. Subsequently, the antibody complex was introduced to cells for the required incubation period before being analyzed using either the iQue®3 or 5 Platform. The kit also included a reagent for evaluating cellular viability (iQue® Cell Membrane Integrity B/Green dye) and an encoder dye (iQue® Cell Proliferation and Encoding Dye V/Blue) to facilitate tracking of mixed cell populations within the same well. Cells were gated, checked for viability (if dye was included), and live cells were evaluated for an ABI response in either the red (R667) or orange channel (Y586).

Assessment of anti-CD20 biosimilar and associated isotypes

Three isotypes of a human anti-CD20 biosimilar were tested for internalization in human B and T cell lines, specifically NALM6, WIL2-NS, and Jurkat. The isotypes included anti-hCD20-hIgG1 biosimilar, hCD20-hIgA2 (natural isotype), and hCD20-IgG4S228P (engineered mutation in the hinge region). Anti-β -Gal IgG1 served as a negative control. The alternative isotypes, IgA2 and IgG4, are known to have altered effector functions compared to the IgG1 isotype. Before seeding, the cells were stained with varying concentrations of the non-perturbing Encoding Dye (V/Blue) to enable individual identification of cell populations when multiplexed in a single well. Cells were seeded at 7,000 per well per cell types (21,000 total per well) in 96-well v-bottom plates and combined with pH dye-conjugated antibodies (ranging from 0.12 to 15 µg/mL) and membrane integrity dye (B/Green). The plates were incubated in dark at 37°C for either 2 or 20 hours to analyze the internalization characteristics of each isotype. Following incubation, the cells were analyzed using the iQue® 5 VYBR Platform.

THP-1 monocyte to dendritic cell differentiation

To generate immature dendritic cells (iDCs), THP-1 cells were seeded at 250,000 cells per well in a 6-well plate pre-coated with PLO (0.01%). Differentiation was initiated by adding GM-CSF and IL-4 cytokines to the RPMI media (Table 1). For mature dendritic cells (mDCs), THP-1 cells were seeded in serum-free RPMI media, and differentiation was induced using a cytokine cocktail containing GM-CSF, IL-4, TNF-α, and ionomycin (Table 1). The cells were incubated for seven days, with the medium exchanged and fresh cytokines added every two days. The differentiation process was monitored using the Incucyte® Live-Cell Analysis System (Incucyte® System). On day seven, the supernatant was discarded, and cells were harvested using TripleE and a cell scraper to ensure effective cell lifting, followed by washing with RPMI media. The cells were then transferred to a 96-well v-bottom plate at 40,000 cells per well for the internalization assay and subsequent assessment on the iQue® Platform.

Antibodies were selected to characterize the different cell phenotypes. A positive control antibody (CD71) was included alongside a negative IgG1 isotype. For iDCs and mDCs, CD11b, CD83, and CD54 were chosen. All antibodies were pre-conjugated with the Incucyte® pH orange mouse IgG1 reagent before being added to the plated cells at a specified final test concentration (1 µg/mL). The cell plate was incubated at 37°C for 24 hours before analysis on the iQue® 5 Platform. No cell health dye was included in this assessment.

Characterization of Antibody Drug Conjugates

Three anti-HER2-hIgG1 antibodies were characterized: a Trastuzumab biosimilar, Kadcyla® (Trastuzumab emtansine; a therapeutic-grade ADC based on Trastuzumab and the chemotherapy drug emtansine (also known as DM1)) and Enhertu® (Trastuzumab deruxtecan, a therapeutic-grade ADC based on Trastuzumab and the chemotherapy drug deruxtecan (or DXd)). An Anti-β-Gal hIgG1 mAb was used as an isotype control.

<u>iQue® Live-Cell Antibody Binding</u>

Test antibodies (10 µL) were incubated with 20 µL of mixed high HER2 expressing AU565 cells and HER2 negative MDA-MB-468 cells at 7,500 cells per well in 96-well V-bottom plates for 30 minutes on ice. MDA-MB-468 cells were pre-labeled with Encoding Dye (V/Blue) to distinguish from unlabeled AU565s. Following incubation, plates were washed with PBS + 2% FBS, the media removed, and cells resuspended by shaking on the iQue® plate shaker (2,000 RPM). Fluorophore-conjugated secondary antibody (Table 3) was mixed with 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® Platform. 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.

<u>Antibody Internalization and Cytotoxicity</u>

Unlabeled target cells or cells labeled with Incucyte® Nuclight Green Lentivirus were seeded overnight in 96-well flat bottom plates at 5,000 cells per well before test antibodies were added at a range of concentrations. The antibodies that had been labeled using a pH-sensitive probe from the iQue® Human Antibody Internalization Kit (as previously described). After 3-6 days, 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® Platform. The percentage of live target cells or extend of internalization was quantified using the iQue Forecyt® software.

Results

Increased flexibility of iQue® 5 HTS platform to support enhanced workflows

The iQue® 5 HTS Platform provides enhanced flexibility to accommodate complex workflows. The addition of a fourth laser (violet, blue, yellow, red) improves fluorochrome compatibility, allowing for the use of a broader range of reagents. The iQue® Antibody Internalization Reagent (ABI reagent, pH Red) included in the iQue® Internalization Kit utilizes the red laser and is compatible with both the legacy iQue® 3 and the iQue® 5 Platforms. The introduction of the yellow laser also supports the use of the Incucyte® Fabfluor pH orange (Fabfluor pH orange reagent). Users can select either reagent on the iQue® 5 Platform to achieve comparable internalization data (as illustrated in Figure 1). Jurkat T cells or NALM6 B cells were combined with pre-complexed antibodies using either the mouse ABI reagent or the mouse pH orange reagent. The histograms demonstrate similar internalization responses for both pH red and pH orange reagents (Figure 1A and B).

Internalization quantification reveals that CD71, a generic cell marker, is active in both cell types, while a T-cell specific marker (CD3) is detected in Jurkat cells and a B-cell specific marker (CD19) is detected in NLAM6 cells as expected (Figure 1C).

Additional features of the iQue® 5 Platform include improved clog detection and management, as well as the ability to adjust gains on individual channels for greater flexibility. For this set of experiments no clogs were detected on the system across the test plates. For the components we tested for ABI assays there was no requirement for adjustment of the default gains.

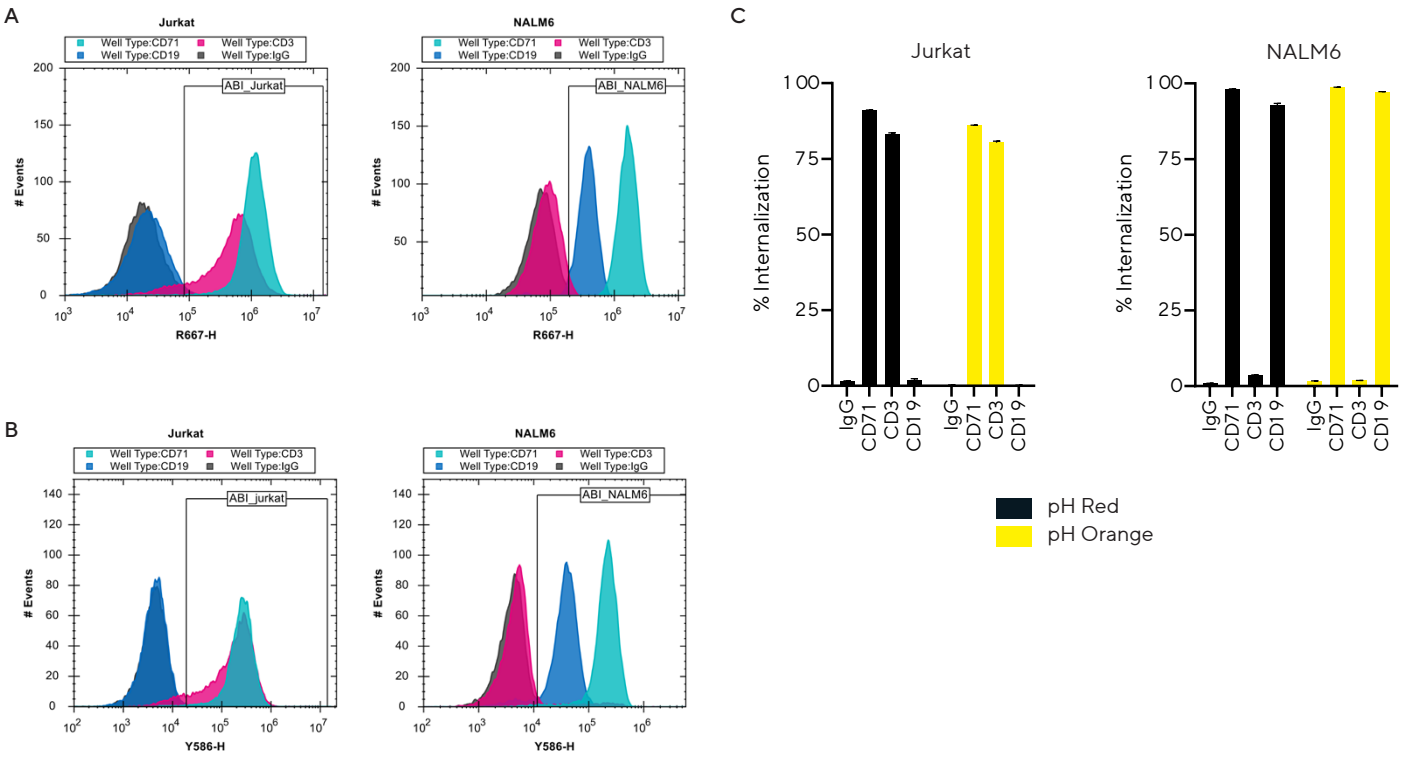


Figure 1. Increased flexibility of reagents compatible with iQue® 5 Platform. ABI data generated with various antibodies (4 µg/mL) in Jurkat T and NALM6 B cells using either (A) the iQue® mouse ABI reagent or (B) Incucyte® pH orange mouse reagent. (C) Bar graphs to show % internalization measured with each reagent. Data shown as mean ± SEM of 4 replicates.

Case study 1: Isotype testing for antibodies

In drug discovery programs modifications can be applied to antibodies to investigate their effects on antigen interaction, functional response, or physiological kinetics. Many of these modifications can be introduced through post-translational changes, such as glycosylation, which can significantly impact the response of mAbs. In this case study we modeled these effects *in vitro* using a family of modified CD20 isotype mAbs. All modifications were made in the constant region of the antibody, including a natural isotype IgA2 and an engineered mutation IgG4 (S228P), both of which result in altered effector functions.

Initially cells were evaluated for CD20, revealing that both Jurkat T and NALM6 B cells have no expression, while WIL2-NS cells exhibit high expression (Figure 2A). NALM6 and WIL2-NS cells were stained with different concentrations of encoder dye before being mixed with unstained Jurkat cells in the same well. Membrane integrity dye was added, followed by the pre-complex antibody and human ABI reagent. Cells were incubated for either 2 or 20 hours before analysis on the iQue® 5 Platform.

Singlet live cells were gated (Figure 2B), and the different cell populations were identified using the encoder dye (Figure 2C). Each cell type was assessed for an internalization response by gating based on the isotype negative response (Figure 2C). At 2 hours, only the CD20-positive WIL2-NS cells showed an ABI response (23% for the IgG1 isotype), meanwhile the CD20-negative cell types did not show any response even at the highest concentration tested (15 µg/mL, Figure 2D). By 20 hours, the response in WIL2-NS cells increased to 57%, with no specific response observed in Jurkat or NALM6 cells. WIL2-NS cells displayed a concentration-dependent increase in ABI with both the native IgG1 and the IgG4 isotype, but no response with the IgA2 isotype. Although both IgG4 and IgA2 are reported to have impaired antibody-dependent cellular cytotoxicity and phagocytosis functionality, only the IgA2 exhibited a reduced response in this internalization assay.

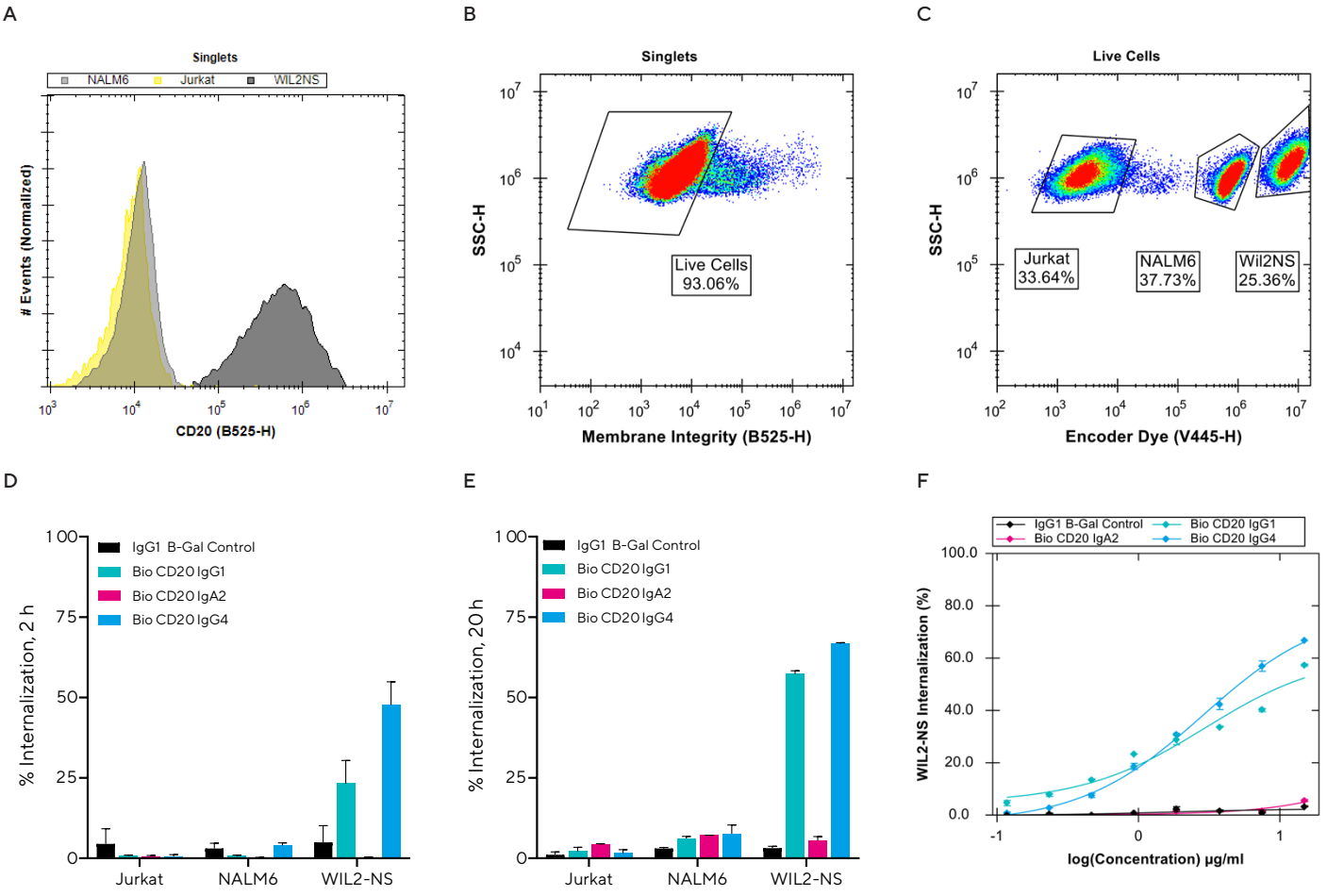


Figure 2. Multiplex assay to investigate antibody isotypes. ABI data generated with different anti-CD20 isotypes in a multiplexed cell assay workflow. CD20 expression profile of test cells (A). Gating strategy showing live cells (B) and encoder populations (C) at 2-hour time point. Internalization responses across cell types and test antibodies (15 µg/mL) at 2 (D) and 20 hours (E). Concentration response curves for CD20 isotypes in WIL2-NS cells at 20 hours (F). Data shown as mean ± SD of 2 replicates from representative experiment.

Case study 2: Characterization of Monocyte to Dendritic Cell Differentiation

Dendritic cells (DCs) and macrophages play a crucial role in modulating tissue immunity, leveraging their abilities in phagocytosis, cytokine production, antigen presentation, and immune cell activation for adoptive cell therapies. This case study used antibody internalization to examine changes in surface marker expression as THP-1 monocytes differentiated into either immature or mature DCs.[6,7] After seven days in cytokine cocktails, THP-1 cells exhibited distinct morphologies: iDCs were rounded, and proliferating, while mDCs appeared flatter, more stellate suggesting increased adherence and reduced proliferation (Figure 3A). The reduced proliferation in mDCs resulted in the collection of fewer events in the final ABI assessment.

When internalization of 5 antibodies was examined, a diverse profile emerged. At 24h, undifferentiated THP-1 cells were positive only for the generic cell marker CD71, showing a clear response in 96% of cells (Figure 3B). iDCs internalized CD71 (93%), CD11b (23%), CD83 (15%), and CD54 (46%), though the response to CD54 was heterogeneous, indicated by a broad peak. mDCs exhibited a stronger internalization response, with distinct peaks shifted compared to negative control for all test antibodies, indicative of a mature phenotype (ABI >60%). Bar graphs illustrate response profiles using two metrics: MFI for peak response position, and % internalization via a specific response gate (Figure 3C). The data indicates that while both immature and mature DCs show internalization with all antibodies to varying degrees, the peak fluorescence response shows greater differences compared to controls in mature DCs.

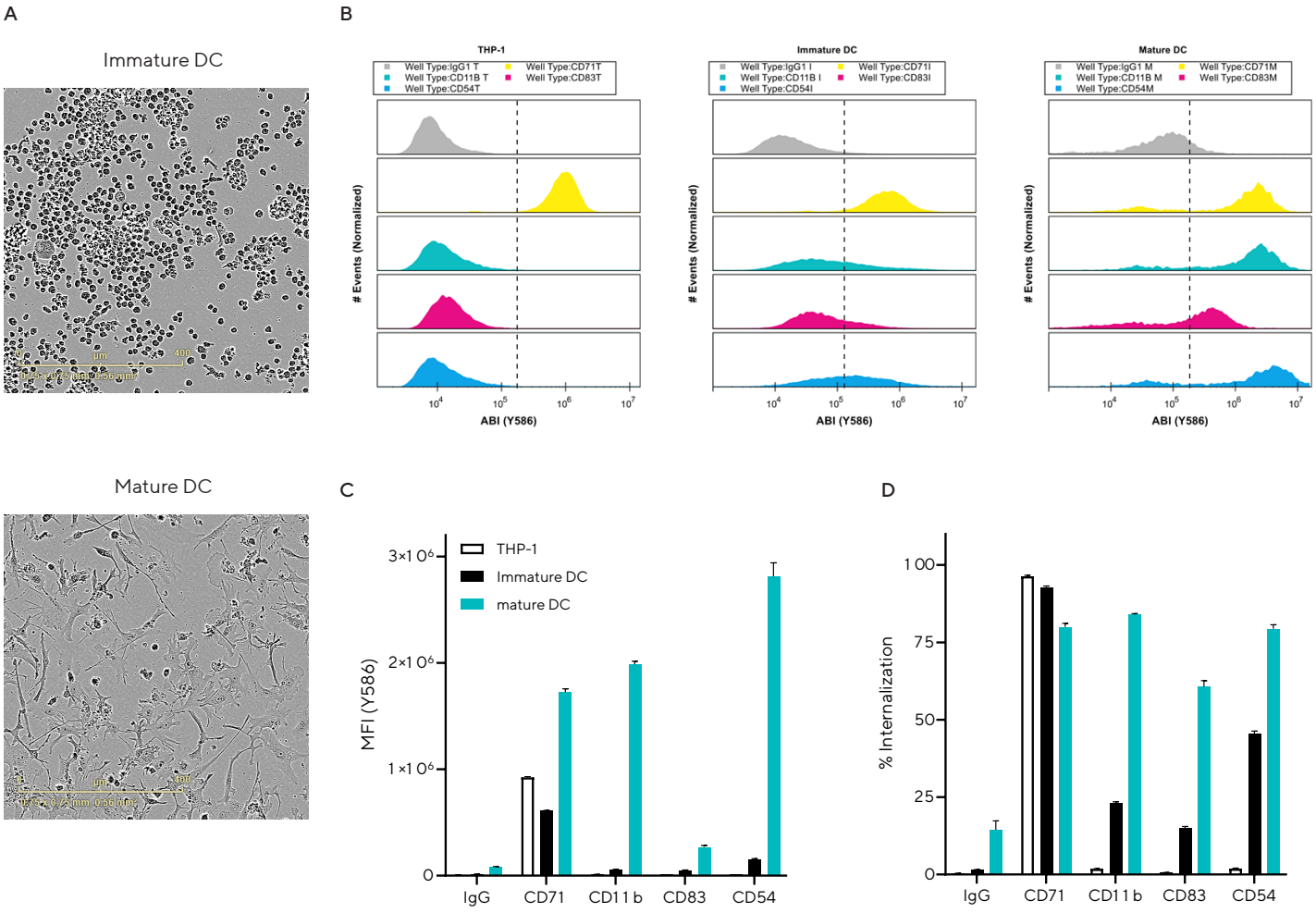


Figure 3. – Phenotypic Characterization of Dendritic Cells. Images show morphological differences post-differentiation (A). Histograms display the different ABI profiles for THP-1 monocytes, iDC and mDCs for a range of test antibodies (B). Dotted line represents internalization gate position. Bar graphs show MFI (C) or internalization (D) responses profiles across cell types. Data shown as mean ± SEM of 4 replicates.

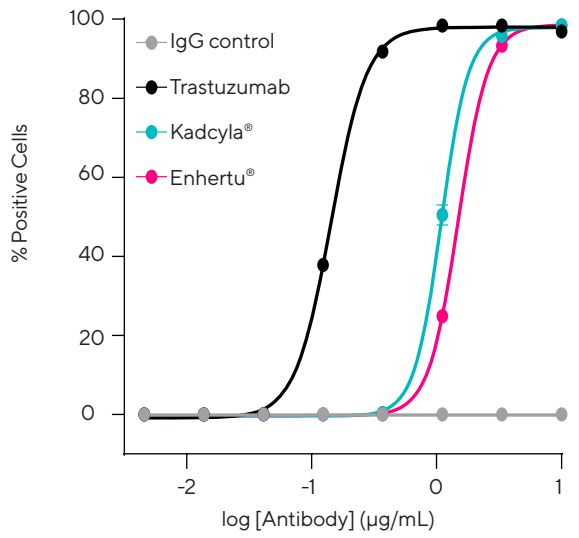
Case study 3: Characterization of Antibody Drug Conjugates

This case study examines the binding and internalization characteristics of three related antibodies: Trastuzumab, Kadcyla®, and Enhertu®. Trastuzumab is an anti-HER2 mAb, while Kadcyla® and Enhertu® are ADCs derived from Trastuzumab. Despite sharing a common backbone, they differ structurally. Kadcyla® carries the cytotoxic payload emtansine (DM1) [8] and uses a non-cleavable linker, whereas Enhertu® is linked to deruxtecan (Dxd) [9] with a cleavable linker. [10]

During ADC development, maintaining high specificity and affinity in antigen binding is crucial. The iQue® Platform was used to generate live-cell binding curves and potency values (Figure 4A and B). Trastuzumab showed higher potency in high HER2-expressing AU565 cells with an EC_{50} of 0.14 µg/mL, while Kadcyla® and Enhertu® had comparable EC_{50} values of 1.1 and 1.5 µg/mL, respectively. None of the antibodies bound to HER2-negative MDA-MB-468 cells, demonstrating their specificity.

ADC internalization into antigen-expressing cells is vital for cytotoxic payload delivery, followed by payload release through linker degradation or lysosomal metabolism, targeting antigen-expressing cells and minimizing off-site toxicity. Using the iQue® Human ABI Kit, AU565 cells were incubated with antibodies labeled with the pH-sensitive dye. After six hours, Kadcyla® and Enhertu® showed comparable internalization with EC_{50} values of 0.75 and 0.93 µg/mL, respectively (Figure 4C). The EC_{50} for internalization of Trastuzumab was 0.68 µg/mL, but its maximal internalization was lower, with only 46% of cells above the MFI threshold compared to 94% and 96% for the ADCs.

A



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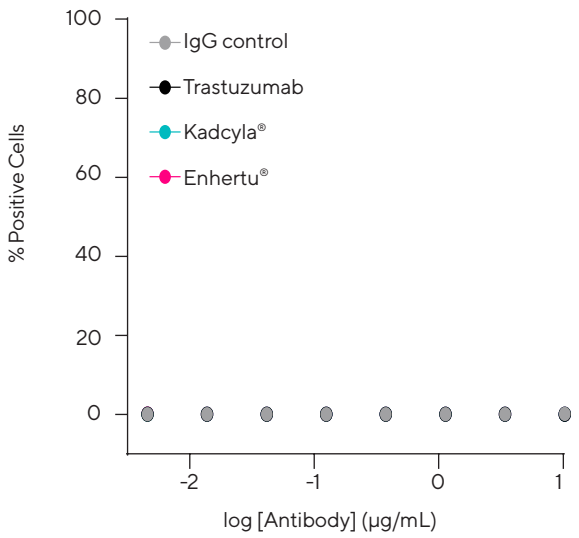

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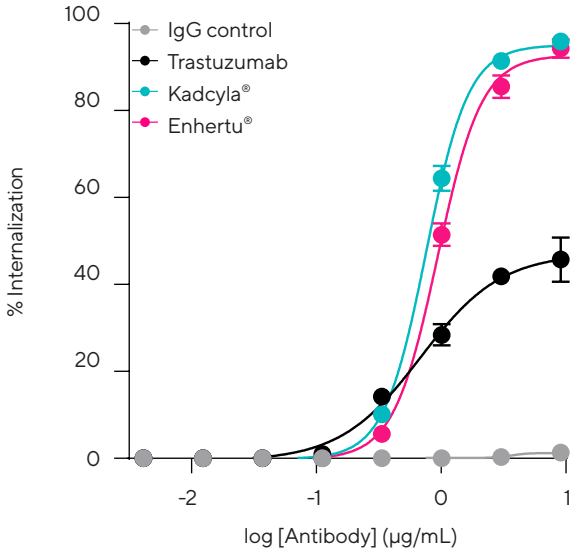

D

Cell Culture	Binding EC_{50} (µg/ml)	ABI EC_{50} (µ/mL)
Trastuzumab	0.14	0.68
Kadcyla®	1.1	0.75
Enhertu®	1.5	0.93

Figure 4. Binding and Internalization Assessment of Trastuzumab, Kadcyla® and Enhertu®. (A, B) Curve showing % of AU565 and MDA-MB-468 cells above a defined MFI threshold for fluorophore-conjugated secondary antibody binding to test antibodies as measured using iQue® direct live-cell binding assay. (C) Antibodies were labeled with the pH sensitive dye from the iQue® Human ABI kit then added to AU565 cells alongside a membrane integrity dye. The percentage of live cells above a defined MFI threshold for internalization after 6 hours is plotted. (D) Table of binding potency and EC_{50} values for internalization for each antibody. Data shown as mean ± SEM of 3 replicates.

ADC cytotoxicity was assessed after a four-day treatment, using a membrane integrity dye and iQue® Platform for cell count analysis (Figure 5). Nuclight Green Cells were used, which enabled a combined selection of green cells that were negative for the membrane integrity dye (Figure 5A). After four days, high HER2 expressing AU565 cells showed increased cell death with both ADCs, with Kadcyla® having a two-fold lower EC_{50} (0.075 µg/mL) compared to Enhertu® (0.16 µg/mL). Trastuzumab had minimal impact on AU565 cell growth, highlighting that without the addition of the cytotoxic payload, Trastuzumab activity is reliant on the presence of immune cells to cause death. HER2-negative MDA-MB-468 cells showed minimal death, reinforcing ADC specificity (Figure 5B).

A further study explored Enhertu's unique "bystander" activity, detailed in another application note.

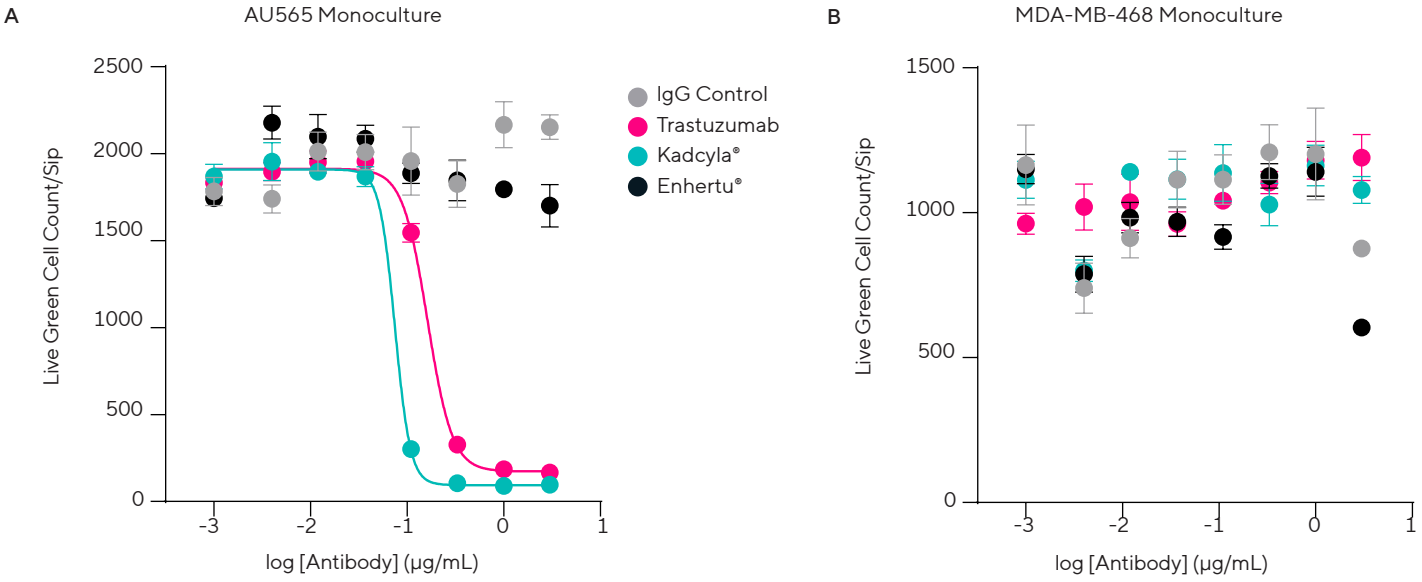

Figure 5. anti-HER2 ADCs induce death of high HER2 expressing cells. Incucyte® Nuclight Green Lentivirus labelled cells (high HER2 expressing AU565s or HER2 negative MDA-MB-468s) were seeded in monoculture with a range of concentrations of antibody. After 96 hours, cells were lifted, labeled with viability dye and analyzed using the iQue®. Graphs show iQue® counts of live, green (A) AU565 and (B) MDA-MB-468 cells. Data shown as mean ± SEM of 3 replicates

Conclusion/Summary

The iQue® HTS Cytometry Platform proves to be a versatile tool in the study of ABI, offering enhanced capabilities for complex workflows. Through three different case studies, including the characterization of ADCs and monocyte differentiation, the platform's ability to measure ABI efficiently is demonstrated. The addition of multiple laser lines and improved data analysis features enhances reagent compatibility and workflow flexibility. ADCs, which leverage ABI for targeted cancer treatment, show promising therapeutic potential, as evidenced by the FDA-approved options targeting both solid tumors and hematological malignancies. The research underscores the importance of optimizing ABI-related factors, such as antigen affinity and intracellular trafficking, for successful mAB development. Overall, the iQue® Platform's advanced features facilitate comprehensive assessments of antibody binding, internalization, and cytotoxicity, contributing to the advancement of targeted cancer therapies.

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