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Application Note

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A High Throughput Multiplexed Antibody Internalization Assay

Carrie Weldon and John O'Rourke
Essen Bioscience, Inc., formerly IntelliCyt Corporation, Part of the Sartorius Group, Albuquerque, NM 87111, USA

Introduction

Antibodies represent a growing class of drugs with therapeutic applications in oncology, chronic inflammation, and autoimmune diseases[1]. The beneficial, and often dramatic therapeutic effects of antibodies are the result of their high specificity and affinity to target antigens, and ability to directly affect cell physiology and immune-mediated functions. However, the binding of antibodies to their targets may also be associated with unwanted downstream toxicities, such as cytokine release syndrome (CRS)[2]. Efficacy can also be influenced by other factors such as internalization, drug transport from the intracellular compartment to the cytosol, and capacity to elicit the desired response. Multifaceted characterization of antibodies for therapeutic optimization proves challenging due to small sample size and low throughput of methods such as flow cytometry and confocal microscopy.

Introduction (continued)

Antibody internalization and associated kinetics are critical characteristics that impact antibody therapeutics and pharmacokinetic profiles and influence the mechanism of action (MOA) of antibody drugs.[3] For example, antibody-drug conjugates (ADCs) may require rapid internalization to deliver their payload directly into cells to avoid systemic dissemination. However, rapid antibody internalization can also be detrimental to immune-mediated killing mechanisms such as antibody dependent cell cytotoxicity (ADCC)[4] or complement-dependent cytotoxicity (CDC)[5]. Antibody design and function becomes particularly important in the context of discovering underlying reasons for drug resistance, even when a viable lead has been identified.[6]

During biologic drug discovery, antibodies are engineered to increase their biological potencies; to customize configurations (i.e. antibody fragments or bi-specific); to optimize conjugations to non-protein components; and to increase serum half-life. These modifications can have profound effects on antibody activity and specificity, as well as antibody internalization. This necessitates thorough in vitro, high throughput screening of antibodies during the early discovery process to quickly identify the most suitable drug candidates for further development.

In vitro antibody screening, performed by traditional methods such as FACS platforms, are labor intensive, require considerable training, have limited throughput and requires large amounts of reagents. Likewise, microscopy techniques have limited throughput due to acquisition time.[7]

The ability to quickly profile and compare large sets of antibodies and characterize their effects on targets for key attributes, such as antibody internalization within the same sample, could vastly reduce the time required for lead generation and expedite the development of potential therapeutic candidates.

Assay Principle

This application note describes a high throughput, multiplexed, no-wash assay that measures antibody internalization, antibody specificity, and cell health from a single, 10 µL sample. This assay is amenable to profiling large numbers of antibodies in 384-well plate formats. Antibodies are easily labeled with the novel, pH-sensitive dye Intellicyt® Antibody Internalization Reagent. The labeled antibodies have little fluorescence at neutral pH, but become highly fluorogenic at low pH when they are internalized and processed through the acidic lysosome/endosome pathway. Cell viability can also be measured using MultiCyt® Membrane Integrity Dye (Intellicyt) to assess general cell health and antibody function. Using the same sample, cell specificity can be characterized using encoding dyes (cell lines) or directly conjugated fluorescent antibodies (cells). The MultiCyt® QBeads PlexScreen Secreted Protein Assay Kit (Intellicyt) can also be incorporated to perform more detailed antibody assessments on the sample, such as cytokine release. Data acquisition is performed using the Intellicyt® iQue Screener PLUS platform, which combines high throughput sampling, flow cytometry detection, and plate-level analytics that deliver rich content with sampling times of less than 20 minutes for a 384-well plate. The integrated ForeCyt® software enables data analysis and visualization.

As illustrated here, the Intellicyt platform provides a comprehensive, integrated solution that rapidly profiles antibody internalization and other critical antibody characteristics using small sample volumes, and analyzing multiple cell types within the same well. The combination of non-perturbing and validated reagents for multiplexing, no-wash protocols, high throughput capabilities, flexibility for robotic interface, and integrated software for multi-parametric data analysis and visualization can accelerate antibody discovery, development, and screening of antibody drug candidates for potential drug efficacy and toxicity.

General Methods and Materials

Cell Culture and Reagents
Jurkat cells (human T-cell line) and Raji cells (human B-cell line) (ATCC) were grown in RPMI-1640 + 10% FBS (Life Technologies). Test antibodies were purchased from BioLegend (human CD3, CD19, CD22, and CD45) and Sigma (human CD20, CD71, and control mouse IgG). Antibody Internalization Reagent, MultiCyt® Membrane Integrity Dye (B/Green), Violet Encoding Dye (V/Blue), and MultiCyt® QBeads PlexScreen Secreted Protein Assay Kit were from Intellicyt. Mouse FabFluor-488 Green labeling reagent (IncuCyte) and Dynole® 2-24 (Abcam) were used for internalization inhibition experiments. CD3, CD14 and CD20 antibodies (BioLegend) were used for the immunotyping studies.

Antibody Labeling and Reagent Preparation
The test antibodies (3X final assay concentration) were labeled at a molar ratio of 1:3 in growth media with Intellicyt® Antibody Internalization Reagent. For temperature inhibition experiments they were labeled with FabFluor-488 Green and incubated for 15 minutes at 37° C. An 8 point, 2-fold dilution series was created for validation assays with a top antibody concentration of 1 µg/mL. For additional studies, a 12-point, 2-fold dilution series was used with a top antibody concentration of 8 µg/mL. MultiCyt® Membrane Integrity Dye (B/Green) was prepared at a final assay concentration of 3X. For encoding studies, Raji cells (2 million/mL in PBS) were added 1:1 to Violet Encoding Dye and incubated for 15 minutes at room temperature. Cells were washed, centrifuged and re-suspended at 3X concentration in media. Unstained Jurkat cells were prepared at a 3X concentration in media.

Antibody Internalization Experiments
Cells were added into 384-well, v-bottom plates at a final concentration of 106 cells/mL along with Internalization Reagent-conjugated antibodies and Membrane Integrity Dye in a final volume of 30 µl. All samples were done in triplicate. Data was acquired 2 hours after addition of conjugated antibodies. For temperature inhibition experiments, cells and Internalization Reagent- or FabFluor-488 Green-conjugated CD71 antibody were cooled to 4° C before incubation and held at 4° C for 2 hours prior to data acquisition. For chemical inhibition experiments, cells were re-suspended in serum-free media before treatment with either 1% DMSO (vehicle control) or 10 µM Dynole 2-24 in 1% DMSO. The Internalization Reagent-conjugated CD71 antibody was then added and incubated at 37° C for 2 hours prior to data acquisition. For the time course experiments, data were acquired every hour for 4 hours, and the plate was returned to the incubator between acquisitions.

Data Acquisition and Analysis
Data was acquired on the Intellicyt® iQue Screener PLUS using 1 second sips corresponding to approximately 2000 cells/well. Dose response curves and EC50 calculations were automatically generated using ForeCyt software. Antibody internalization was assessed in the RL1 channel (675/30 nm), cell viability in the BL1 channel (530/30 nm) and encoded cells in the VL1 channel (445/45 nm). This panel was chosen to eliminate the need for color compensation in this assay. The BL1 channel was also used for measuring FabFluor-488 Green-labeled antibody in the temperature inhibition experiments.

Primary Cell Specificity and Cytokine Detection
PBMCs from two donors (Astarte Biosciences) were cultured overnight in RPMI-1640 with 10% FBS, 1% Non-Essential Amino Acids, 1% Sodium Pyruvate, and 1% Pen/Strep. Cells were prepared at a 4x concentration before assay. A 12-point dilution series of Internalization Reagent-conjugated antibodies (IgG1, CD45, CD19 or CD22) was prepared with a top concentration of 8 µg/mL. Equal volumes of cells, Internalization Reagent-conjugated antibodies, MultiCyt® Membrane Integrity Dye (B/Green) and media were added to each well for a final volume of 40 µL. The plate was centrifuged briefly to ensure liquid was at the bottom of the well, mixed for 10 seconds at 3000 rpm, and incubated at 37°C. After 2 hours, 10 µL of the cell assay was removed to a fresh 384-well plate for cytokine analysis. Ten microliters of an optimized cocktail of fluorescently labeled antibodies were added to the cells to identify T-cells (CD3), B-cells (CD20), and monocytes (CD14), and the cells were incubated for an additional hour at room temperature. Data were acquired on the Intellicyt iQue Screener PLUS. A 10-plex panel of QBeads® (Intellicyt; IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IFNγ, TNFα, and GM-CSF) was used for relative quantitation of the PBMC supernatants.

Results

Proof of Concept

As proof of concept studies, Jurkat and Raji cells were incubated with various concentrations of Antibody Internalization Reagent labeled anti-CD71 (positive control) or mouse IgG1 (negative control) and cells were analyzed 2 hours post antibody addition. Jurkat and Raji cells were first gated for viability, followed by assessment for antibody internalization. Gating on viable cells dramatically reduced the background of the antibody internalization signal. As seen in Figure 1A, the median fluorescent intensity (MFI) of antibody internalization (RL1) was plotted versus antibody concentration. For both Raji and Jurkat cells, a concentration-dependent increase in MFI was observed for the anti-CD71 treated population but little to no signal was measured in the negative control (mIgG1). The percent of cells positive for antibody internalization for each cell type was calculated using ForeCyt software (Figure 1B). CD71 was highly expressed on the cell surface and nearly 100% of all cells were positive for CD71 antibody internalization at the low antibody concentrations. However, MFI levels did not plateau at the highest antibody dose, suggesting that saturating CD71 antibody levels were not achieved.

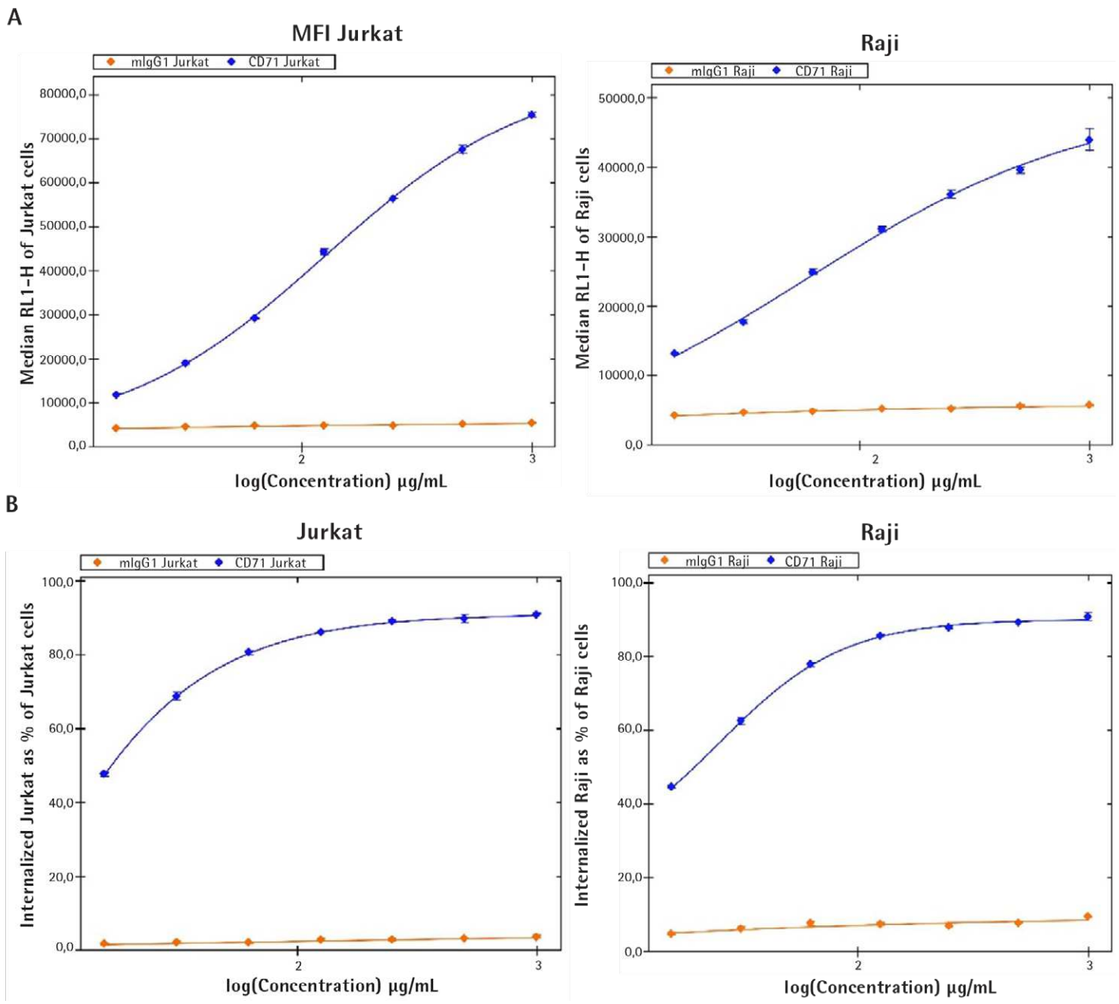


Figure 1. Antibody Internalization Controls. (A) MFI and (B) percent positive at a single, 2-hour time point. Dose response curves for an 8-point serial dilution of Internalization Reagent-conjugated mouse IgG1 (negative) or anti-CD71 (positive) with a top concentration of 1 µg/mL. For both Jurkat and Raji cells, dose dependent internalization was observed for anti-CD71 but not for mIgG1. Differences in MFI between the cell types may reflect differences in the amount of cell surface CD71.

Inhibition of internalization was demonstrated in Jurkat cells using temperature as well as the endocytosis inhibitor Dynole 2-24 (Figure 2). When incubated 4°C for 2 hours, Internalization Reagent-labeled anti-CD71 showed little to no internalization (RL1), as compared with the 37°C control (Figure 2A). The FabFluor-488 Green labeled anti-CD71 showed consistent, high levels of CD71-positive cells (BL1) in both 37°C and 4°C conditions (Figure 2B), indicating receptor binding irrespective of internalization. FabFluor-488 is not pH dependent, and remains green whether inside or outside the cell. When treated with Dynole 2-24, little to no concentration- dependent increase in MFI (RL1) was observed, as compared with the vehicle control (Figure 2C).

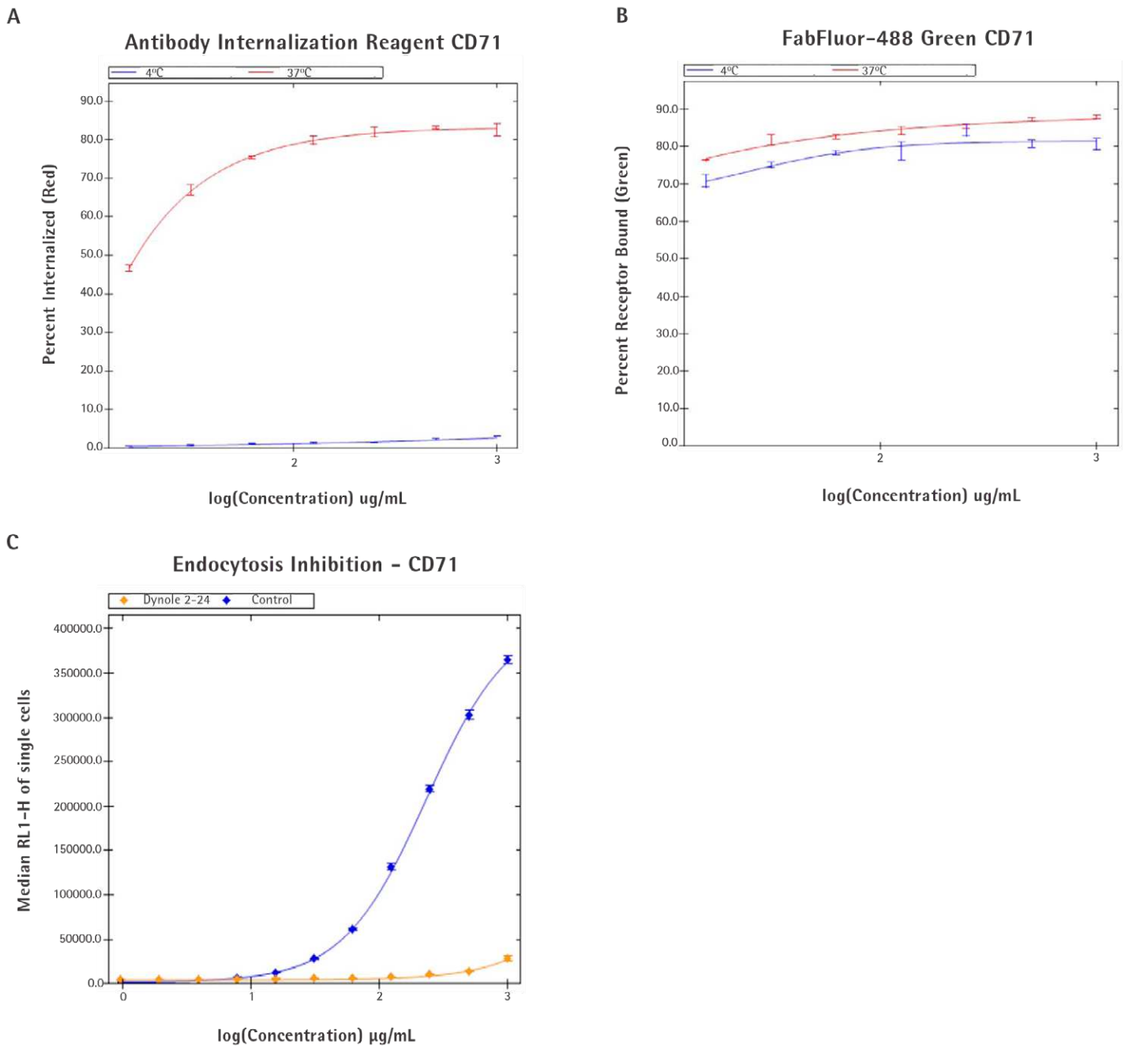


Figure 2. Antibody Internalization Inhibition. (A) Antibody Internalization Reagent- and (B) FabFluor-488 Green-labeled anti-CD71 incubated with Jurkat cells in 4°C and 37°C conditions, and (C) Dynole 2-24 treated and control Jurkat cells. An 8-point serial dilution with a top concentration of 1µg/mL of Internalization Reagent-labeled CD71 antibody demonstrated a dose-dependent increase in the percent cells internalized for the 37°C condition only. The FabFluor-488 Green labeled CD71 antibody showed no difference in the percent green between cold and warm conditions, indicating receptor binding independent of internalization. Treatment with the endocytosis inhibitor Dynole 2-24 showed near-complete inhibition of internalization of the Internalization Reagent-labeled anti-CD71 as compared with the vehicle control.

To investigate the specificity of antibody internalization, Jurkat T-cells or Raji B-cells were incubated with anti-CD3 (T-cell marker), anti-CD19 or anti-CD22 (B-cell markers) or the mIgG negative control. As seen in Figure 3, Jurkat cells showed rapid, concentration-dependent internalization of anti-CD3, but not anti-CD19 or anti- CD22. Raji cells rapidly internalized anti-CD19 and anti-CD22, but not anti-CD3.

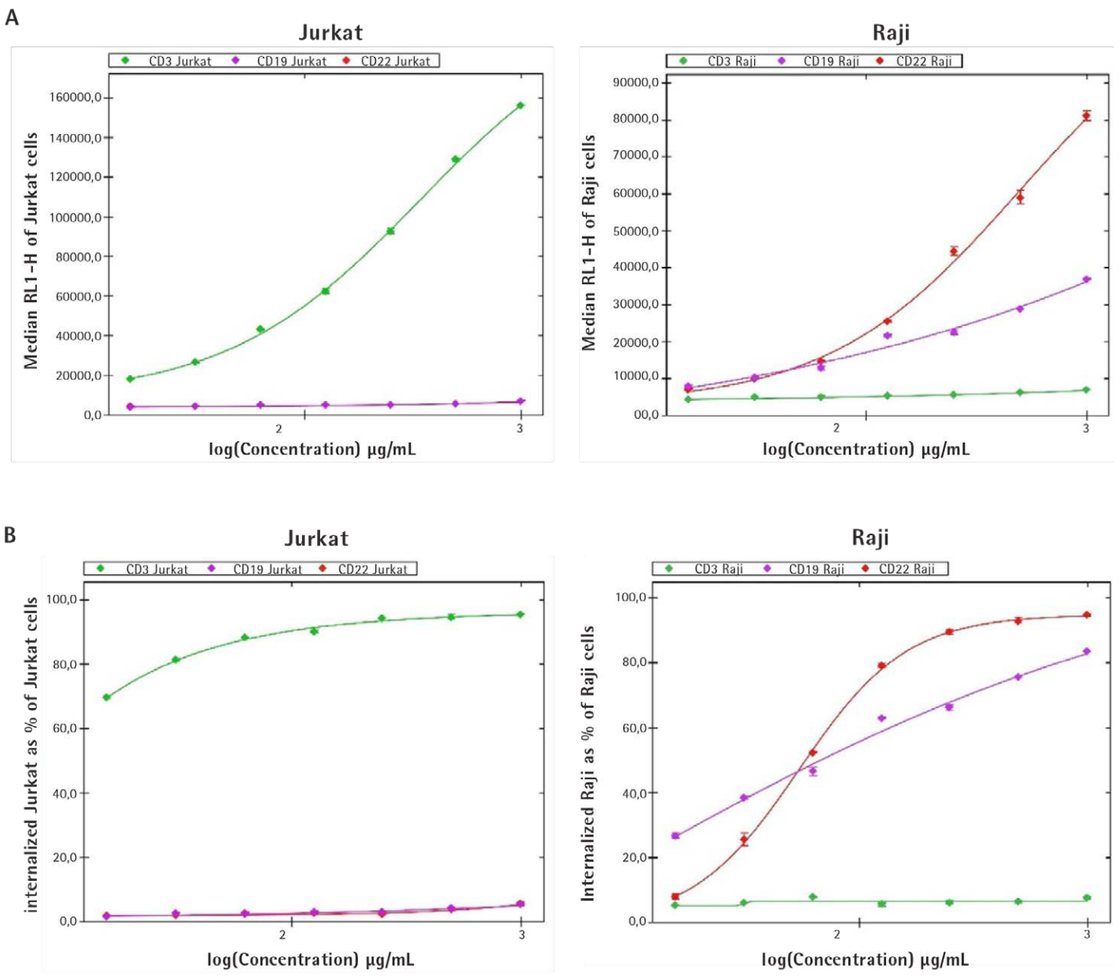


Figure 3. Antibody Internalization Specificity. (A) MFI and (B) percent positive at a single, 2 hour time point. Dose response curves for an 8-point serial dilution of Internalization Reagent-conjugated anti-CD3 (T-cell marker), anti-CD19, or anti-CD22 (B-cell markers) with a top concentration of 1 µg/mL. Jurkat cells (a T-lymphocyte cell line) showed a dose-dependent increase in internalization of anti-CD3, but not the two B-cell markers. Conversely, Raji cells showed a dose-dependent increase in internalization of anti-CD19 and anti-CD22, but not anti-CD3.

We next investigated the time course of antibody internalization. Jurkat and Raji cells were incubated with MultiCyt® Membrane Integrity Dye (B/Green) and the Internalization Reagent-labeled antibodies as indicated. The antibody range was increased 8-fold, and the assay plate was measured at 1, 2, 3, and 4 hours post antibody addition.

Antibody concentrations as high as 8 µg/mL did not achieve an EC50 max, but there was a slight increase non-specific antibody internalization. While a small increase in MFI was observed over time, internalization with all tested antibodies was nearly complete by 1 hour (Figure 4).

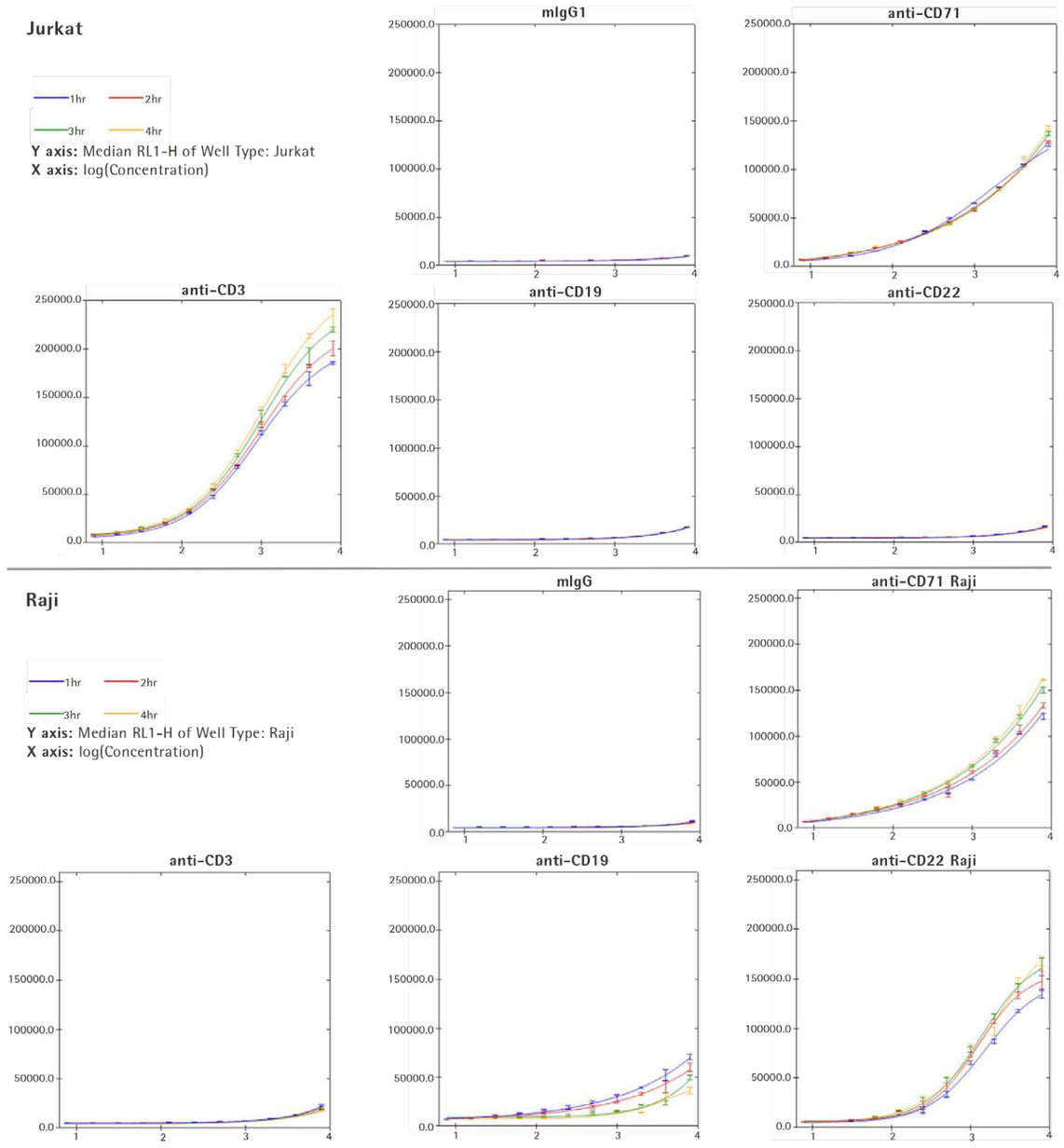


Figure 4. Specificity Time course with expanded antibody dosage. Jurkat top and Raji bottom. A 12-point serial dilution of each of the Internalization Reagent-conjugated antibodies with a top concentration of 8 µg/mL, MFI measured at 1, 2, 3, and 4 hour time points. Only slight difference in antibody internalization (MFI) was observed in the dose response curves between 1 and 4 hours.

Assaying multiple cell types in the same sample well
Site-directed mutagenesis, or other antibody engineering techniques, may alter antibody specificity, necessitating assays confirming antigen specificity. As a proof of concept, multiplexed studies were performed using barcoded cell lines. Raji cells were stained with violet encoding dye and mixed with unstained Jurkat cells, then incubated with antibodies as described above. Figure 5A, shows the gating strategy. First, viable cells were identified, then Raji and Jurkat cells were spectrally separated on the VL1 channel.

Antibody internalization was subsequently assessed for each cell line. Dose response curves were generated for the specificity markers for each cell type (Figure 5B). Jurkat cells showed internalization of anti-CD3, but not anti-CD19 or anti-CD22, whereas the Raji cells internalized anti-CD19 and anti-CD22, but not anti-CD3. Importantly, little difference was observed when the cells were assessed for internalization alone or when mixed, showing that multiplexing does not interfere in the antibody internalization assay.

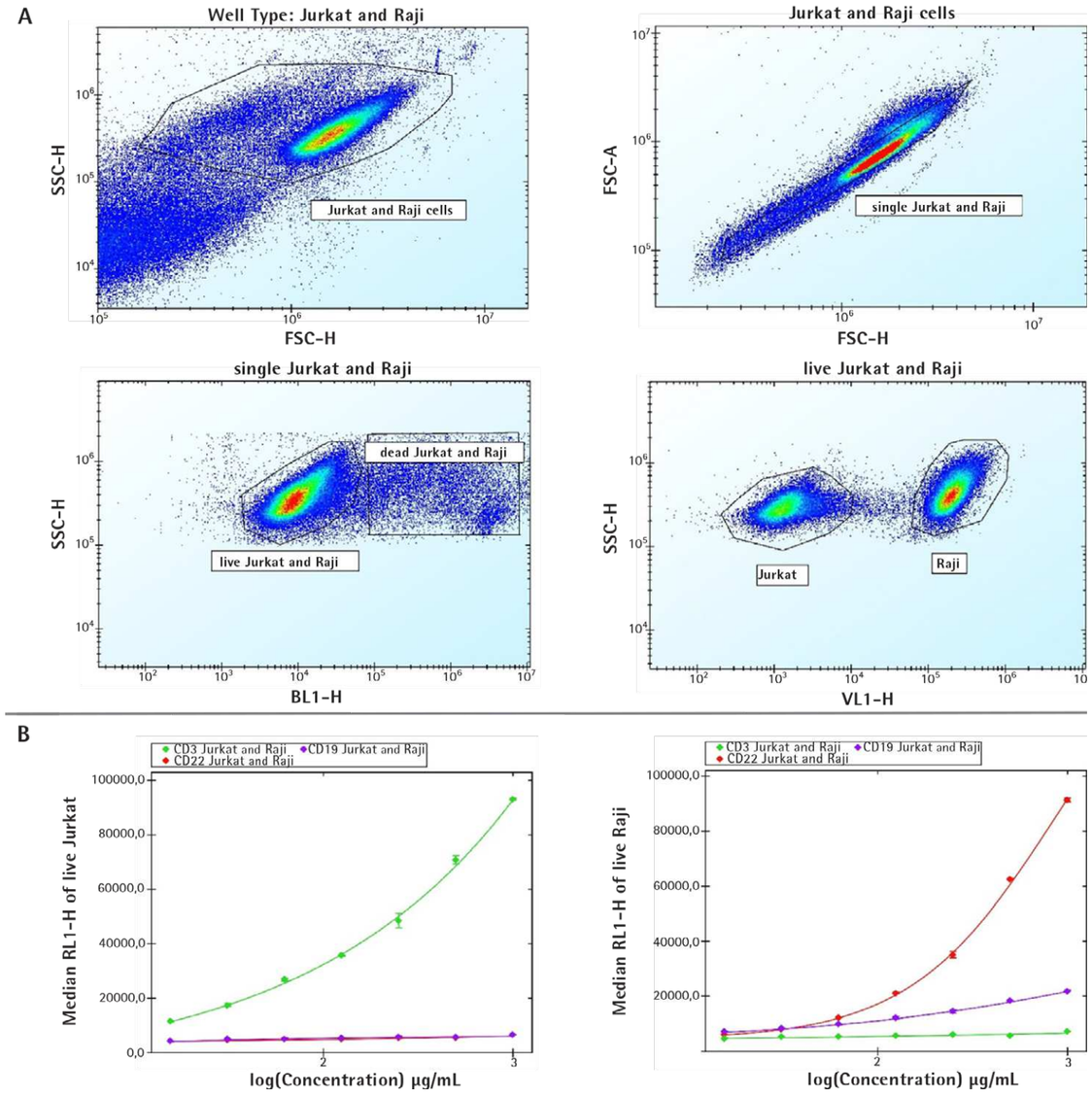


Figure 5. Multiplexing with encoder dye. Violet-encoded Raji cells and unstained Jurkat cells in the same sample well with a 8-point serial dilution of Internalization Reagent-conjugated specificity antibodies with a top concentration of 1 µg/mL, MFI at a single, 2 hour time point. **(A)** Gating strategy used to separate the two cell types. **(B)** Antibody internalization for mixture of cell types shows the same specificity and relative MFI as when run separately.

Primary Cell Internalization and Cytokine Release Assay
More complex immune modeling systems can yield increased information during target and non-target specific antibody internalization. As a proof of concept, we used a PBMC model to measure the specificity of antibody internalization and subsequent cytokine release. PBMCs from two donors were mixed with viability dye and an increasing amounts of the indicated antibodies. Two hours after antibody addition, a 10µL aliquot of the cell assay was transferred to an assay plate for cytokine analysis using QBeads®. A cocktail of fluorescent antibodies were added to characterize specific PBMC subsets. Viable cells were then separated into T-cell, B-cell and monocyte populations by gating on CD3, CD20 or CD14 positive cells (Figure 6A). Antibody internalization was determined for each cell population and dose response curves for each of the Internalization Reagent-conjugated antibodies were generated (Figure 6B). Antibodies against the pan-hematopoietic marker CD45 were internalized in all cell types whereas the B-cell markers (anti-CD19 or anti-CD22) were only internalized in the B-cell population. CD14 positive monocytes from both donors showed uptake of all antibodies, including the mouse IgG negative control. CD14 on monocytes can bind the Fc portion of IgG with subsequent internalization.

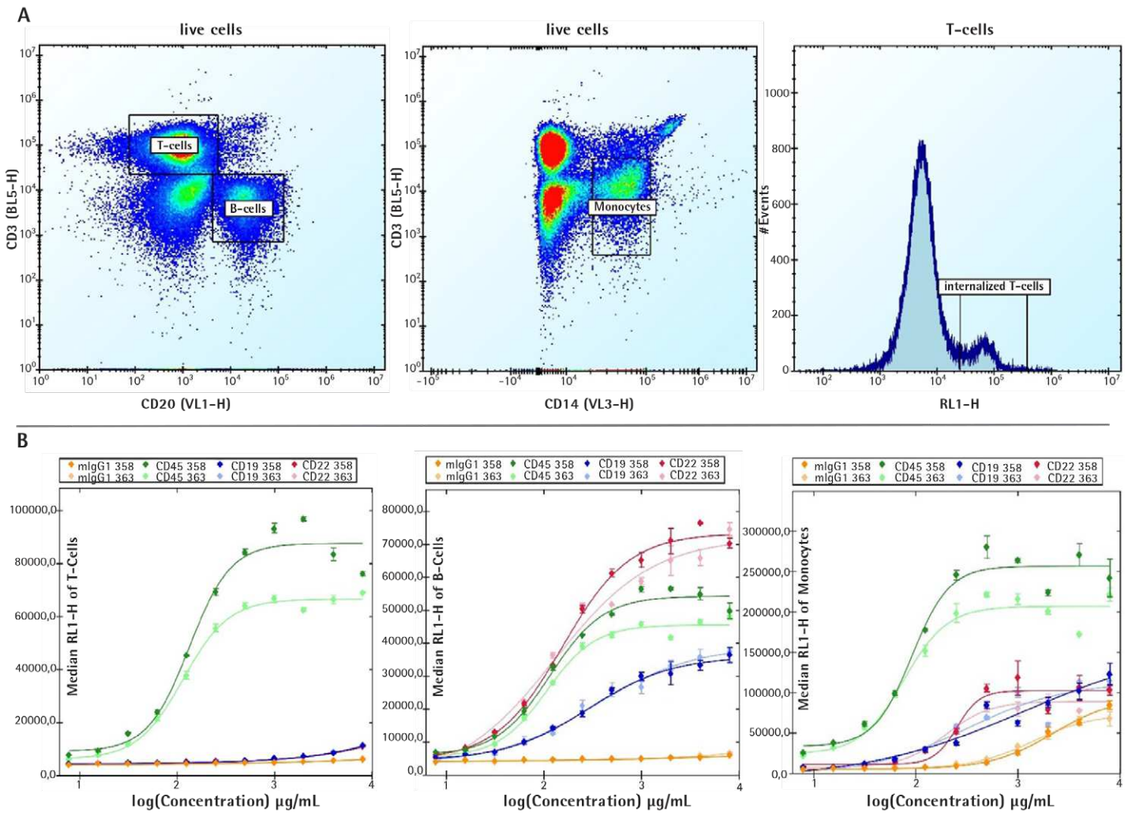
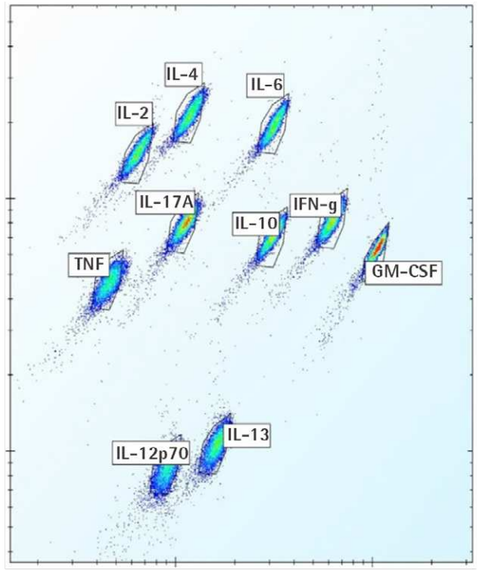


Figure 6. Primary Cell (PBMC) Specificity. PBMCs from two different donors (358 and 363) were incubated for 2 hours with a 12-point dilution series of Internalization Reagent-conjugated mouse IgG1 (negative control), anti-CD45 (positive control), anti-CD19 (B-cell specific), or anti-CD22 (B-cell specific), and BL1 viability dye. A cocktail of fluorescently labeled anti-CD3 (T-cell positive, BL5), anti-CD20 (B-cell positive, VL1), and anti-CD14 (macrophage positive, VL3) was added to the cells to identify the different cell types and incubated for another hour at room temperature before acquisition on the Intellicyt iQue Screener PLUS. (A) Gating strategy used to separate the cell types. After gating on viable cells, cell types were separated using fluorescence and antibody internalization was assessed. (B) Dose response curves were generated for each cell type. Cell type specific antibody internalization was observed in both PBMC samples, consistent with previous experiments, with little difference between donors. Monocytes showed a dose-dependent, non-specific antibody internalization.

To assess the possible association of antibody binding and cytokine release, a 10-plex QBeads® panel was used to measure the relative levels of cytokines in each sample (Figure 7A). Analysis of the cytokine levels revealed an antibody specific and dose-dependent increase in IL-6 (~ 5-fold) and TNF (~300-fold), with no increase observed for the negative control mouse IgG1 (Figure 7B). The other cytokines monitored showed no change with increasing antibody binding.

A



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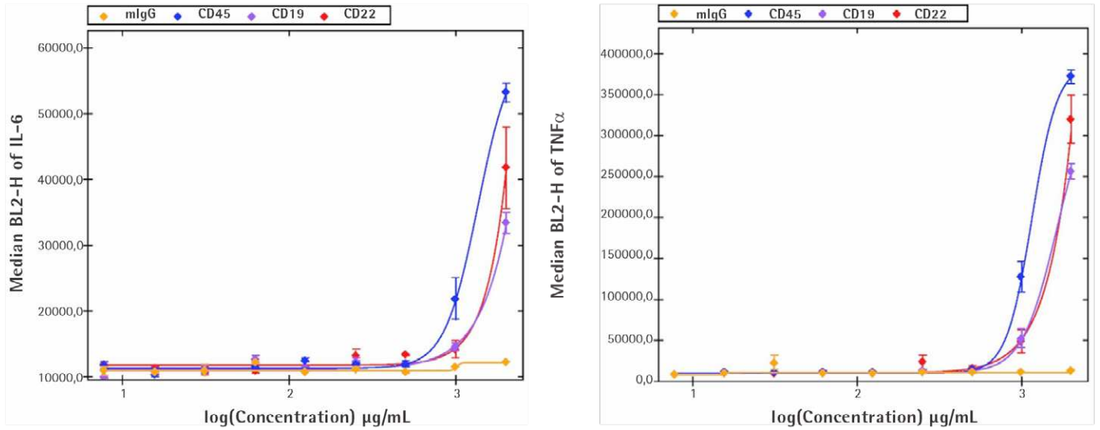

Figure 7. Cytokine detection in PBMC. 10 µl of cell assay from the PBMC internalization study were transferred to a fresh 384-well plate and cytokine levels were measured using a QBeads® assay (A) A 10-plex QBeads® kit was used to measure cytokine levels. (B) IL-6 and TNFα showed an antibody specific and dose-dependent increase, while other monitored cytokines remained unchanged with increasing antibody dose.

Conclusions

- This assay enables the rapid (2-3 hours), simultaneous measurement of important antibody characterization profiles, such as antibody internalization and viability using 10 µL sample volumes.
- Specificity can be demonstrated with either encoding dye or by immunotyping.
- The protocol can be easily multiplexed with other MultiCyt® reagent kits, and QBeads® for measurement of cytokine analysis.
- The validated reagents from Intellicyt coupled with no-wash protocols permit biologically relevant assessments with minimal disturbance to the integrity of antibodies and cells.
- The Intellicyt iQue Screener PLUS platform and associated data analysis and visualization tools are able to rapidly profile antibody internalization in a system that is amenable to high throughput screening for antibody discovery, characterization, and development.

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Essen Bioscience, Inc.,
formerly IntelliCyt Corporation,
Part of the Sartorius Group

5700 Pasadena Ave. NE
Albuquerque, NM 87113
USA

www.intellicyt.com
Email: info@intellicyt.com

USA +1.505.345.9075
fax: +1.866.782.3140