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A High Throughput, Multiplex Antibody Internalization Assay

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Introduction

Many fluorescence based methods are available to assess internalization; however, performing these assays can prove challenging due to complicated workflows, large sample sizes and the low throughput of analytical methods such as traditional flow cytometry or confocal microscopy. To address the challenges of antibody internalization (ABI) screening and profiling studies, an easy to use, mix-and-read pH-sensitive ABI reagent for use with the iQue® platform has been developed (Figure 1). We describe a high throughput, multiplex, no-wash assay that measures ABI, specificity, and cell health from a single 30 µL assay volume in 96- and 384-well formats. Antibodies are easily labeled with the novel, pH-sensitive ABI reagent. The labeled antibodies have little fluorescence at neutral pH, but become highly fluorogenic at low pH when internalized and processed through the acidic lysosome/endosome pathway. Cell viability is simultaneously measured using cell membrane integrity dye, and cell specificity is characterized using encoding dye. Data acquisition is performed using the iQue 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.

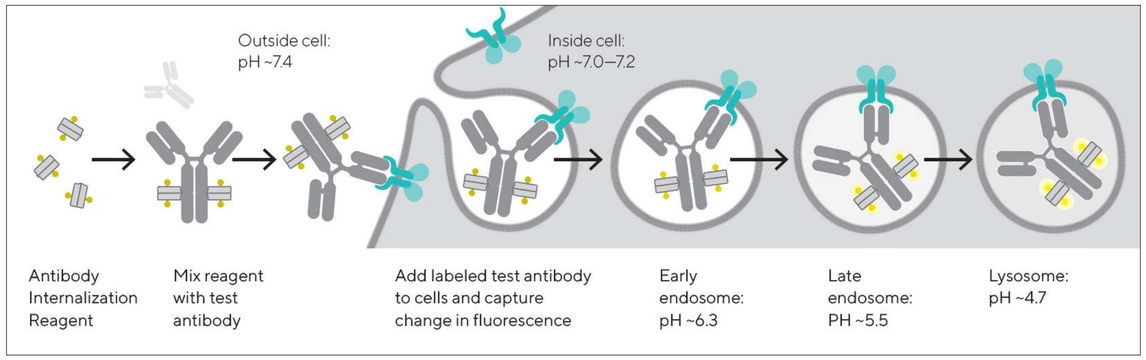

Figure 1: The pH-sensitive fluorescent probe principle. A novel pH-sensitive fluorescent probe enables one-step, no-wash labeling of isotype matched antibodies. A fluorescent signal is generated as internalized antibody is processed into the acidic endosome and lysosome pathway.

1. Experimental approach

To demonstrate the multiplexing capabilities of this novel pH-sensitive dye on the iQue platform, we used Ramos and Raji cells stained with two intensities of violet encoding dye, combined with unstained Jurkat cells. We incubated this for three hours with a serial dilution of dye-conjugated specificity antibodies: isotype-matched anti-CD3 (T cell marker), anti-CD19, anti-CD20, anti-CD22, or anti-CD79b (B cell markers), anti-CD71 (positive control), and IgG (negative control), then added a cell membrane integrity dye before acquiring data. Using this strategy, we were able to identify viable cells, then spectrally separate Ramos, Raji, and Jurkat cells. We then assessed antibody internalization for each cell line. Serial dilution curves were generated for the specificity markers of each cell type (Figure 3).

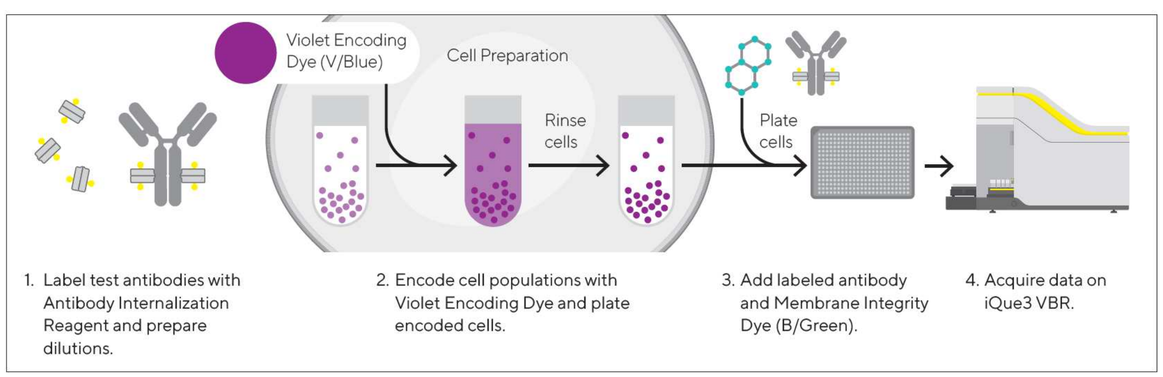

Figure 2: The assay consists of 3 components, each 10 µL: labeled test antibody, cells (encoded or not), and Cell Membrane Integrity Dye (B/Green). Each component is prepared at 3X before addition for a final concentration of 1X and an assay volume of 30 µL.

2. Results

As expected, 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. Only Ramos cells showed a concentration-dependent increase in internalization of anti-CD79b, an ADC drug target for non-Hodgkin's lymphoma. In the three-hour assay time frame, we did not observe anti-CD20 internalization, but we did see an increase in the two B cell lines by 24 hours (data not shown). Importantly, we saw little difference when we assessed the cells for internalization alone or when we mixed them. This result shows that multiplexing positive and negative cell lines does not interfere with the ABI assay. Compared to performing a series of singleplex assays, a multiplexed assay approach enables you to analyze multiple readouts (internalization, viability, cell type) from a single well, decreasing the number of tests needed to perform a comprehensive functional characterization of the antibody candidate.

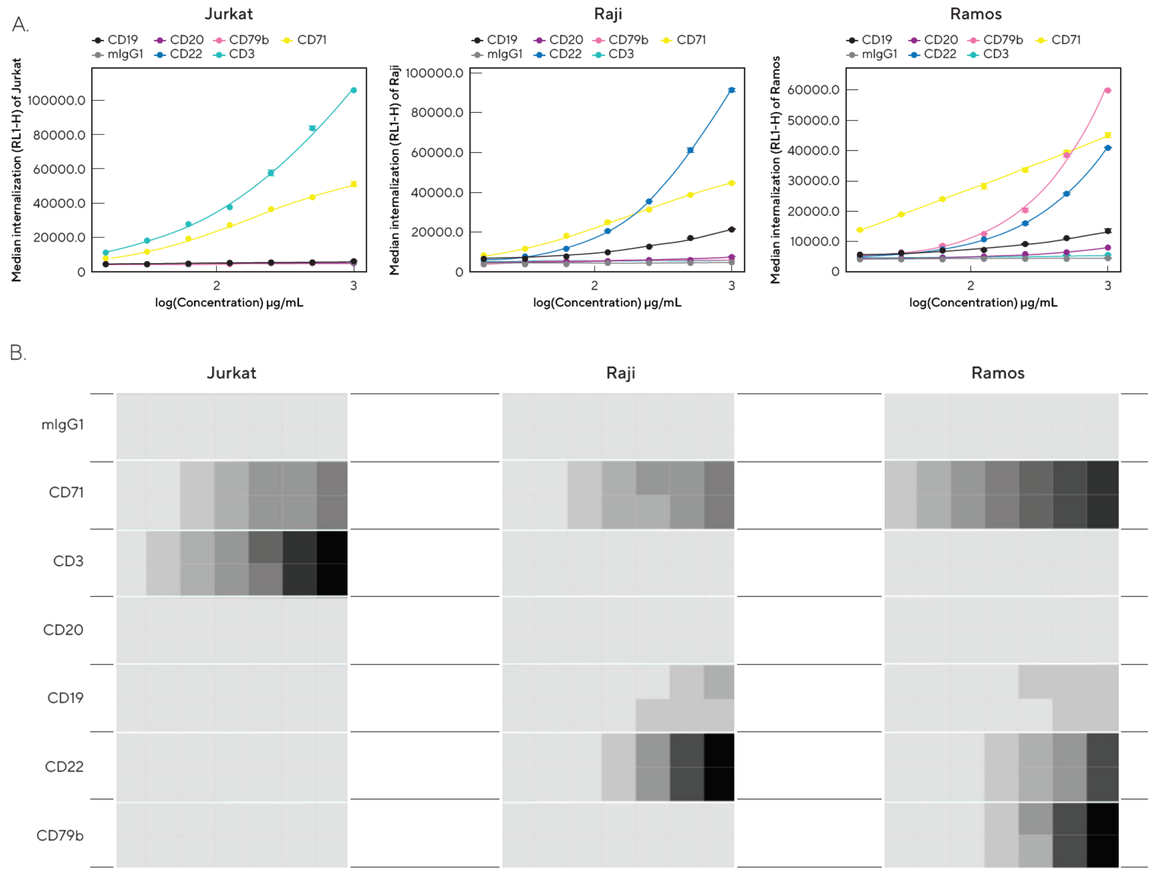

Figure 3: (A.) Serial dilution curves for internalization-labeled antibodies with a top concentration of 1 mg/mL in different cell types after a three-hour incubation. (B.) Multiplexed positive and negative cell lines may be used in an ABI assay to generate high-content data in one assay. Median fluorescent intensity (MFI) for Internalization Reagent-labeled antibodies after three hours. A serial dilution of each antibody with a top concentration of 1 mg/mL was prepared and incubated with encoded Jurkat, Raji, and Ramos cells in the same well. Jurkat cells (a T lymphocyte cell line) show a concentration dependent increase in internalization of anti-CD3, but not the two B cell markers. Conversely, Raji cells show a concentration-dependent increase in internalization of anti-CD19 and anti-CD22, but not anti-CD3. Ramos cells show a concentration-dependent increase in internalization of anti-CD79b, an ADC drug target for non-Hodgkin's lymphoma.

3. Conclusions

- iQue platform and integrated Forecyt® software provide an integrated solution that rapidly profiles antibody internalization and other critical antibody characteristics using data analysis and visualization with plate-level analytics to accelerate antibody discovery, development, and screening of antibody drug candidates for potential drug efficacy and toxicity
- Uses a novel, pH sensitive dye to identify antibody internalization in a simple, plate-based format
- Rapid, simultaneous measurement of internalization, specificity (using encoding dye or phenotyping antibodies), and viability using 10 µL sample volume
- Flexibility to combine with other validated reagents (such as Qbeads) for multiplexed, no-wash, high throughput capabilities