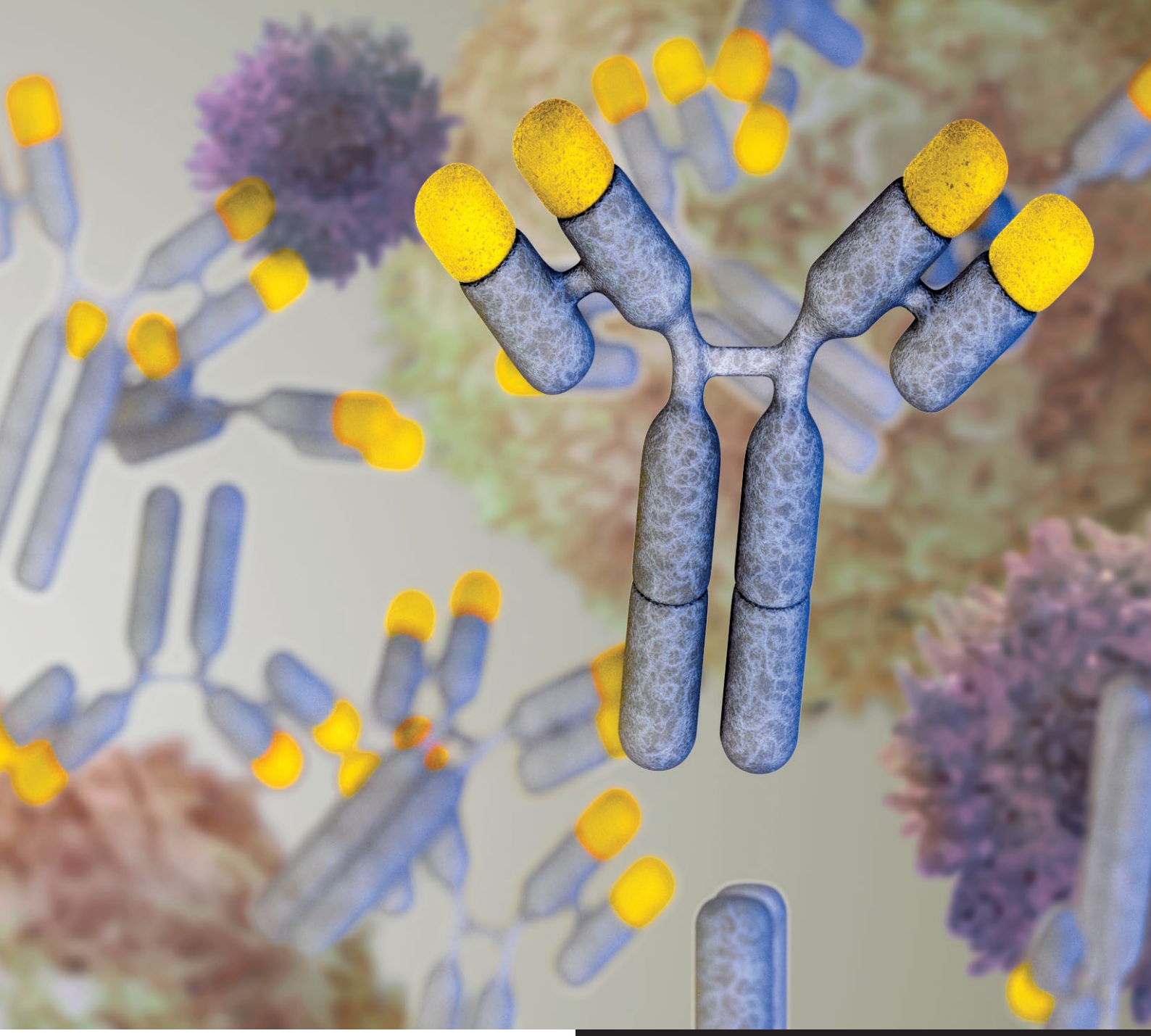

Intellicyt® iQue3

Accelerated Antibody Discovery Application Compendium

Simplifying Progress

SARTORIUS

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Faster, Smarter Flow Cytometry to Advance Therapeutic Antibody Discovery

Introduction

Monoclonal antibodies (mAbs) still remain a focus of biopharmaceutical research. In the discovery process, you need to make crucial decisions, such as selecting your best clones and identifying your best drug candidates. This can be challenging, especially when using traditional screening methods.

One such traditional screening method is the enzyme-linked immunosorbent assay (ELISA). ELISA has historically been used to screen hybridomas and other libraries for antibody binding to extracellular targets. Color change from an indirect enzyme/substrate reaction shows antibodies that bind to the immobilized antigen.

ELISAs have historically been a staple of antibody screening labs, but they have several disadvantages:

- ELISAs are not ideal for screening antibodies that bind to cell surface antigens because these antigens must be extracted from the cell membrane and purified, which often leads to disruption of conformational epitopes that can be important targets for therapeutic antibodies.
- Testing a single antigen may require secondary and sometimes tertiary screens with control antigens to confirm specificity and/or cross-reactivity.
- ELISA protocols are labor intensive because multiple wash steps are required to remove or minimize unbound antibodies and detection reagents that give a background signal, resulting in longer screening workflows.

In addition, ELISA or Bio-Layer Interferometry (BLI) methods only evaluate immunoglobulin G (IgG) titer; they tell you nothing about the actual cells you are testing. Cells can appear to be productive because of high IgG secretion, but they may not be the most healthy and vigorous cells you want to take forward into the next step of your cell line generation process.

Multiplex High-Throughput Screening for Antibody Discovery With the Intellicyt® iQue3 Advanced Flow Cytometry Platform

Recent advances in assay technology, however, are beginning to transform antibody discovery. These advances give you the ability to perform high-throughput, multiplexed, and multiparameter assays. Using advanced assays allows collection of more data early in the screening process, which builds your confidence in potential hits, and increases the likelihood that you choose candidates that will successfully proceed through downstream steps in antibody development.

The Intellicyt® iQue3 advanced flow cytometry platform (Figure 1) is a suspension-based, high-throughput screening system that can be used to perform multiplexed screens of antibody binding to either cell surfaces or circulating target antigens. In addition, the iQue platform allows high content assays to evaluate the effects of your lead candidates in multiplexed cell-based and secreted protein assays.

This application compendium shows how you can advance and accelerate antibody discovery by illustrating some of the applications of Sartorius' groundbreaking iQue platform.

Your benefits for mAb development when using the iQue platform include:

- Multifactorial and actionable results for more insight and better decisions
- Reduction in the potential for error and, thus, experiment time
- Quick and efficient evaluation of critical productivity attributes for each clone

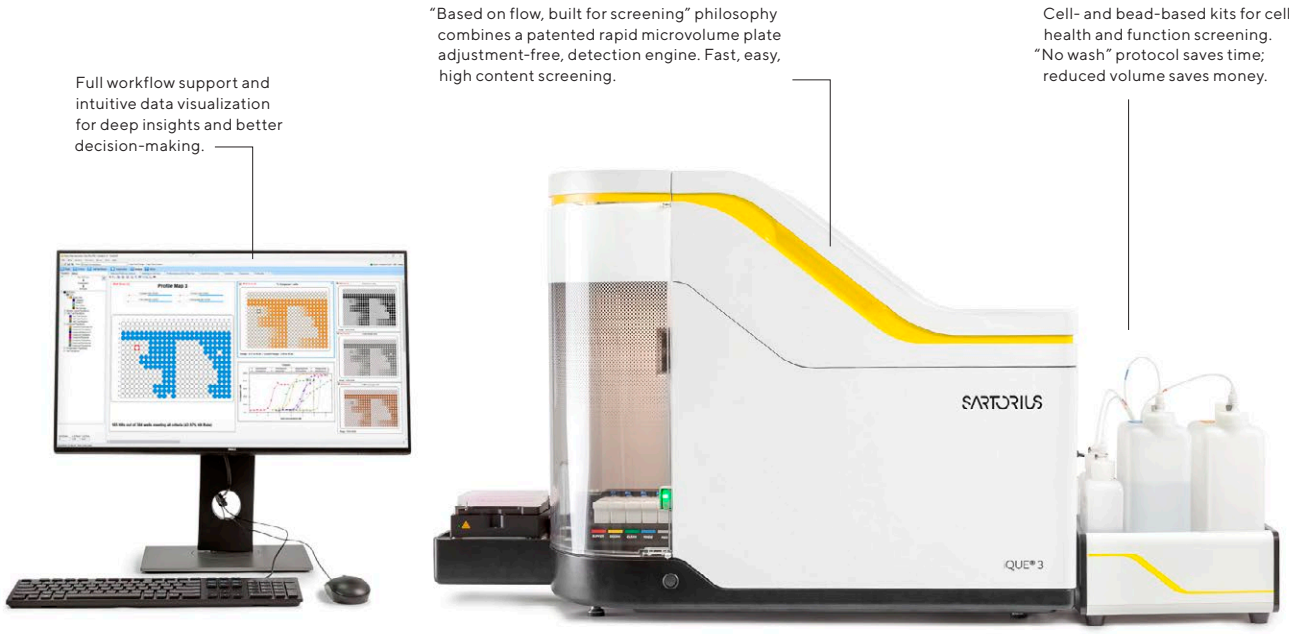

Figure 1: The Intellicyt® iQue advanced flow cytometry platform. The iQue platform features easy-to-use instruments, software, and reagent kits that are optimized to work together and designed to conserve precious samples, use less reagent, and minimize time-to-answer.

Rapid, Microvolume, High-Throughput Sampling Technology

The Sartorius iQue platform's rapid, microvolume, high-throughput sampling technology based on flow cytometry delivers rich content with sampling times of 5 minutes for a 96-well plate and under 20 minutes for a 384-well plate. An entire plate of data is processed at one time because samples are delivered to detectors in a patented air-gap delimited stream.

Powerful Multiplexing Approach Through Encoding Technology

The iQue advanced flow cytometry platform performs high-throughput assays with cells and beads in suspension, enabling a powerful multiplexing approach through encoding technology. With encoding technology, scientists can combine multiple populations of cells or beads bearing different antigens of interest into the wells of assay plates, and screen against multiple target antigens, in the same experiment. This greatly increases the power and robustness of antibody screening campaigns and allows for an enhanced screening workflow (Figure 2).

Why Encode?

1. **Simultaneously test antibodies for binding to target and control antigens.** This provides an internal reference control within each well, which improves confidence in hits. Also, including control antigens in primary screens can lessen the need for secondary counter screens, which streamlines the overall antibody screening workflow.

2. **Screen for cross-reactivity to related antigens.** Encoding allows scientists to include multiple antigens in their primary screens to search for cross-reactive antibodies that bind to families of target antigens such as cytokines. This technique can also be used to screen for antibodies that bind to a target antigen from multiple animal species. This is important when developing therapeutic candidates specific for human target antigens that can also be used in animal models of toxicity and efficacy.

3. **Combine multiple projects into a single screening campaign.** Encoding can also be used to screen a single library against multiple unrelated antigens. Assay plates can be set up containing encoded cells or beads presenting different antigens of interest. Multiple antibody libraries can then be tested in the same screen, greatly improving the overall workflow.

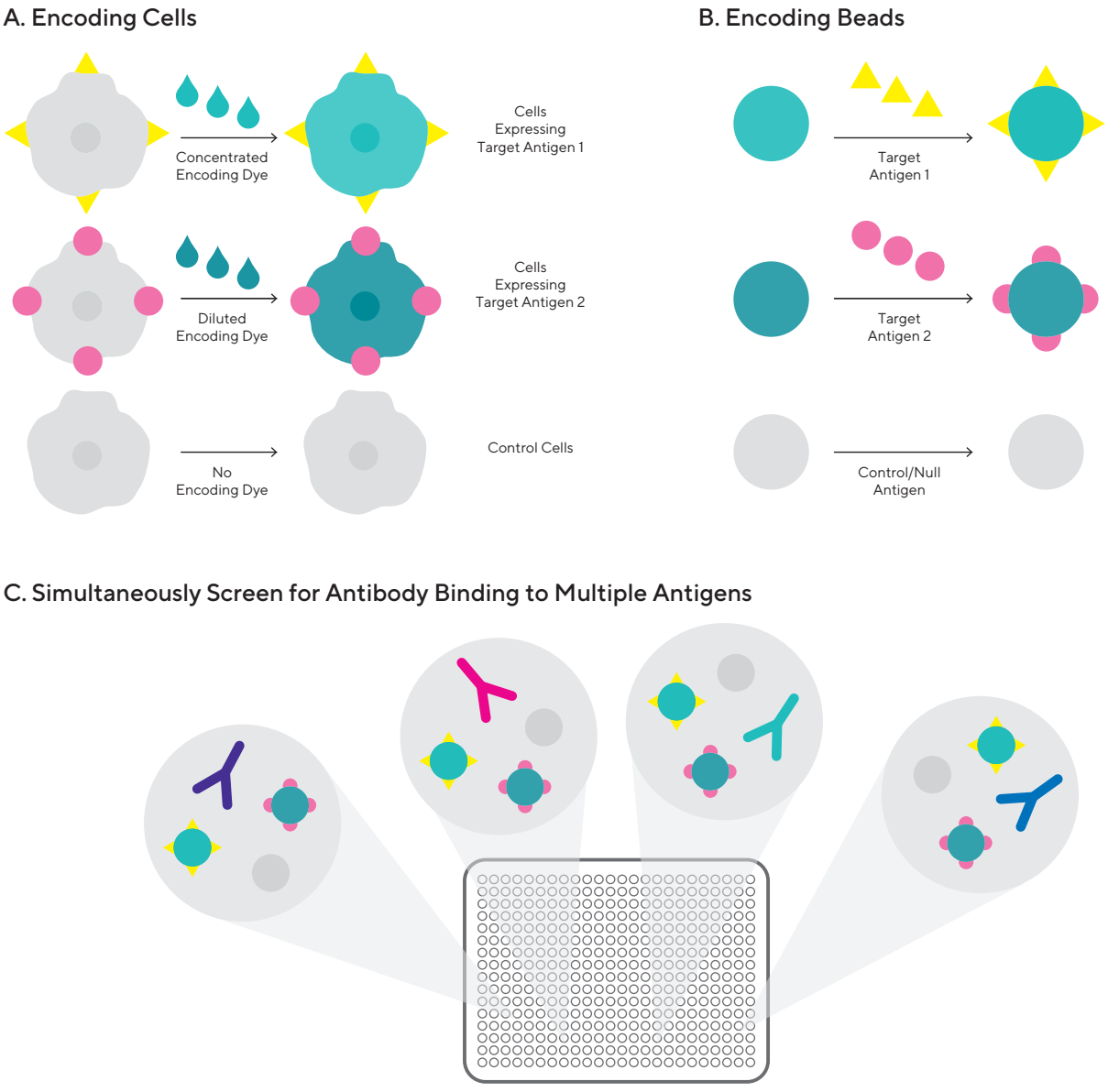

Figure 2: Encoding technology for cell- and bead-based antibody screening. Cellular and soluble antigens can be multiplexed with encoding technology. For cellular antigens, cells expressing the various homologs are encoded with dye (A). Soluble antigens are attached to dye-encoded beads (B). The encoded cells or beads are combined and added to the screening plate (C) which contains the antibody library, and binding of the antibodies to the different antigens is measured.

For a real-world case study of how encoding technology enabled scientists at XOMA to screen for antibodies that bind to a target antigen from multiple animal species, see **Multiplexed Antibody Characterization for Preclinical Testing in Animal Models** below.

Integrated Software Solution

The iQue advanced flow cytometry platform includes ForeCyt® Software to produce actionable IgG clone data by generating dynamic visualization tools such as plate heat maps, histograms, plots, dose response curves, and profile maps. A multiplate analysis feature, called Panorama, generates an analytical "big picture" that automatically compares, identifies, and ranks the IgG clones at the screening campaign level (Figure 3) across multiple plates of an experiment, as well as multiple plates in an experiment over several days. In addition, criteria threshold slider bars adjust data on the fly for real-time "what if" analyses of plate data with the click of a mouse. Panorama provides a capability to instantly dial in an optimized set of IgG clones to move forward, eliminating the weeks or months of loading, reloading, and recalculating data required by legacy platforms.

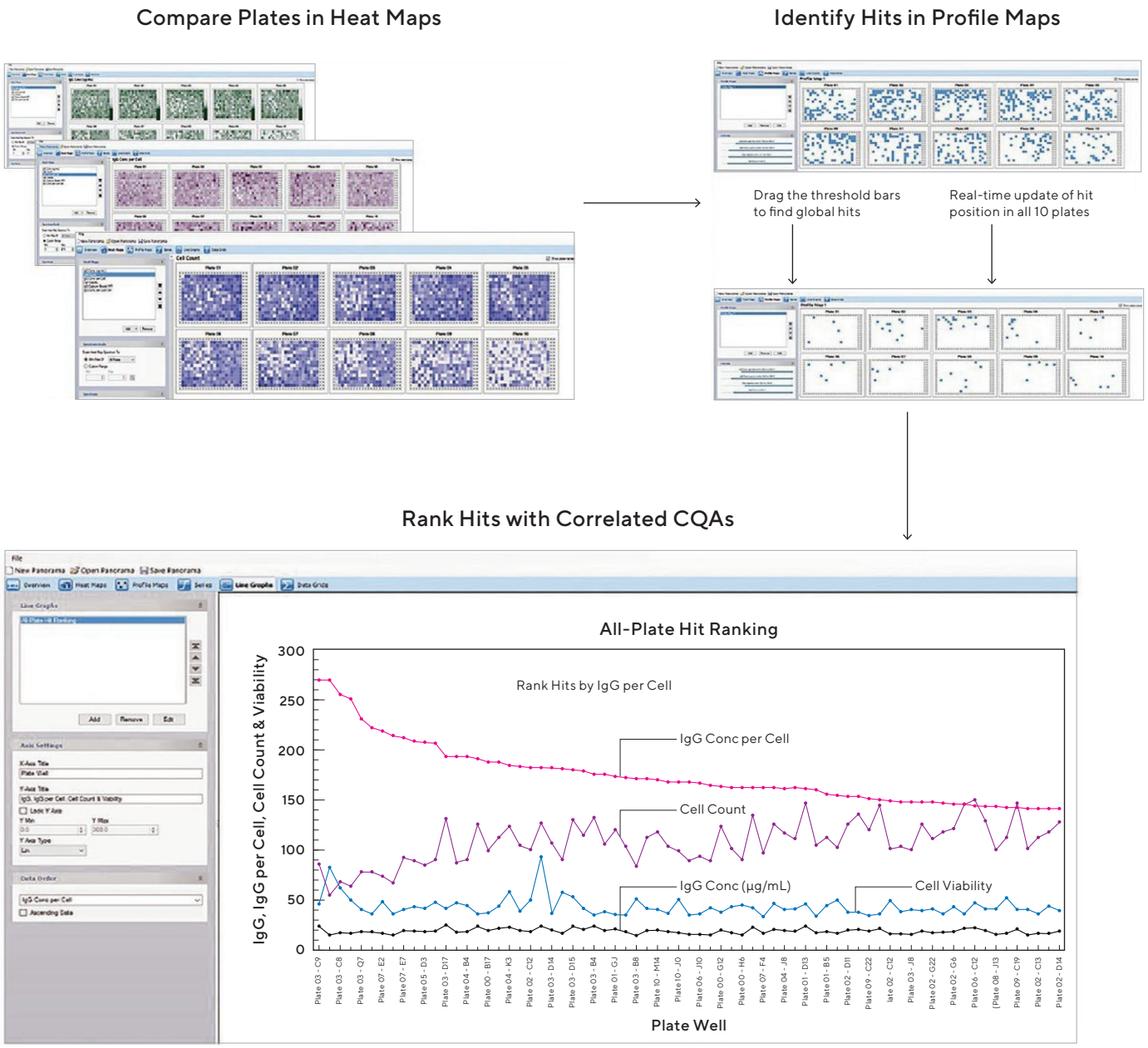

Figure 3: Cross-plate data analysis identifying best productive IgG clones in screening Critical Quality Attributes (CQAs).

Conclusion: Reduce Errors and Time to Actionable Results

Clone discovery and optimization is reported to take an average of 6 to 12 months, and vigorous clone candidates are rare. Reduce your uncertainty in clone selection by using multiple cell attributes—not just IgG concentration—to choose the optimal clones for your antibody development program.

The iQue platform gives simultaneous reporting of multiple cell attributes (Figure 4), ensuring you choose the most robust clones to move forward into the next phase of mAb development. The iQue platform saves you time and effort, while providing valuable information on clone productivity, by combining separate, time consuming steps into one during your cell line screening process.

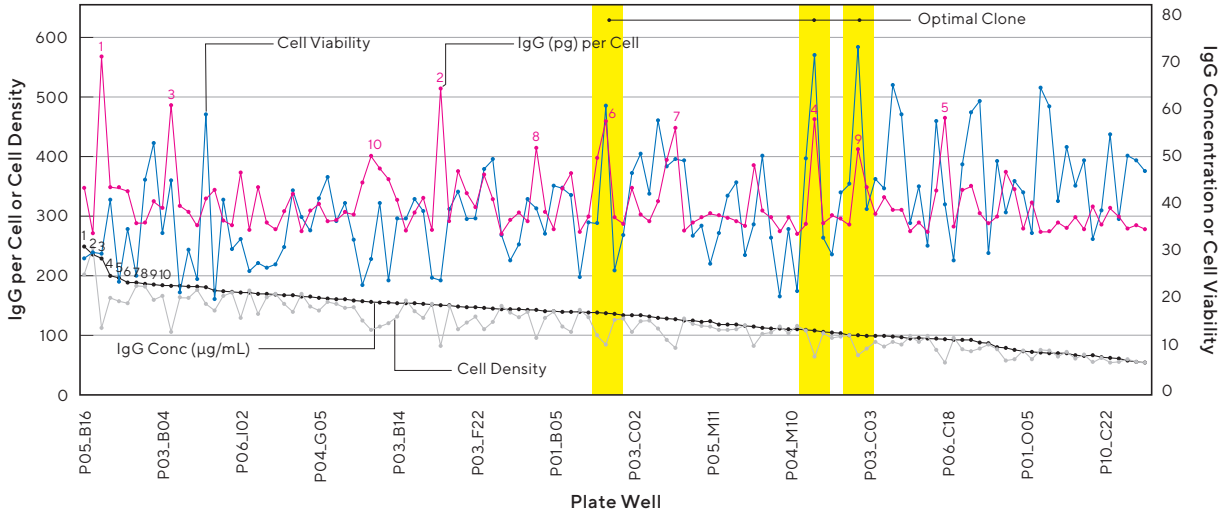

Figure 4: Simultaneously screening for IgG titer and cell health attributes reduces error by ensuring you choose the optimal clone candidates. The blue line represents IgG concentration, and the red line shows IgG quantitation per cell. The gray line demonstrates that while the IgG concentration declined there are still several viable clones. The yellow shading shows clones that have high IgG titers on a per cell basis. Such clones could be interesting candidates for downstream processing, and would likely have been excluded based solely on IgG titer.

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Applications of the iQue® Advanced Flow Cytometry Platform

Accelerate Hybridoma Screening during mAb Candidate Identification

The next generation of antibody drugs focuses on more challenging therapeutic targets, which necessitates the use of high-throughput methods to perform large screening campaigns and powerful software capable of analyzing these large multiplexed data sets easily.

The iQue® Platform Advances and Accelerates Your Production of mAbs Through Improved Hybridoma Screening

The iQue platform is a high-throughput sampling technology based on flow cytometry that delivers rich content with sampling times of under 20 minutes for a 384-well plate. The iQue platform eliminates the dead volume of traditional flow cytometry, allowing for assay miniaturization, saving precious cells and reducing assay costs. ForeCyt software quickly converts large, raw, multiplex data into easy-to-visualize and interactive graphics with seamless navigation from plates to wells to cells. Its Panorama feature analyzes large data sets across multiple plates, eliminating the need for tedious data export from individual sample wells.

Streamlining Antibody Discovery at ImmunoPrecise Antibodies (formerly ModiQuest Research) with the iQue Advanced Flow Cytometry Platform

Methods

Hybridoma Generation: Splenic B-cells were fused with myeloma cells by electrofusion, cultured in 96-well plates, and grown in hypoxanthineaminopterin-thymidine (HAT) media to select for fused cells. 9,600 hybridomas from two mice selected through antibody titer assays were cultured in 96-well plates. Supernatants were reformatted into 25 384-well plates and analyzed on the iQue platform 14 days after culture.

Data Acquisition and Analysis: All data were acquired using an iQue platform and analyzed using ForeCyt software version 6.1. Heat maps and profile maps for the multiplate studies were generated using the Panorama feature of the ForeCyt software.

Results

Supernatants from the hybridoma clones were mixed with the three encoded cell lines used for the target titer assays to measure target binding and specificity. Figure 5 shows the antibody binding heat maps for the three cell populations. Mouse 2 shows many more specific binders than Mouse 1, reflecting possible differences in type of antigen used for immunization. The profile maps show hits (~0.15% in Mouse 1 and ~1% in Mouse 2) in which hybridoma supernatants bound specifically to antigen expressing cells (in blue) above the user-defined criteria.

Multiplexed Hybridoma Screen

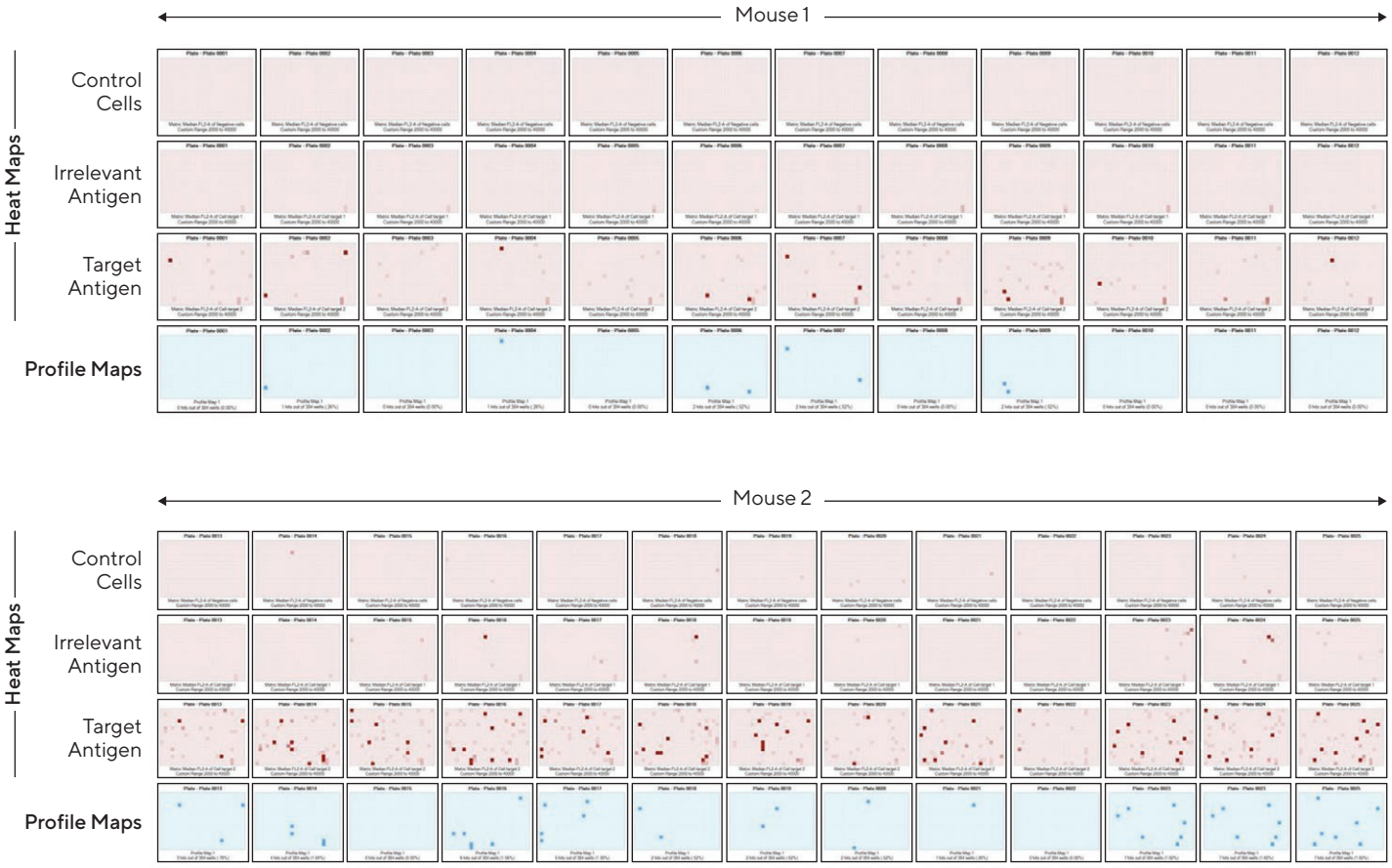

Figure 5: Results from the entire screen visualized using the Panorama feature in Forecyt Software. Three cell lines were color coded with the fluorescent Cell Encoder dye, mixed together and dispensed into wells of 384-well plates. Hybridoma supernatants were added to each well, followed by a fluorescent detection antibody, and plates were analyzed with the Intellicyt iQue platform. 9,600 hybridomas were tested from 2 different mice immunized with cells expressing target antigen. The entire screen was completed in 1 day. Heat maps (red) showing results for antibody binding to control cells, cells expressing target antigen and cells expressing a related but irrelevant antigen. Profile maps (blue) combine the binding results to the three cell types, showing antibodies that bind to cells expressing the target antigen, but not to cells expressing an irrelevant antigen or to negative control cells.

Summary

Multiplexed cell analysis on the iQue platform combines multiple assays into a single study, which simplifies and accelerates your hybridoma screening workflow. Further, assay miniaturization reduces costs and saves your precious antibody supernatant for use in additional confirmatory or functional studies.

References

Senutovitch N. *et al.* **A Faster Solution to Hybridoma Screening: Harnessing High-Throughput Flow Cytometry with an Integrated Software Solution.** BioProcess International 2019 Sept; 17(9).

Streamlining the Antibody Discovery Workflow at ModiQuest Research. Sartorius Application Note 2018.

Multiplexed Antibody Characterization for Preclinical Testing in Animal Models

Antibody therapeutics are unique in that they specifically bind to their target antigen. However, this specificity can introduce challenges to downstream discovery and development. Pre-clinical testing, for example, may be difficult when a lead candidate is selected because it specifically binds to a human target antigen but then has limited ability to bind to the same antigen from other animal species. Therefore, pre-clinical testing of efficacy and toxicity in animal models often requires surrogate molecules that react with the antigen in animal models but which may have different characteristics than the human-specific lead candidates.

One approach to overcome this obstacle is to engineer into the lead candidates the ability to cross-react with the target antigen from multiple animal species. When done early in the discovery process, hundreds or thousands of candidates are still available for evaluation. The multiplexing capabilities of the iQue can be used to incorporate antibody cross-species reactivity into the screening phase of your discovery process.

iQue High Capacity Flow (HCF) Approach at XOMA (US) LLC for Multiplexed Antibody Characterization

Methods

Five different CHO-K1 cell lines expressing the parent, mouse, rat, human, or cynomologous monkey target receptor were differentially labeled using five different concentrations of a cell labeling dye that is dedicated on the FL4 channel of the iQue platform. All five of the labeled cell lines were combined in a single test tube in equal proportions, and then distributed into 96-well plates (Figure 6).

Primary and secondary detection antibodies were added to each well and incubated in separate steps. After antibody staining, the plates were immediately analyzed on the iQue platform.

Results

Twelve different antibodies previously shown to bind to the target receptor were tested against these five different cell lines under four different conditions: 1) at pH 6 in the presence of the ligand, 2) at pH 6 in the absence of a ligand, 3) at pH 7.4 in the presence of ligand and 4) at pH 7.4 in the absence of ligand. The binding curves of the antibodies to each cell line were assessed. Figure 7 shows these dose response curves for the antibodies against each cell line under each condition.

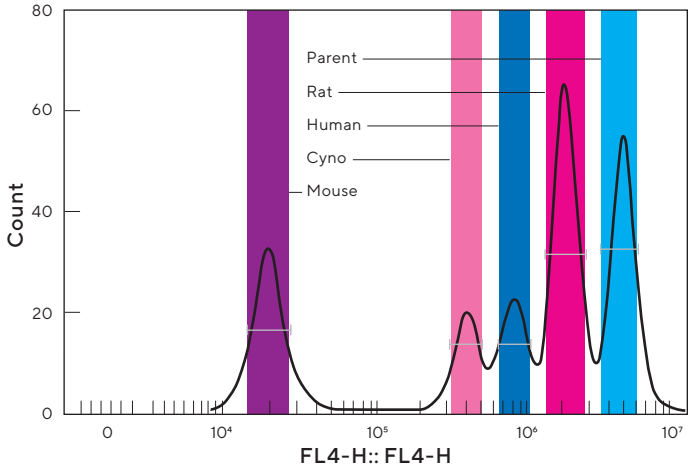

Figure 6: Five different cell lines show different levels of fluorescence, allowing easy segregation. Identification of color-coded cell populations across different species. From a 1D plot of FL4 fluorescence intensity, five individual cells populations with non-overlapping fluorescence can clearly be segregated.

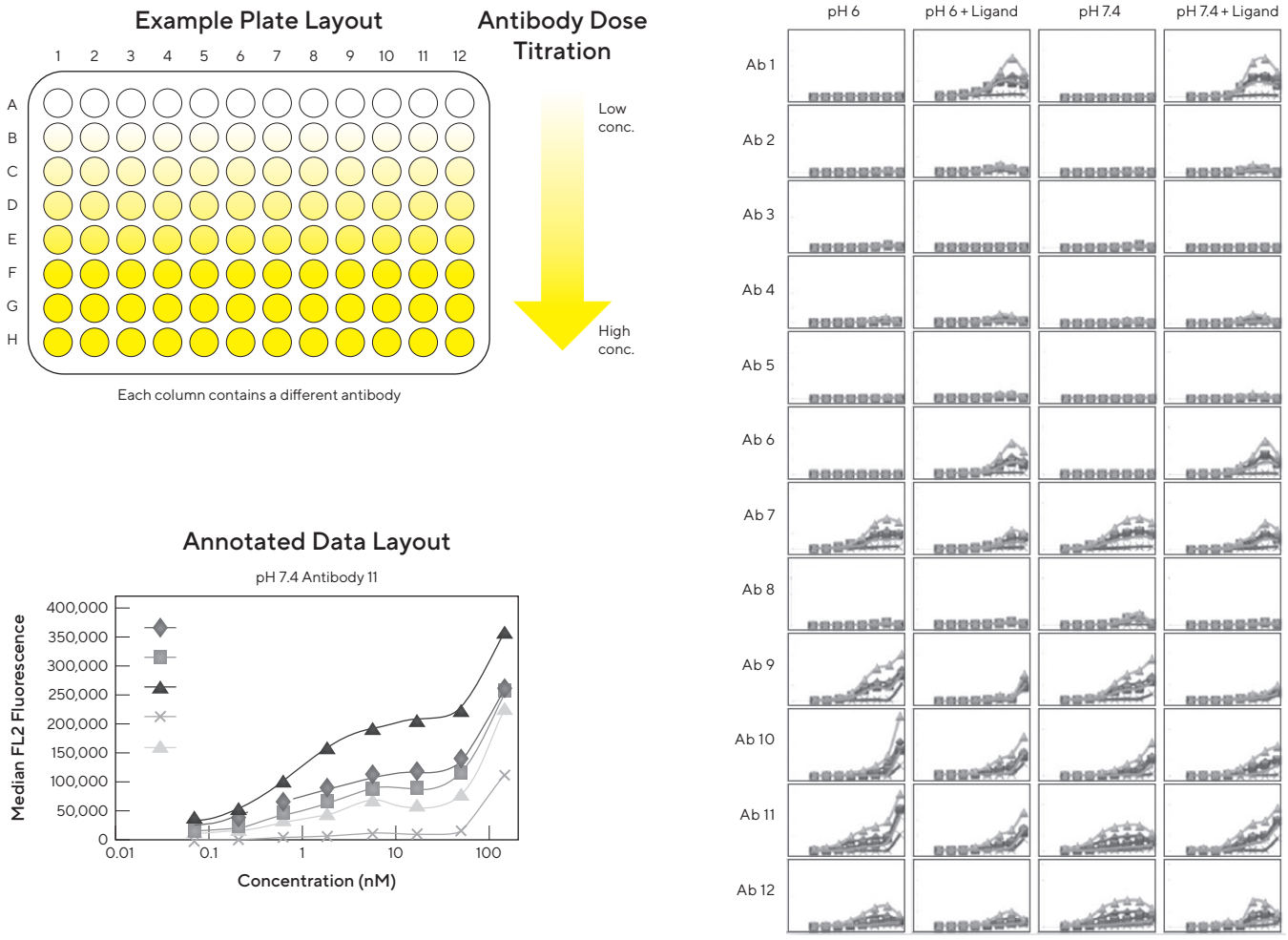

Figure 7: Multiplexing allows analysis and quantification of antibody binding to receptor for each cell population and species individually. A combinatorial approach facilities the collection of large data sets. Example plate layout showing 12 antibodies tested in 8-point dose responses per plate. Four conditions were tested in this assay: pH 6, pH 7.4, with and without ligand for each. Dose response and binding affinity curves for five different species receptors were determined, as shown in the annotated data layout. Differences in antibody reactivity are easily determined. For example, Antibody 1 only reacts in the presence of ligand.

Summary

The iQue advanced flow cytometry platform allowed the combination of five binding assays into one, which significantly reduces the amount of reagent and time required to perform one study. Here, each cell line expressed the target receptor from a different animal species and was used to evaluate binding of 12 antibodies under four different conditions, for a total of 1,920 data points.

Alternatively, this cell color-coding concept could be applied to a primary hybridoma screen to search for antibody candidates that react with the target antigen from humans, as well as from other species that may be used in downstream animal models of efficacy and toxicity.

References

Watson, S.R. **Multiplexed Antibody Characterization: Combinational Evaluation of Species Cross-Reactivity Using a High Capacity Flow (HCF) Approach.** Sartorius Application Note 2018.

Comprehensive, Integrated Solution to Rapidly Profile Antibody Internalization

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.

Intellicyt® Antibody Internalization Reagent is a novel, pH-sensitive dye that allows profiling large numbers of antibodies in 384-well plate formats. Antibodies labeled with this reagent 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, a Sartorius brand) 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).

Methods

The test antibodies (human CD3, CD19, CD20, CD22, CD45, CD71, and control mouse IgG) were labeled at a molar ratio of 1:3 in growth media with Intellicyt Antibody Internalization Reagent. Jurkat cells (human T-cell line) or Raji cells (human B-cell line) were added to 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.

Data was acquired on the iQue platform using 1 second sips corresponding to approximately 2,000 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).

Results

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 8A, 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 8B). 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.

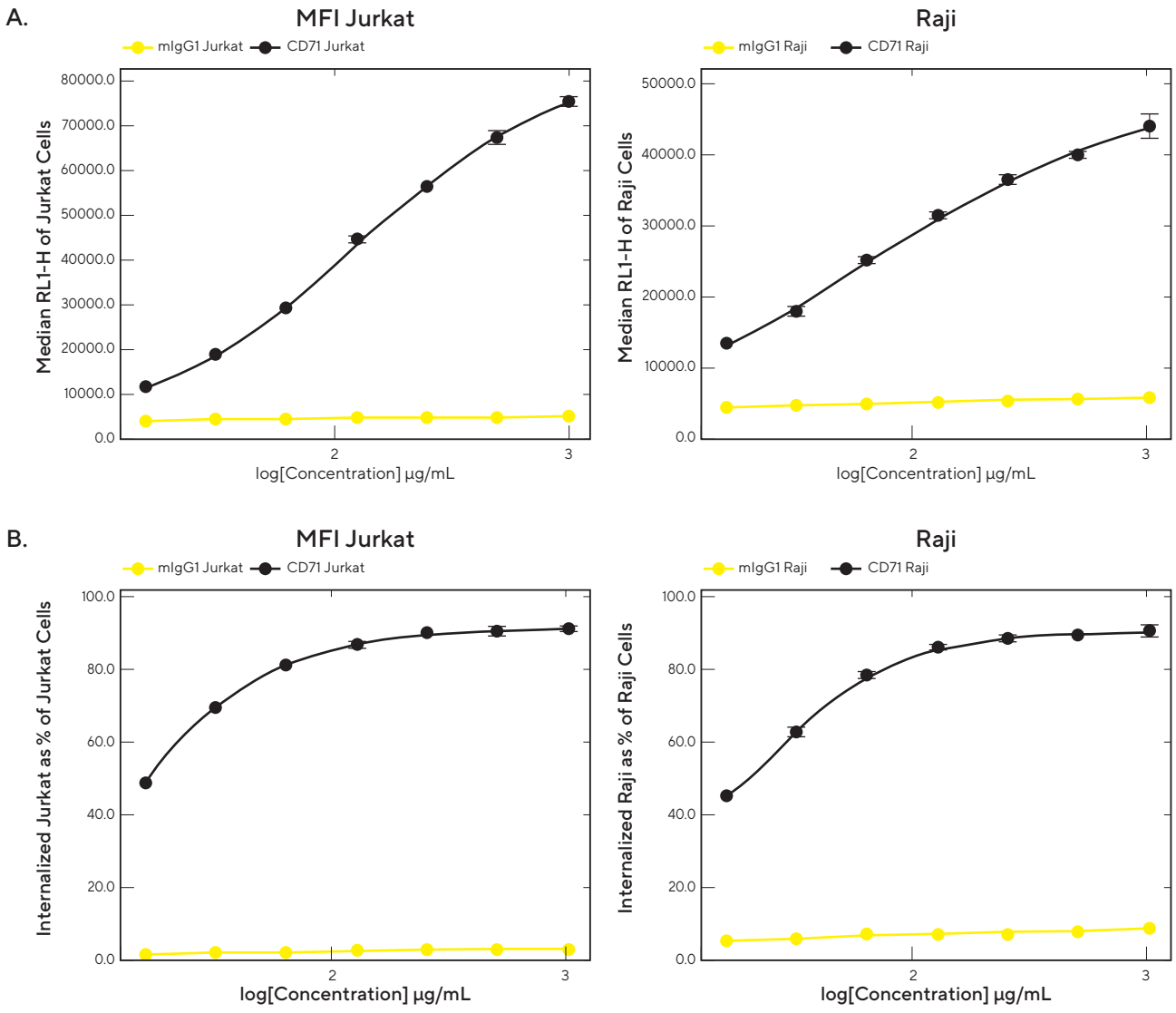

Figure 8: Antibody internalization controls. (A) MFI and (B) percent positive at a single, 2-hour time point. Dose response curves for an 8-point serial dilution of Internalization Reagent-conjugated mouse IgG1 (negative) or anti-CD71 (positive) with a top concentration of 1 µg/mL. For both Jurkat and 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.

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 9A 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 9B). 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.

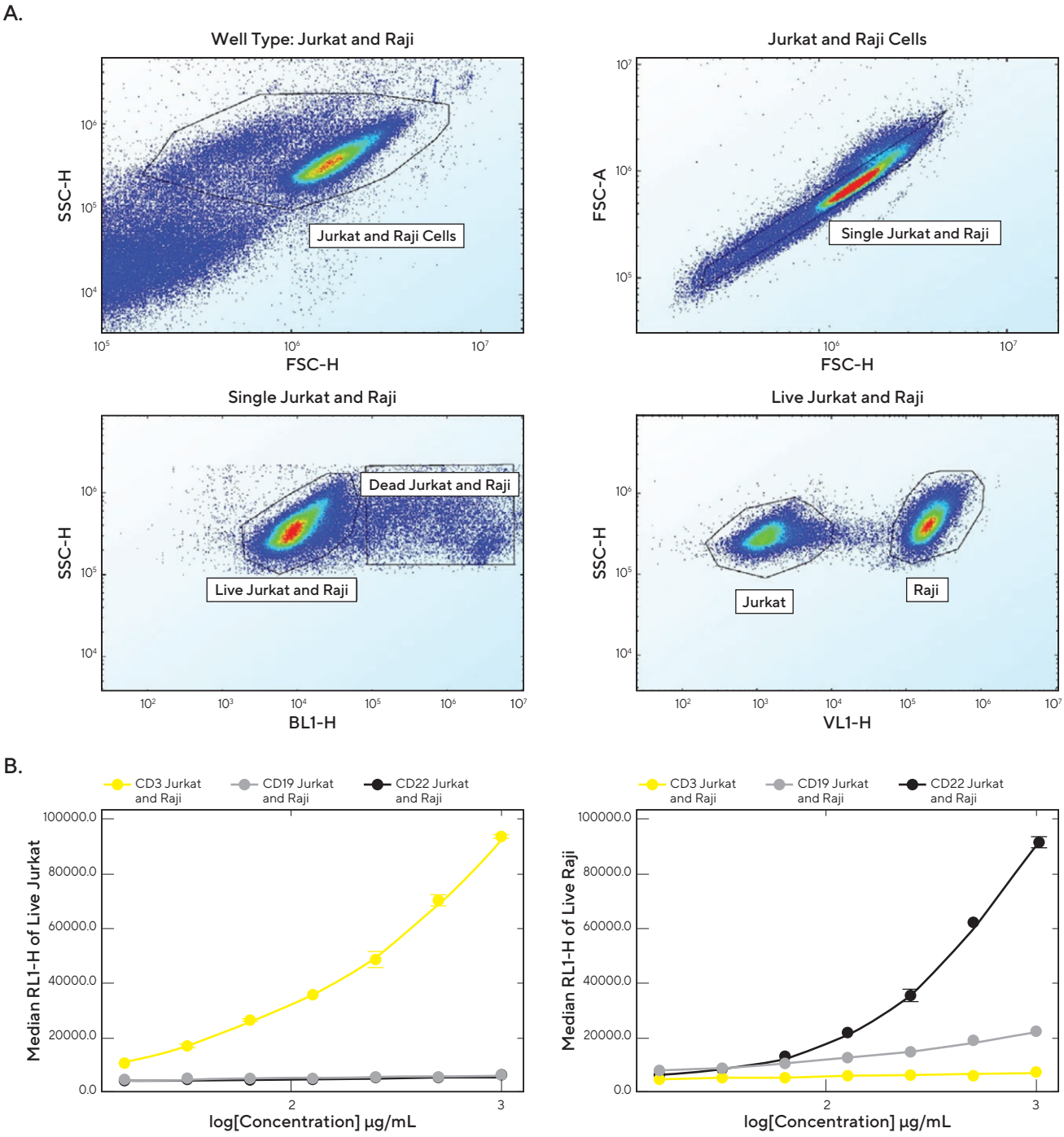

Figure 9: Multiplexing with encoder dye. Violet-encoded Raji cells and unstained Jurkat cells in the same sample well with a 8-point serial dilution of Internalization Reagent-conjugated specificity antibodies with a top concentration of 1 µg/mL, MFI at a single, 2 hour time point. (A) Gating strategy 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.

Summary

The iQue advanced flow cytometry platform provides a comprehensive, integrated solution that rapidly profiles antibody internalization and other critical antibody characteristics using small sample volumes, and allows analysis of multiple cell types within the same well.

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Weldon, C. and O'Rourke J. **A High-Throughput Multiplexed Antibody Internalization Assay**. Sartorius Application Note 2019.

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