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White Paper

March 2020

Keywords or phrases:
Antibody Internalization, Live-Cell Analysis, Advanced High-Throughput Flow Cytometry, Antibody-Drug Conjugate (ADC), Antibody Screening

Antibody Internalization: Advanced Flow Cytometry and Live-Cell Analysis Give Rich Insights During Antibody Profiling

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Introduction

The natural characteristics of antibodies, such as high binding affinity, specificity to a wide variety of targets, and good stability, make them ideal therapeutic candidates for many diseases. Monoclonal antibodies (mAbs), in particular, deliver promising therapeutic results in several different disease areas, such as autoimmunity, oncology, and chronic inflammation. Researchers' abilities to improve the breadth of antibodies have been aided by innovative technologies for antibody discovery, for instance, through humanization of mouse antibodies and phage display. However, advanced antibody design techniques create the need for new screening methods so that lead candidates can be quickly and effectively identified as early in the development process as possible.

Part of an effective screening strategy is to identify the desired therapeutic goal for your candidates. For instance, binding and rapid internalization are desirable properties for antibody-drug conjugates (ADCs), as cells must be selectively targeted and killed via delivery of a cytotoxic payload. In contrast, if the goal is to induce antibody-dependent cell-mediated cytotoxicity (ADCC), the antibody must remain bound to the cell surface, rather than being internalized, in order to activate an immune response.

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When designing ADCs, one key attribute to predicting efficacy is the antibody internalization (ABI) rate and the associated kinetics. These internalization kinetics are influenced by factors such as the epitope on the target antigen, affinity of the ADC-antigen interaction, and intracellular trafficking. Evaluating for these factors is critical throughout the antibody screening pathway (Figure 1); however, here, we concentrate on evaluating antibodies' ABI during functional profiling via rate comparisons during the development process, as well as multiplexed mechanistic studies later in the screening process.

Traditionally, antibody screening workflows have required labor intensive, time consuming, end-point-only methods, such as FACS, ELISA, or microscopy. One solution to address these screening challenges is to use advanced screening methods that offer maximum insight through high-content data. Herein, we demonstrate an advanced antibody screening method that focuses on speed and insight by using a novel, pH-sensitive reagent for characterizing ABI via both advanced flow cytometry and live-cell imaging.

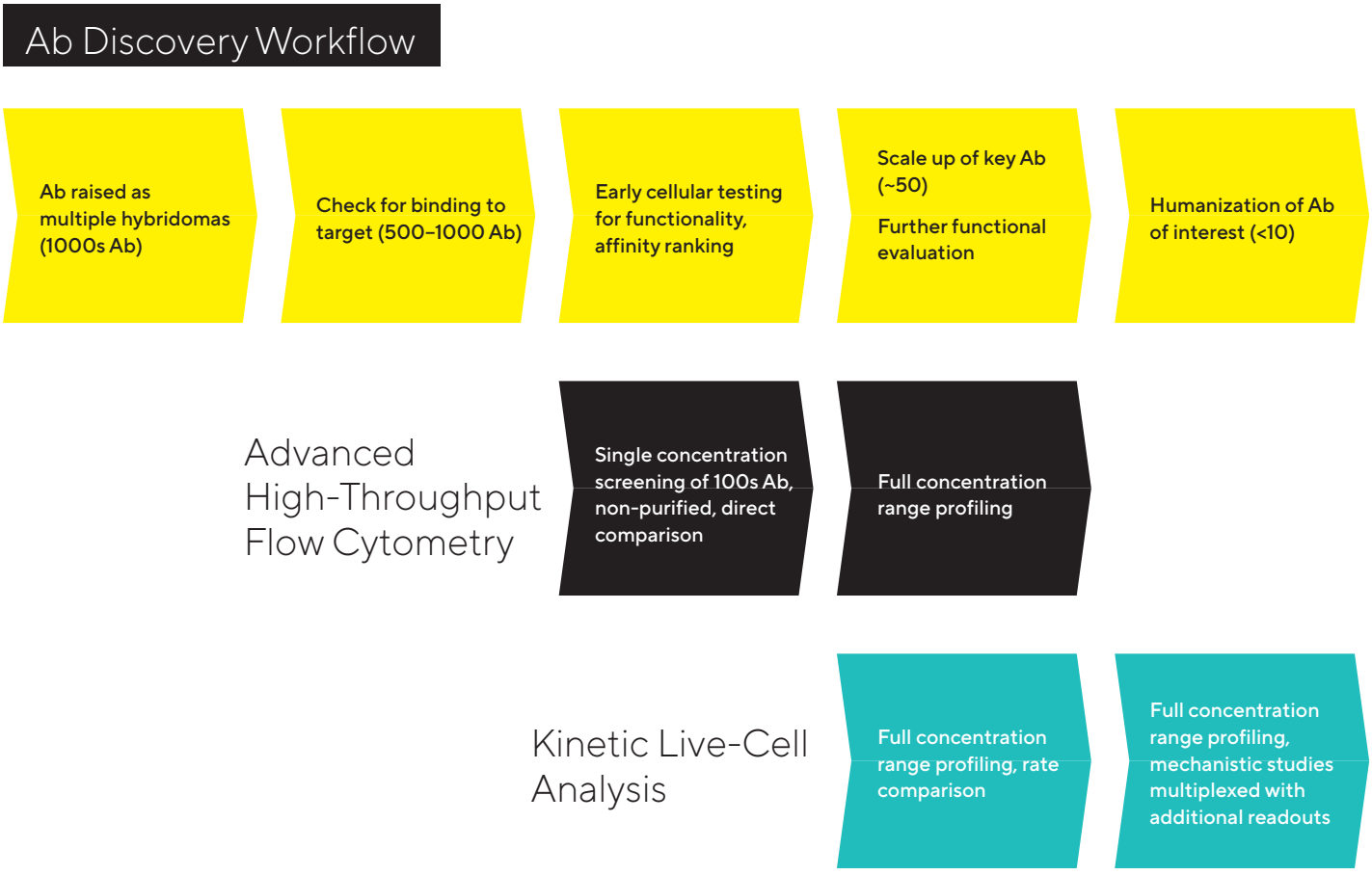

Figure 1: Inclusion of ABI in the screening pathway for antibodies. Selecting for ABI throughout the antibody screening pathway, whether during functional profiling and rate comparisons or mechanistic studies.

Challenges with Traditional Antibody Screening Workflows

Monoclonal antibodies (mAbs) are large (about 150 kDa), complex biologic molecules that require post-translational modifications for their activity (Chames *et al.,* 2009). Therefore, researchers face several challenges when engineering and producing mAbs as therapeutics. Engineering antibodies to optimize their biological potencies during the discovery phase can address many of these challenges. However, these attempts to optimize one attribute can have profound and unintended consequences on other antibody attributes. For instance, optimizing an antibody's specificity may negatively affect activity (Tiller and Tessier, 2015).

One way of simultaneously optimizing multiple antibody properties is by using mutagenesis to produce large screening libraries. However, large screening libraries necessitate a thorough *in vitro,* high-throughput screening method to quickly identify the most suitable drug candidates for further development early in their discovery process.

Workflows for antibody screening commonly include fluorescence-activated cell sorting (FACS), enzyme-linked immunosorbent assays (ELISA), and microscopy (such as confocal) techniques. Yet, these *in vitro* antibody screening methods have several drawbacks: (1) they can be labor intensive with limited throughput, (2) they do not allow direct, head-to-head comparisons of antibodies, and (3) they need large amounts of reagents.

For instance, even though FACS can be used to sort hundreds of thousands of cells based on their fluorescence properties, it cannot be used to perform high-throughput, time-dependent studies on individual cells because the cells must be sorted into single colonies before any further analysis can be performed (Doerner *et al.,* 2014).

ELISA, on the other hand, is adaptable to high-throughput screening (Saeed *et al.,* 2017), and thus has historically been used to screen hybridomas and other libraries for antibody binding to each of its targets. ELISAs are performed by coating a single target antigen onto the wells of assay plates, followed by the addition of individual samples from an antibody library (for instance, from hybridoma or phage display). Antibodies that bind to the immobilized antigen are detected by a color change due to an indirect enzyme/substrate reaction.

In addition to its adaptability to high-throughput screening, ELISA is rapid, consistent, and relatively easy to analyze (Saeed *et al.,* 2017). However, ELISA has several disadvantages that can limit its successful use in a modern antibody screening lab. First, primary screens that test binding to a single antigen often require subsequent secondary and sometimes even tertiary screens with control antigens to confirm their specificity and cross-reactivity. Second, ELISA is not the best method for screening antibodies that bind to cell surface antigens because these antigens are extracted from the cell membrane and purified before adsorbing to the plastic ELISA plate. Extracting antigens from the membrane often leads to disruption of conformational epitopes that can be important targets for therapeutic antibodies. Finally, to minimize background signal, ELISA requires multiple wash steps to remove unbound antibodies and detection reagents, resulting in long, labor-intensive screening workflows.

Also, although ELISA can provide data on the immunoglobulin G (IgG) titer, it offers no reflection on how the therapeutic antibody candidates affect the health of the test cells. i.e. how quickly or efficiently they induce death. Thus, candidates that appear to be productive based on ELISA screening may be carried forward into the next step of the production process even though they are unideal candidates in terms of function or vigour.

Microscopy techniques, in contrast to FACS, are useful for single-cell analysis, as well as for such localization and temporal studies as antibody internalization and live-cell imaging for monitoring individual cell behavior. Microscopy techniques, however, have limited throughput due to data acquisition time, and they have limited multiplexing capabilities. Thus, for antibody discovery, some other method, preferably high-throughput, is generally used for the initial selection and screening of large libraries, followed by a more thorough functional analysis of a smaller set of antibody candidates using microscopy (Doerner *et al.,* 2014).

Like FACS, microscopy techniques also require labeling each antibody with a fluorescent tag, which must be separated from the free label via a column or wash step because analysis requires robust isolation of internalized antibodies from those outside the cells. To aid isolation of the positive signal, researchers often resort to perturbing techniques, such as washing cells, using blocking dyes, and reducing the temperature to slow cellular activity. However, cells can be lost during washing steps, and the associated reductions in temperature perturb the cellular environment.

A further drawback to almost all the techniques used for antibody screening is that they only enable end-point analysis, which means that multiple experiments are required to follow an antibody attribute, such as internalization, over time.

The best approach to address all the limitations of traditional antibody screening is to combine data from advanced screening methods; using advanced methods, researchers can choose their candidates based on a thorough evaluation of all relevant characteristics, such as IgG titer, cell health, internalization, and their associated kinetics early in their discovery process.

A Simple Way of Labeling Antibodies for Addressing Challenges with FACS, ELISA, and Microscopy

One of the challenges presented above with FACS, ELISA, and microscopy techniques is the requirement for wash steps to minimize background signal. Among other undesirable effects, such as increased time to results, this need for wash steps also increases reagent requirements (and cost) and makes cell loss inevitable. However, an advanced method for labeling cells could reduce reagent requirements, as well as simplify protocols when analyzing antibody internalization.

ABI Assays Made Simple, With No-Wash Labeling and Low Reagent Requirements via a Novel, Ph-Sensitive Reagent

The key to this simple, no-wash protocol is a novel reagent composed of Fc-region targeting Fab fragments conjugated to a pH-sensitive fluorescent probe (Nath *et al.*, 2016). This type of novel reagent enables a generic, one-step, no-wash labeling protocol for all isotype-matched, Fc-containing test antibodies when optimized for use on specific instruments. Figure 2 shows how this reagent works: labeled antibodies are added to cells, and a fluorogenic signal is produced as the Fab-Ab complex is internalized and processed via acidic (pH 4.5-5.5) lysosomes and endosomes.

Antibodies of interest are quickly and effectively labeled, with low reagent requirements, by incubating in growth media with this novel, pH-sensitive dye (Figure 3). Cells are then added to 384-well plates, along with the dye-conjugated antibodies, and incubated again. This reagent, when used with an advanced flow cytometry platform, provides a comprehensive, integrated solution for rapid profiling of antibody internalization and other critical antibody attributes using small sample volumes in 96- or even 384-well plate formats (Riedl *et al.*, 2016). Much of the data acquisition and analysis, including generation of serial dilution curves and EC_{50} calculations, is automated with the help of advanced software packages, for example, as included in the iQue® advanced flow cytometry platform.

Functional Profiling for Comprehensive Cell and Antibody Characterization Early in the Process with Multiplex Analysis

Combining the above-described pH-sensitive dye with other reagents in one assay on an advanced flow cytometry platform enables simultaneous analysis of a variety of cell and antibody characteristics within the same workflow. For instance, you can measure cell viability using a membrane integrity dye to assess general cell health, as well as cell death due to cargo delivery, when optimizing ADCs. Or you can characterize cell specificity using encoding dyes (for cell lines) or directly conjugated fluorescent antibodies (for complex cell models with a variety of cell types). Other reagents, such as those for assessing cytokine release, are also available for more detailed antibody assessments on the sample. Therefore, analysis with an advanced flow cytometry platform can be optimized to deliver rich content very quickly while only using a low sample volume.

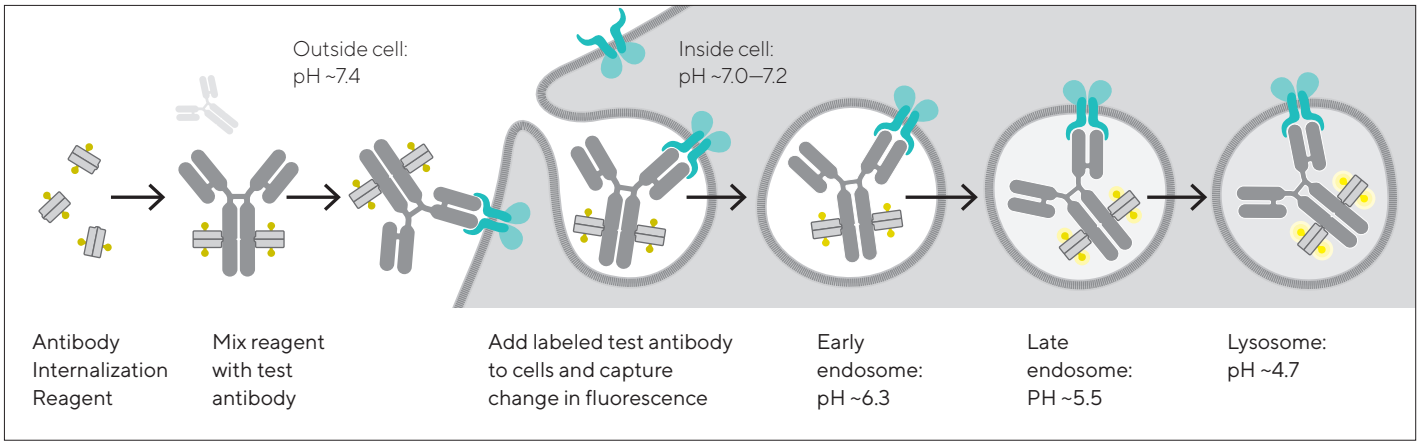

Figure 2: The pH-sensitive fluorescent probe principle. A novel pH-sensitive fluorescent probe enables one-step, no-wash labeling of isotype-matched antibodies. A fluorescent signal is generated as internalized antibody is processed into the acidic endosome and lysosome pathway.

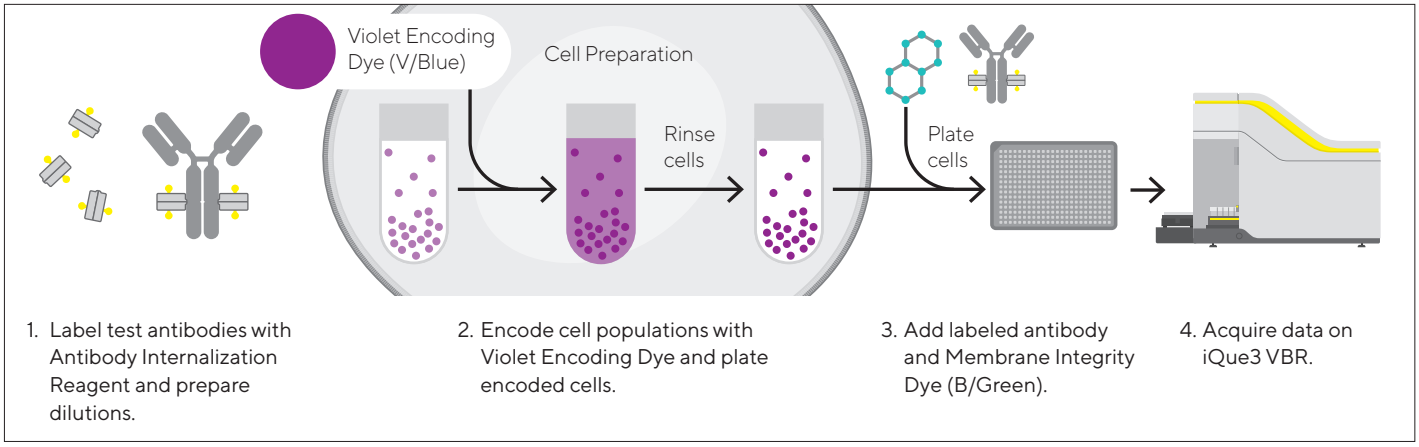

Figure 3: The assay consists of 3 components, each 10 µL: labeled test antibody, cells (encoded or not), and Cell Membrane Integrity Dye (B/Green). Each component is prepared at 3X before addition for a final concentration of 1X and an assay volume of 30 µL.

Sartorius produces such a pH-sensitive reagent for use on our iQue advanced flow cytometry platform. The combination of non-perturbing and validated reagents for multiplexing, no-wash protocols, high-throughput capabilities, flexibility for a robotic interface, and integrated Forecyt® software for multiparametric data analysis and visualization expedite the process of screening drug candidates for potential efficacy and toxicity to accelerate antibody discovery and development.

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 as a T cell marker, anti-CD19, anti-CD20, anti-CD22, or anti-CD79b as B cell markers, anti-CD71 as a positive control, and IgG as a negative control), then added a cell membrane integrity dye before acquiring data from the plate. 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. We generated series dilution curves for the specificity markers of each cell type (Figure 4A). 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 antibody internalization 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 Ab candidate.

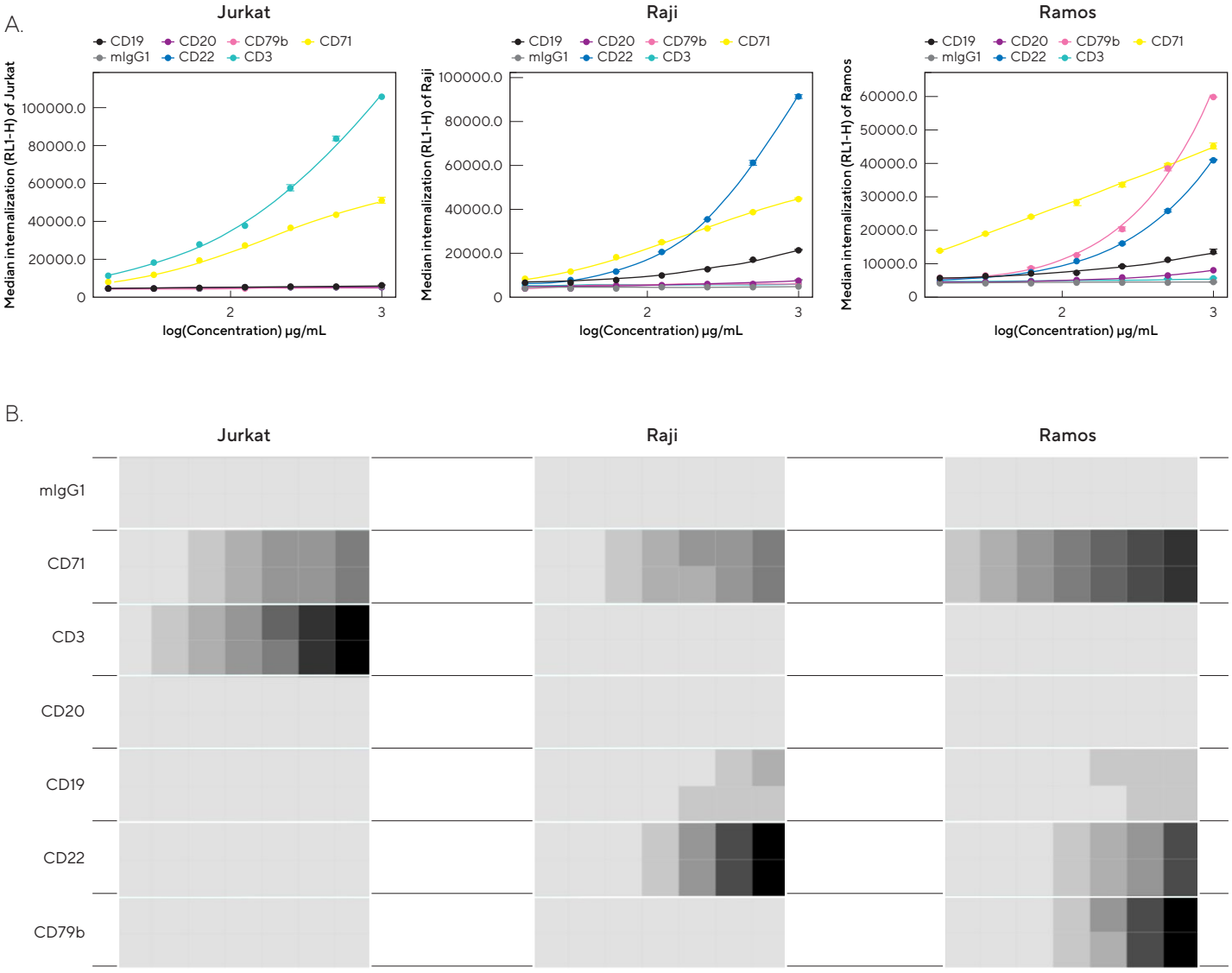

Figure 4: Serial dilution curves for internalization-labeled antibodies with a top concentration of 1 mg/mL in different cell types after a three-hour incubation. Multiplexed positive and negative cell lines may be used in an ABI assay to generate high-content data in one assay. Median fluorescent intensity (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.

Full Concentration Profiling with Live-Cell Imaging and Analysis

A pH-sensitive dye-coupled antibody fragment designed according to the same principle as that used above (Figure 2) can also be optimized and used in other instruments. For instance, using this pH-sensitive reagent in a real-time, live-cell imaging system such as the Incucyte® Live-Cell Analysis System, allows visualization and automatic quantification of the full time-course of ABI. This combination of reagent and platform thus provides a simple method for directly profiling and comparing ABI for a large number of antibodies (10–100s at a time in a miniaturized format).

To demonstrate the power of the pH-sensitive dye approach for high-throughput antibody internalization assays in a real-time, live-cell analysis system, we performed a head-to-head comparison of the internalization properties of six different commercially available anti-CD71 antibodies into HT1080 fibrosarcoma cells. We labeled the anti-CD71 with our pH-sensitive reagent before adding to cells in 96-well plates. We then captured the internalization signal in a live-cell analysis system every 30 minutes over 12 hours using a 10X magnification.

The plate view in Figure 5A shows clear positive and negative control responses in column 11 and 12, with concentration-dependent responses for each antibody across the two plates (antibodies 1a and 1b are the same clone from two different sources).

We found that three antibodies (Ab1a, Ab2, and Ab1b) gave internalization signals that we detected at low concentrations (< 0.05 µg/ml) (Figure 5). Reassuringly, Ab1a and Ab1b gave similar internalization responses. Antibodies 3, 4, and 5 were internalized more weakly and only at higher concentrations. From the control responses, we calculated a mean Z' value of 0.82 (two plates: 0.75, 0.87), indicating high robustness for this microplate assay.

These data show our method is suitable for comparing the internalization of multiple antibodies at a single target, or one antibody in various cell types. The assay precision and workflow is such that it would be possible to compare 100s of different antibodies at once, and further throughput could be achieved through miniaturization to a 384-well format.

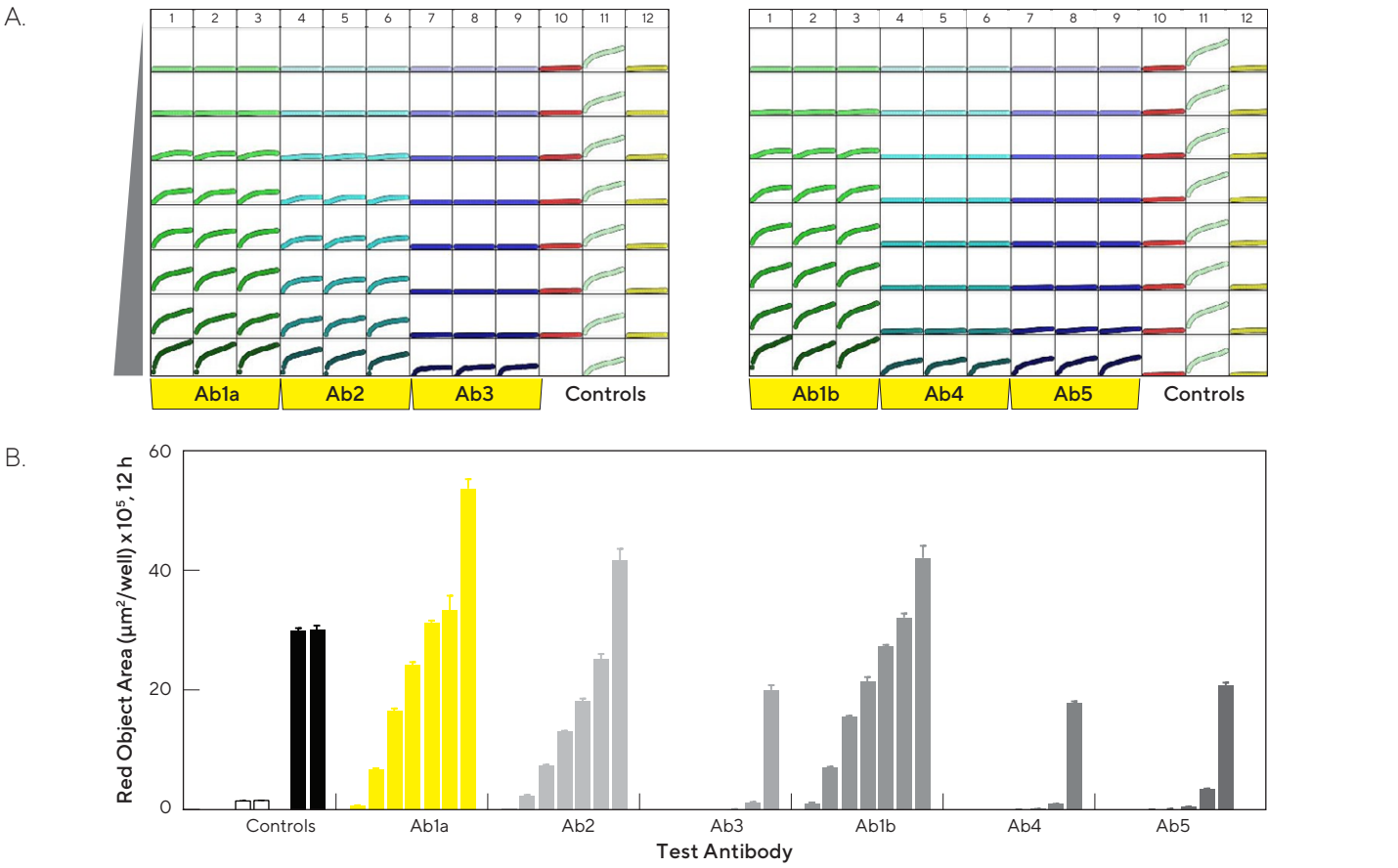

Figure 5: Screening test of Abs for internalization. The pH-sensitive reagent is suitable for high-throughput antibody internalization assays in a real-time, live-cell analysis system.

Showing a Simple Pharmacological and Kinetic Quantification of Antibody Internalization Using Herceptin

In addition, we experimented to determine EC_{50} values for the internalization of a clinically used monoclonal antibody, Herceptin (Trastuzumab). We constructed a concentration-response curve by labeling Herceptin with the pH-sensitive reagent, then serially diluting (1:2) before adding to BT-474 cells.

In BT-474 Her2-positive breast carcinoma cells, we saw definite time and concentration-dependent internalization of Herceptin over 48 hours. From an area under the curve (AUC) time-course analysis, we calculated the EC_{50} value for internalization at 323 ng/mL ≡ 2.1 nM (Figure 6). Our calculated EC_{50} value is similar to the known KD value for Herceptin for its target receptor (approximately 5 nM).

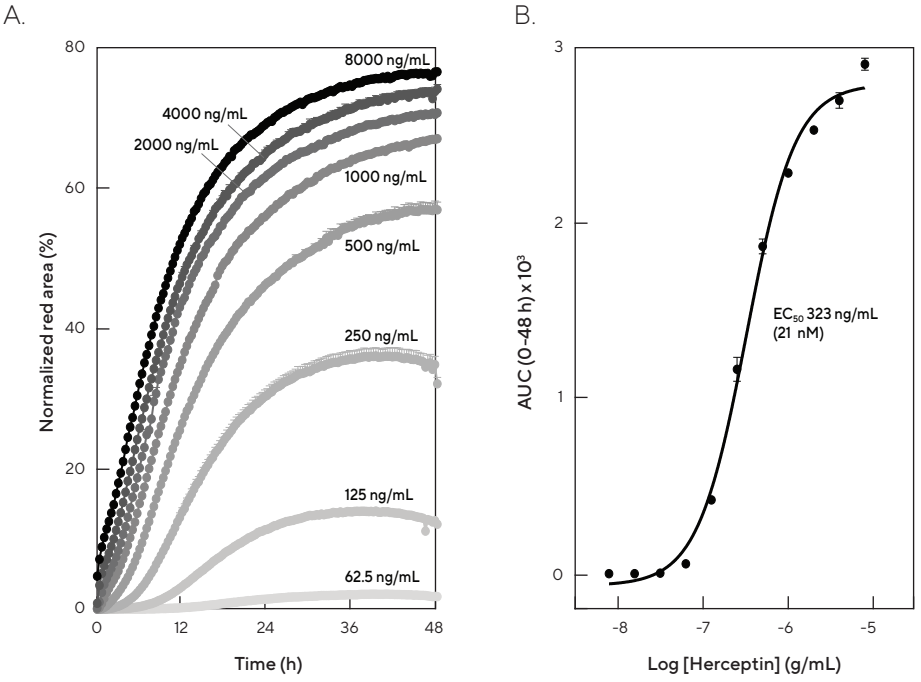

Figure 6: Quantitative pharmacological analysis of pH-sensitive dye-labeled Herceptin shows time and concentration-dependent internalization and an EC_{50} value of 2.1 nM. Quantitative pharmacological analysis of pH-sensitive dye-labeled Herceptin. BT-474 Her2-positive cells were treated with increasing concentrations of pH-sensitive dye-labeled Herceptin. The time course graph displays an increasing normalized red area over time with increasing Herceptin concentrations (A). Area under the curve analysis of this response displays a clear concentration dependent response with an EC_{50} of 323 ng/mL (B). All data shown as a mean of 3 wells ± SEM, time course data shown as normalized red area.

Speed and Insight through Advanced Antibody Screening

Quick and accurate identification of suitable drug candidates is key to the development of therapeutic antibodies. ABI is an essential part of the selection criteria for ADC candidates. It can be used for functional profiling and rate comparisons, as well as mechanistic studies when coupled with additional multiplexed readouts, thus reducing the time required for lead generation. To illustrate the capabilities of advanced antibody screening, we have described our solution for efficiently interrogating libraries of candidates early in the screening process.

For comprehensive cell and antibody characterization, we used our novel, pH-sensitive reagent and the iQue advanced flow cytometry platform. This combination is best for screening and early full concentration profiling because it enables simultaneous analysis of a variety of cell and antibody characteristics within the same workflow. For quantitative, pharmacological analysis and direct, head-to-head comparisons of ABI, we used a version of our novel, pH-sensitive reagent and the Incucyte live-cell analysis system. This combination is useful for further functional profiling that requires spatial and temporal resolution. This advanced antibody screening process is a simple, fast, and insightful way to identify candidates that meet your therapeutic goals early in the drug discovery process.

Combining Advanced Flow Cytometry and Live-Cell Imaging and Analysis for Complete Antibody Profiling: A Real-World Screening Strategy Employing Our pH-Sensitive Dye and Instrument Platforms

Researchers at LifeArc used the iQue platform and Incucyte Live-Cell Analysis System as part of their strategy to develop a new ADC targeting neuroblastoma. Neuroblastoma is a rare cancer that nevertheless is the most common extra-cranial solid tumor in children, with a 5-year survival rate of 50% for patients with high-risk disease. The researchers found they were able to use these systems in combination, not only for screening, but also for assay development, lead candidate profiling, and characterization. They found that the iQue platform offered fast, high-content analysis, while the Incucyte live-cell analysis system offered kinetic, image-based analysis. Combining data from the two systems gave them a complete antibody profile.

In brief, the researchers identified anaplastic lymphoma kinase (ALK) as a target for their therapeutic approach because level of ALK expression correlates with disease stage and ALK antibodies show surface expression in patient samples. In collaboration with colleagues at Mt. Sinai, researchers at LifeArc produced 1,152 candidate hybridoma clones that they were able to narrow to 53 candidates that ELISA and flow cytometry showed bound to ALK. Using the advanced flow cytometry capabilities of the iQue platform, the researchers were able to further narrow this to 20 candidates that bound ALK at the surface of cells, since this is a critical attribute of the mechanism of action of ADCs.

Of particular interest, these researchers used internalization assays on both the iQue and Incucyte cell analysis platforms to further narrow their lead candidates to two. Critically, they gained kinetic imaging data and high-content analysis using multiplexed cell viability assays early in their ADC discovery process. Using advanced screening methods that incorporate a novel, innovative reagent for characterizing ABI via both advanced flow cytometry and live-cell imaging, they gained maximum insight through high-content data and were able to make critical decisions early in their process, thus saving time, material, and money.

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Version: 03 | 2020