APPLICATION NOTE

Multiplexed Antibody Characterization

Combinational Evaluation of Species Cross-Reactivicty Using a High Capacity Flow (HCF) Approach

Susan R. Watson, Xoma Corporation, 2200 Powell Street, Suite 310, Emeryville, CA 94608

Key Features and Benefits

Features	Benefits
• Five color-coded cell lines combined in each well	• Engineering reactivity that can be used in animal models of efficacy and toxicity into lead candidates
• Internal (negative) assay control in every well	• More robust and reliable screening data
• Condensed format for multiplex experiments	• Resources reagent use and assay variability

Introduction

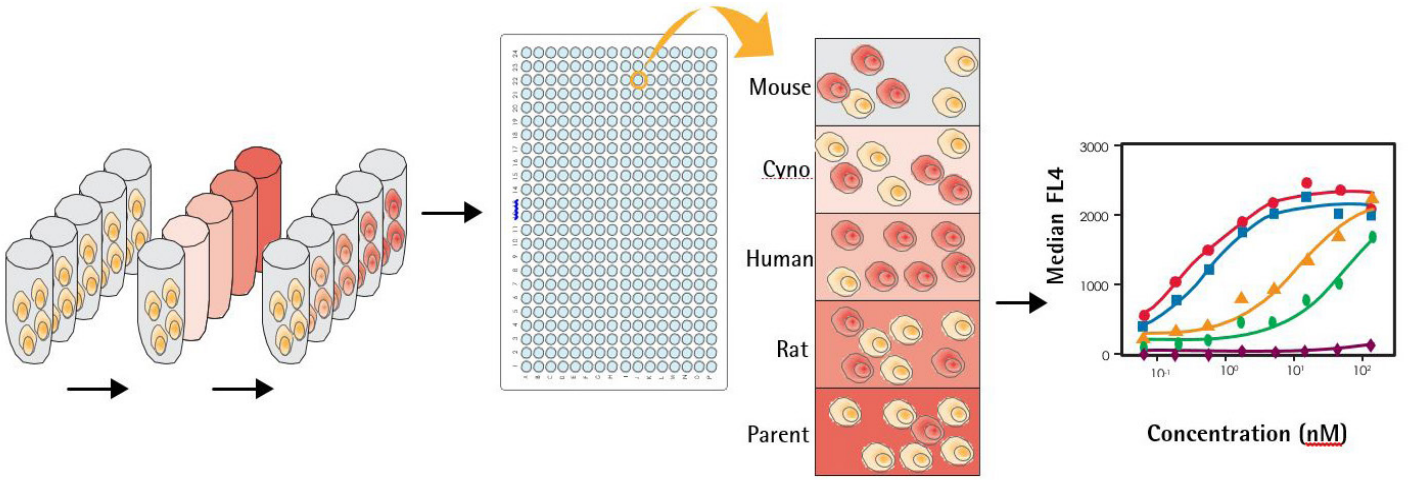
Therapeutic antibodies represent one of the fastest growing areas of the pharmaceutical industry. XOMA (US) LLC, uses proprietary technology to discover develop and manufacture novel antibody therapeutics in areas including cardio- metabolic, inflammatory, autoimmune and infectious disease, and cancer. This case study describes how XOMA is incorporating IntelliCyt's screening platform to drive the success of their antibody engineering efforts.

The discovery and development of antibody therapeutics is a time-intensive and costly process that involves screening of large candidate libraries, functional characterization of lead molecules in vitro, and preclinical testing in animal models of efficacy and toxicity[1]. Antibody therapeutics are unique in that they are exquisitely specific in terms of binding to their target antigen. While clearly an advantage as a characteristic of a targeted therapeutic modality, this specificity can introduce challenges in downstream discovery and development processes. For example, a lead candidate that is selected for its specific binding to a human target antigen may have limited ability to bind to the same antigen from other animal species, making pre-clinical testing difficult. Because of this, pre-clinical testing in animal models of efficacy and toxicity often requires the use of surrogate molecules that react with the antigen in animal models, but likely 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. Ideally this would be done early in the discovery process, when hundreds or thousands of candidates are available for evaluation. The multiplexing capabilities of the IntelliCyt Screening System, which utilizes a flow cytometry detection modality, can be used to incorporate cross species reactivity of antibodies into the screening phase of the discovery process[2].

Using the multiplexing capabilities of a high capacity flow (HCF) screening approach, different cell populations can be distinguished by pre-labeling each population with a different intensity of a fluorochrome, establishing a unique signature of fluorescence intensity for each population. The color coded cell lines are physically combined into a single suspension, and mixed together with test reagents.

After data acquisition, the cell lines from each sample can be identified and individually evaluated for readout of interest, such 60 as antibody binding. Here we present the development of a species cross-reactivity assay for the IntelliCyt Screening System. In this assay, five different cell types, expressing a target receptor from 40 a different animal species plus the parental cell line (negative control) were simultaneously tested for antibody binding under different 20 test conditions in a 96-well plate format.



1. Add dye to cells at a range of concentration. Incubate at room temperature 5-10 minutes.

2. Wash away unbound dye

3. Combine cells in equal proportions in single sample. Use directly in assay to determine binding specifically

4. Acquire data on Intellicyt Screening Platform; perform downstream

Figure 1. Workflow overview for species cross-reactivity determination using IntelliCyt's Cell Encoder Kit to label and simultaneously assay five different cell lines per sample. Parent cell line, and lines expressing mouse, rat, cynomologus monkey and human receptor are labeled separately and combined into a single sample. The inclusion of the untransfected parental line serves as a negative binding control for every sample. The cell sample is aliquoted in 96-well plates for an antibody binding assay, where primary and secondary detection antibodies are sequentially added. Total acquisition time was approximately 5 minutes per plate, and data analysis rapidly facilitated generation of 8-point binding affinity curves.

Assay Procedure

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 Intellicyt Screening System. This resulted in each cell line exhibiting a different level of fluorescence. All five of the labeled cell lines were combined in a single test tube in equal proportions, and then distrubuted into 96-well plates. The primary detection antibody was added to each well incubated for 45 minutes at 4°C. Plates were centrifuged and washed twice in FACS buffer, then re-suspended in a solution containing diluted PE-conjugated IgG detection antibody. The secondary detection antibody was again incubated for 45 minutes at 4°C, washed twice, and re-suspended in the sample reaction buffer for analysis. All samples were prepared, stained, and assayed in 96-well plates[3]. After staining, the plates were immediately analyzed with the Intellicyt Screening System. 96-well plates were acquired and fully analyzed in 3–6 minutes, and thus the entire assay was completed, with data analyzed, in under 30 minutes.

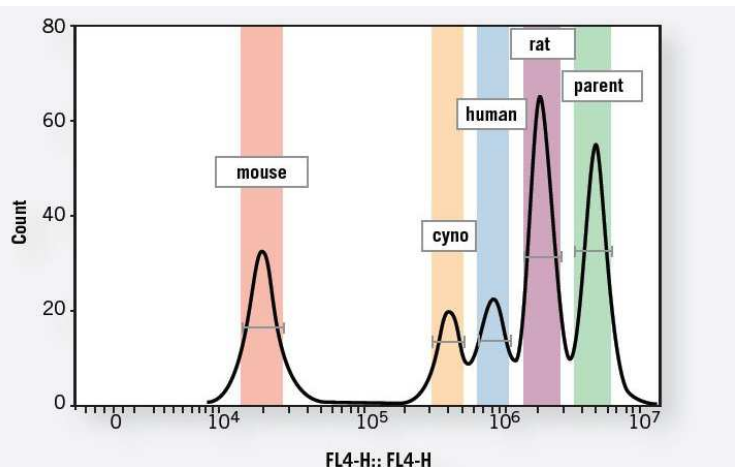


Figure 2. Identification of Color-coded Cell Populations and Determination of Binding Affinities across Different Species Receptors. From a 1D plot of FL4 fluorescence intensity, five individual cells populations with non-overlapping fluorescence can clearly be segregated, allowing rapid deconvolution of data collected from one sample back into the five different cell populations. For each sample, the binding of antibody to receptor can be analyzed and quantified for each cell population and species individually. This multiplexed data set facilitates and streamlines studies such as the determination of binding affinity curves by reducing the number of samples required, as well as the inclusion of a negative assay control in every well.

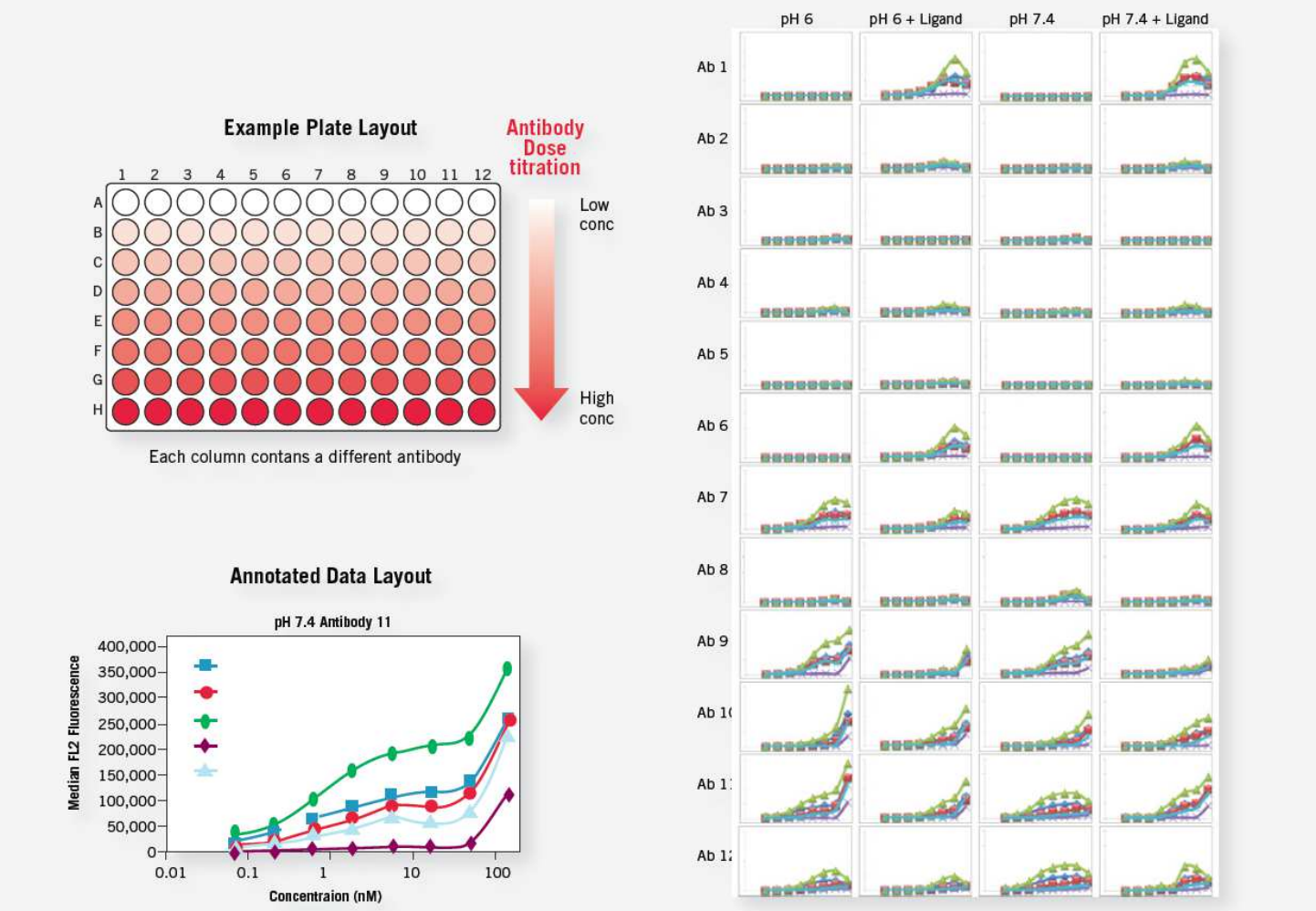


Figure 3. A combinatorial approach facilities the collection of large data sets. Example plate layout showing 12 antibodies tested in 8-point dose responses per plate. Four plates were utilized in this assay: pH 6, pH 7.4, with and without ligand for each pH. This multiplexed data set represents 1920 individual assay data points, collected from four 96-well plates. Dose response and binding affinity curves for five different species receptors can be simultaneously determined. Differences in antibody reactivity are easily determined. For example, Antibody 1 only reacts in the presence of ligand.

Results and Discussion

In this study, 12 different antibodies previously shown to bind to the target receptor were tested against five different cell lines in 8-point dilution curves under four different conditions: i) in the presence of the ligand, ii) in the absence of a ligand, iii) at pH 6 and iv) at pH 7.4. As shown in Figure 2, the color coded cell populations were readily distinguished one from another based on their level of fluorescence in the FL4 channel of the IntelliCyt Screening System. The binding curves of the antibodies to each cell line could then be assessed. Figure 3 shows dose response curves for the antibodies against each cell line under each condition.

Color-coding and combining the cell lines in each well of the assay plates enabled combining five binding assays into one, significantly reducing the amount of reagents required to perform the study. In this study, each cell line expressed the target receptor from a different animal species, and was evaluated in an extensive set of binding experiments. 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.

Summary

The speed of the sampling and data analysis capabilities of the IntelliCyt Screening System combined with the cell color coding can significantl increase the capacity and productivity of labs performing these assays. From a user's perspective, this high capacity flow (HCF) approach enables the use of a combinatorial assay as a high throughput primary screen, greatly reducing time and cost to acquire a large data set. In this case, five cell lines were tested against twelve different antibodies in eight point dose response curves under four different assay conditions. Thus 1920 data points were generated from four 96-well plates, with the sampling and data analysis completed within one hour. By taking full advantage of the multiplexing capabilities of the IntelliCyt Screening System this assay enables high content studies to be performed in a high throughput setting.

References

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