

## ABSTRACT

Antibody discovery is a costly and resource intensive endeavor, and technologies that streamline the discovery process and increase productivity are greatly needed. A key process in the early discovery of new antibody therapeutics lies in the screening of hybridoma and phage display libraries for candidates that bind specifically the target antigen of interest, and not to unrelated antigens. We report here a mix and read screening assay that simultaneously tests the binding of antibodies to both target and control antigens on the surface of cells. Two cell lines, one expressing a target antigen and one expressing a negative control antigen were color coded using a cell labeling dye and mixed together in each well of the assay plates. Antibodies, such as those present in the supernatants from a hybridoma library, were allowed to bind to the cells, and a detection reagent that fluoresces in a separate channel was used to measure the levels of primary antibodies bound to the cells. Using the multiplexing capabilities of the HTFC Screening System we were able to simultaneously discriminate the cell lines based on their color coding, and determine the level of antibodies bound to each cell line. In addition to combining target and control screens together in one, this is a cell based assay which makes it possible to perform primary screens for antibodies that bind to cell surface antigens in their natural conformation on living cells. Combined with the speed of the platform (96 well plates in as little as 3 minutes, 384 well plates in 15 minutes or less), this multiplex cell based screening assay can significantly improve productivity of the hybridoma screening process.

## ASSAY PRINCIPLE

Two cell lines were utilized to simulate a hybridoma screening process and highlight the power of multiplexing different cell types in a single well (Figure 2).

The target cell line, Jurkat 45.01, expresses CD45, whereas the control cell line, Jurkat D1.1 does not. Both cell lines express CD3.

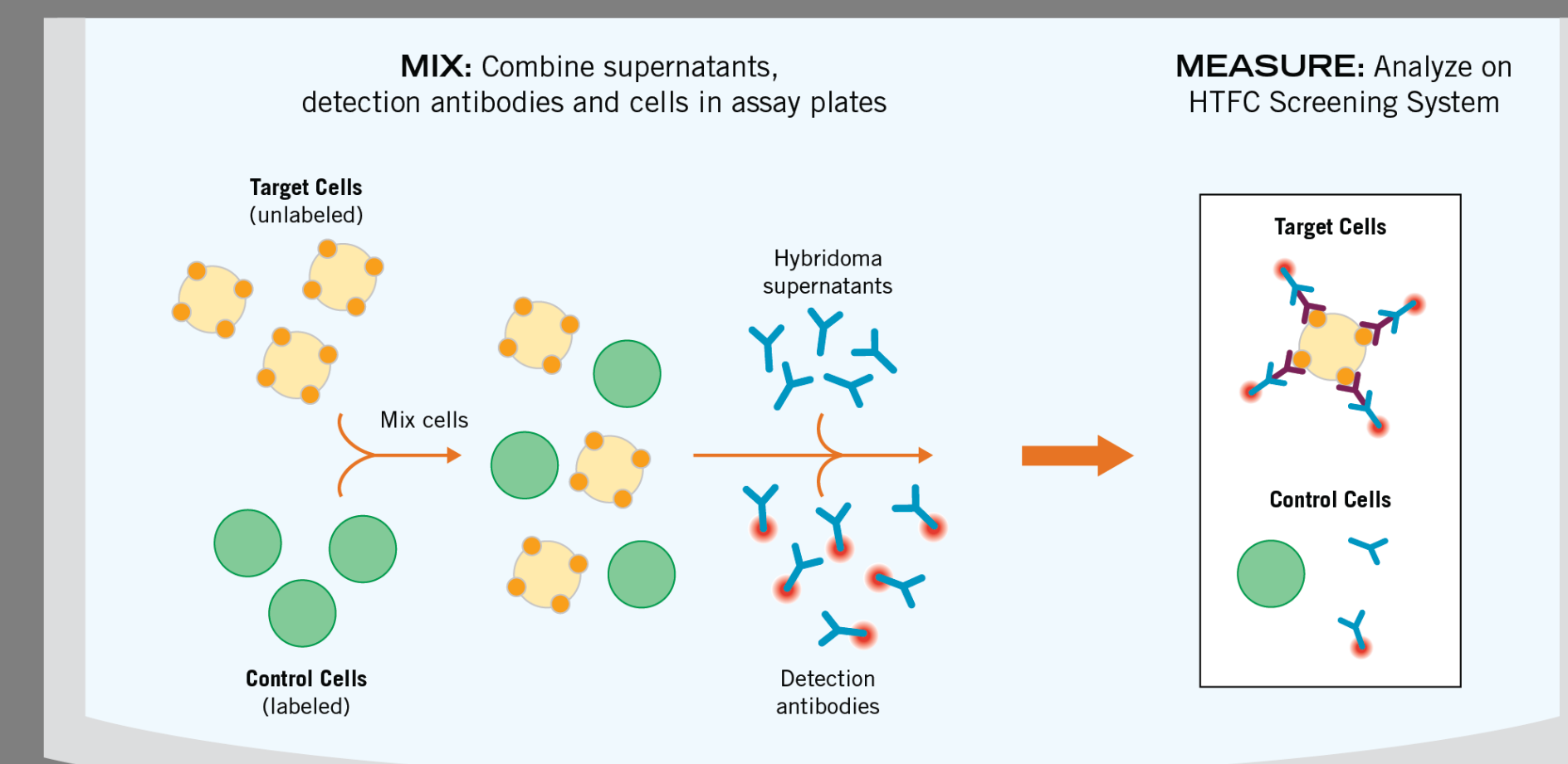
The control cells were labeled with a green fluorescent dye (Calcein AM) while the target cells were left unstained, and both cell types were mixed in equal proportions into a single cell suspension for the assay at a concentration of  $1 \times 10^6$  cells/mL.

A representative assay plate was set up to mimic the presence of both specific and cross reactive antibodies binding to the cells. Specific binding was shown with an anti-CD45 antibody to represent specific hits, as only target cells expressed this antigen. The ability to detect cross reactive antibodies was demonstrated with wells spiked with an anti-CD3 antibody which binds to both target and control cells.

Binding to the control cell signals either cross reactivity or nonspecific binding, essentially providing an internal control for every well of the assay to maximize the success of the screen.

This is a mix and read assay in which the primary antibody was added and incubated with the cells for 30 minutes, followed by the addition of the detection antibody and incubation for a further 30 minutes.

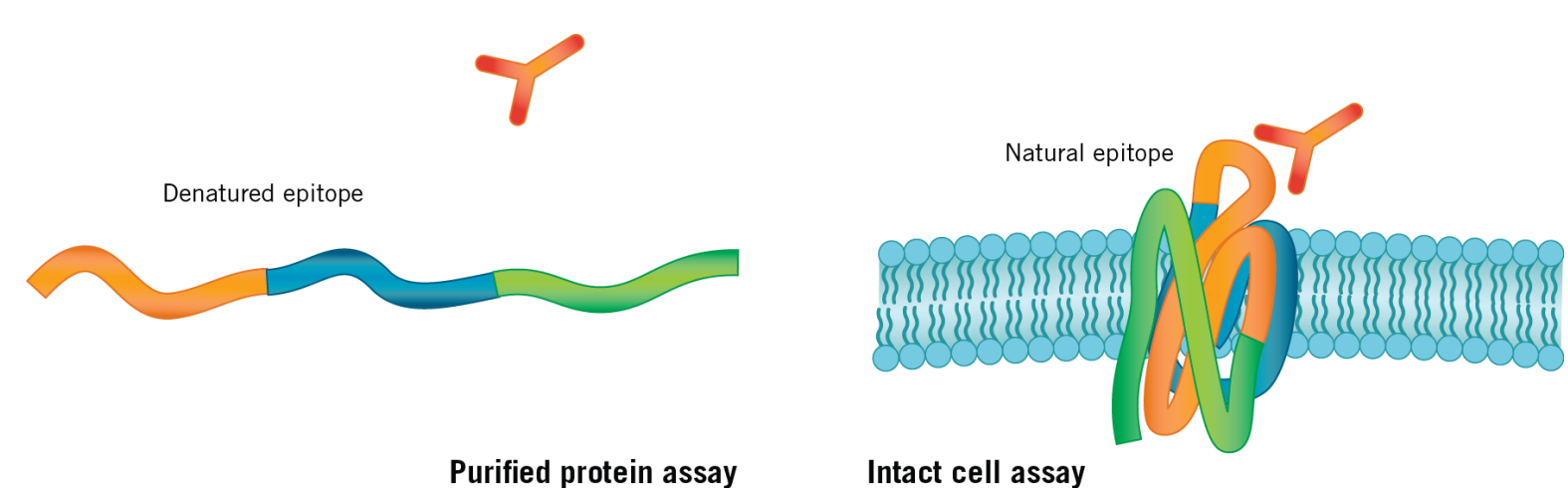
## MIX AND MEASURE WORKFLOW



**Figure 2.** Target cells (unlabeled) and control cells (Calcein labeled) are mixed together, and distributed into all the wells of the assay plates. Test antibodies, such as those present in supernatants from a hybridoma library are added to the assay plates, followed by incubation with a red fluorescent detection antibody. Plates are analyzed on the HTFC Screening System, which allows the detection of binding of antibodies from the hybridoma supernatants to target and control cell populations simultaneously. The assay volume for each well was 20  $\mu$ L. 1  $\mu$ L of the test antibodies was added to each well.

## INTRODUCTION

Antibody engineering is critical to development of new therapies in many fields such as oncology, where monoclonal antibodies are currently the most widely used immunotherapy for cancer (1). Screening hybridoma libraries for antibodies that bind to cell surface antigens is a time and resource intensive process (2). The current screening workflow often involves several individual testing steps, including different biochemical tests that test for binding, specificity, followed by cell based assays. The first step in the process, testing for binding, is traditionally performed during a primary screen using an ELISA assay in which the target antigen is purified, isolated and immobilized on the surface of the assay plates. Once binders are found in the primary screen, subsequent testing is required to ensure that the antibodies do not cross react with other antigens (specificity testing). Positive antibodies are further tested to determine if they bind to the antigen in its natural environment within the cell membrane using cell based binding assays. In addition to being costly and laborious, purified protein assays such as ELISA that utilize denatured antigens can generate false negatives - potential missed hits that would have bound to antigens in their native conformation (Figure 1). IntelliCyt has developed a "mix-and-measure" intact cell assay for primary screening that simultaneously assesses antigen binding and cross-reactivity. This assay reduces workflow steps and increases productivity. Additionally, this approach reduces the potential for false negatives due to testing with denatured antigens.



**Figure 1.** Intact cell based assay allows screening of antibodies to conformational epitopes that may not be available in ELISA and other purified protein assays where the antigens are often unfolded and denatured during the purification process.

## SCREENING ASSAY ON THE HTFC SCREENING SYSTEM

Key Features	Key Benefits
<b>Mix-and-read format</b>	Streamlined, shortened assay protocol reduces cost and variability
<b>Color coded cells combined in each well</b>	Simultaneously test binding of antibodies to target and control cells provides specificity in your primary screen
<b>Screen using antigens in their natural conformation in intact living cells</b>	Ability to detect conformational epitopes leads to fewer false negatives



### HTFC® Screening System

- High throughput suspension assays.
- 2 lasers and 6 detectors provide 4-color fluorescent and 2 light scatter measurements
- Analyze thousands of cells/second
- Sample a 96 well plate in 3 minutes or a 384 well plate in 12 min.
- Sample 1-2ul/well with no sample waste

## CONCLUSIONS

IntelliCyt's mix-and-measure hybridoma screening assay presents a powerful application to optimize the antibody screening process.

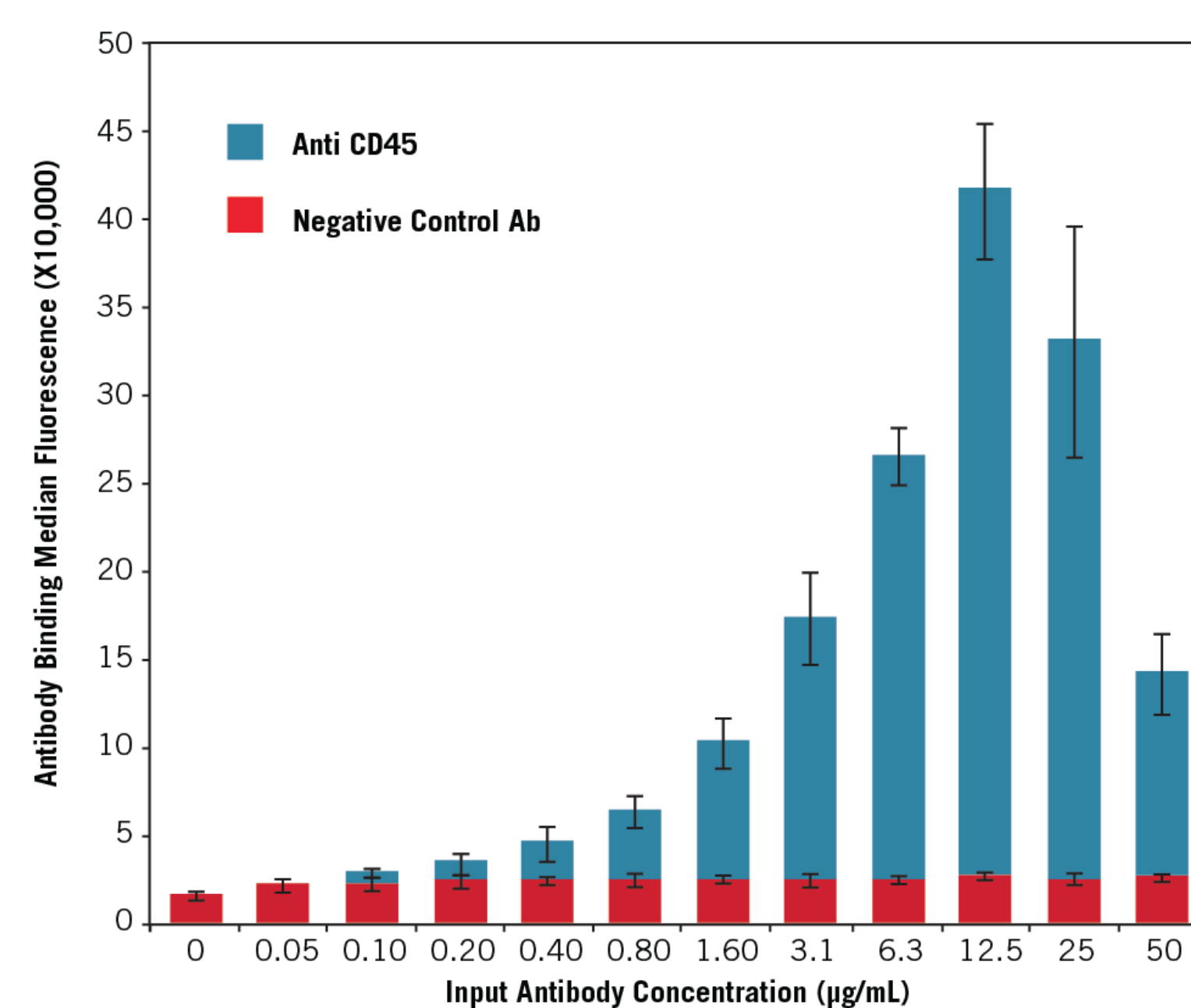
Simultaneously testing antibody binding to target and control cell lines enables users to set up primary screens that combine the ability to test binding and specificity to cell surface antigens in their natural conformation.

Our approach incorporates intrawell controls, adding confidence to screening results and significantly improving productivity of the hybridoma screening process.

Finally, we have demonstrated here a simple implementation of color coding of cells for screening on the HTFC Screening System. Variations of this assay, including the incorporation of additional color coded cell lines in each well could be used to screen cross reactive antibodies that bind to multiple members of a receptor family or to cell surface antigens from different animal species.

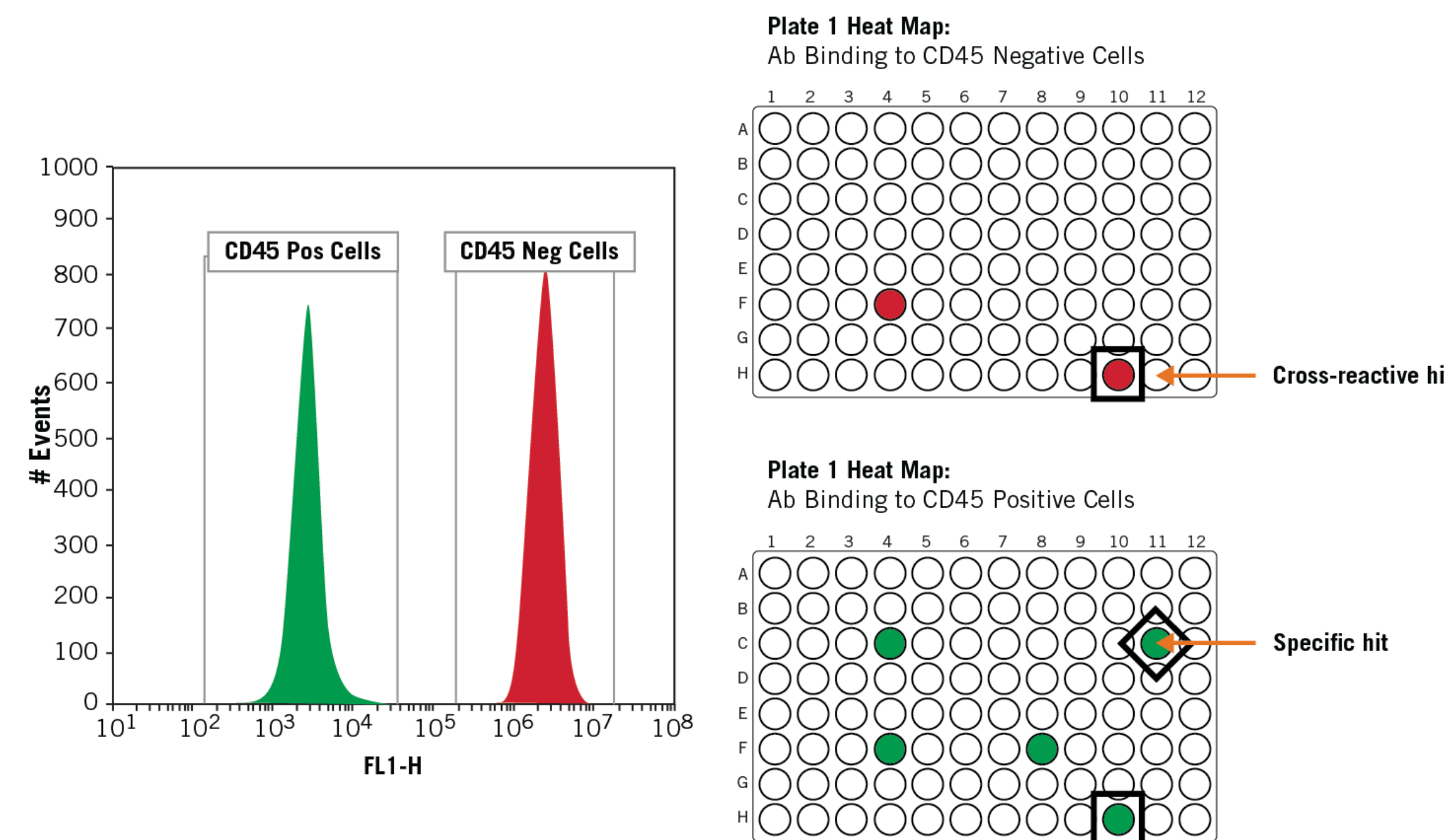
## VALIDATION OF SCREENING ASSAY

### Assay Specificity and Dynamic Range



**Figure 3.** Titrations of anti-CD45 antibody binding to CD45+ and CD45- Jurkat cells. Specific and quantifiable binding can be seen on CD45+ cells. CD45- cells not expressing the receptor show binding levels comparable to a negative control antibody. At concentrations of antibodies present in hybridoma supernatants (1-50  $\mu$ g/mL), this assay exhibits a significant S/B ratio.

### Screening Assay Metrics and "Hits"



**Figure 4.** Target cells (unlabeled) and control cells (Calcein labeled) are mixed together, and distributed into all the wells of the assay plates. Test antibodies, such as those present in supernatants from a hybridoma library are added to the assay plates, followed by incubation with a red fluorescent detection antibody. Plates are analyzed on the HTFC Screening System, which allows the detection of binding of antibodies from the hybridoma supernatants to target and control cell populations simultaneously. The assay volume for each well was 20  $\mu$ L.

## REFERENCES

- Garber, K. New Discoveries Still Abundant in Monoclonal Antibody Research. *JNCI J Natl Cancer Inst* (2000) 92 (18): 1462-1464.
- Chiarella P, Fazio VM., Mouse monoclonal antibodies in biological research: strategies for high-throughput production. *Biotechnol Lett*. 2008 Aug;30(8):1303-10. Epub 2008 Apr 17.

### Significant Signal over Background Signal for Antibody Concentrations between 1 $\mu$ g/mL and 50 $\mu$ g/mL

To determine the specificity and detection range of the assay, an anti-CD45 control antibody was tested in dose response curves against the CD45+ and CD45- Jurkat cells. Figure 3 shows that the antibody specifically bound to the CD45+ cells. A significant signal over background was observed with approximate antibody concentrations between 1  $\mu$ g/mL and 50  $\mu$ g/mL.

After sampling plates on the HTFC Screening System, the data were analyzed using a stepwise process with HyperView software, which facilitates a seamless transformation of raw data into screening results within minutes after data acquisition is complete. With the HyperView software, data from each plate of the screen is analyzed at once. Thus it is a straightforward exercise to separate the color coded cell lines, and identify antibodies that bind specifically to the CD45+ cells and those that bind in a cross reactive fashion to both CD45+ and CD45- cell lines is straightforward.