

## Technique for Loading Cells with BD Horizon™ Violet Proliferation Dye 450 (VPD450)

Jeanne Elia, Monisha Sundarrajan, and David Ernst

BD Biosciences, San Diego

# Technical Bulletin

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### Introduction

Fluorescein diacetate (FDA) and related molecules like carboxyfluorescein succinimidyl ester (CFSE) are non-fluorescent molecules that diffuse into cells. Once inside cells, they are hydrolyzed by intracellular non-specific esterases and covalently bind to cellular components to become fluorescent products. Dead cells and cells without intact membranes do not label with these dyes and are not fluorescent. The BD Horizon™ Violet Proliferation Dye 450 (VPD450) is functionally similar to CFSE since they contain both an esterase-cleavable group and an amine-reactive succinimidyl ester group. VPD450 is particularly suitable for multicolor applications where either green fluorescent proteins or FITC-labeled antibodies are used. The VPD450 dye is excited by the violet laser compared to CFSE and its fluorescein analogs which utilize the same lasers and detectors as GFP, FITC, and Alexa Fluor® 488.

FDA dyes have multiple uses which include in vitro or in vivo cell tracking experiments, fluorescent barcoding, and cell proliferation. In this set of experiments, we have optimized the use of VPD450 for cell proliferation studies. Stimulated cells labeled with VPD450 divide and form new generations of cells. Each new generation of cells has approximately half the fluorescence intensity of the previous generation. These generations of divided cells can be counted by displaying a fluorescence histogram of the V450 channel. The VPD450 dye is excited by the violet laser (emitting at 450 nm in the V450 channel), allowing for multicolor staining with additional markers using the standard blue or red lasers. Combining cell generation analysis with specific cell surface and intracellular markers allows each investigator to determine when key events are taking place in their experimental systems.

When loading cells with the VPD450 dye, there are three key details to have successful labeling: cell concentration, dye concentration, and the physical technique of loading the cells. In this technical note, we address these key considerations and demonstrate the effects of the VPD450 dye labeling on the ability of cells to proliferate or respond to activation signals using techniques such as BrdU incorporation, cytokine production, and cell signaling.

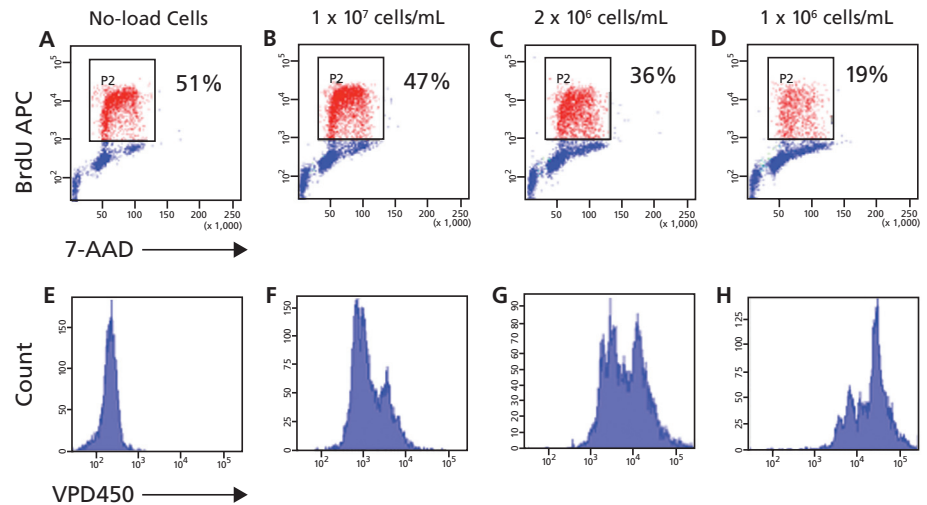


To address each of these conditions, we analyzed both mouse splenocytes and human peripheral blood mononuclear cells (PBMCs). Mouse splenocytes were isolated, made into single-cell suspensions, followed by red blood cell lysis. In the case of human samples, the blood was collected in heparinized tubes, then separated using Ficoll-Paque™ density centrifugation to isolate the PBMCs. All groups were washed twice in 1X Dulbecco's phosphate buffered saline (PBS) to remove any residual proteins from the wash buffers. The cells were transferred into 15-mL polypropylene centrifuge tubes and labeled with VPD450. Optimal conditions were determined for cell concentration, dye concentration, and loading technique. In each experiment, a no-load VPD450 group was set up as a control.

Cells were activated in 6-well plates with either phytohemagglutinin (PHA) or anti-CD3 $\epsilon$ /CD28 antibodies (PBMCs) or with anti-CD3 $\epsilon$ /CD28 antibodies (mouse spleen) for 2–5 days. Cell proliferation was then assessed between VPD450-loaded cells and the no-load (without VPD450) control cells. We further chose to monitor proliferation in the same group of cells by traditional BrdU incorporation using the BD Pharmingen™ APC BrdU Flow Kit (Cat. No. 552598). Thus, BrdU incorporation allowed us to compare the VPD450-loaded samples to the no-load controls for their ability to synthesize DNA in response to stimulation. The APC fluorochrome of the Anti-BrdU antibody (APC BrdU Flow Kit; used for detecting incorporated BrdU) was excited by the red laser and is detected in the APC channel. The 7-AAD (7-Aminoactinomycin D) dye from the same kit was used for staining cellular DNA. It was excited by the blue laser and detected in the PerCP channel. The VPD450 dye was excited using 405 nm violet laser and detected in the BD Horizon™ V450 or Pacific Blue™ channel. Data was collected using a BD™ LSR II flow cytometer and was then reanalyzed using BD FACSDiva™ software.

## Cell concentration

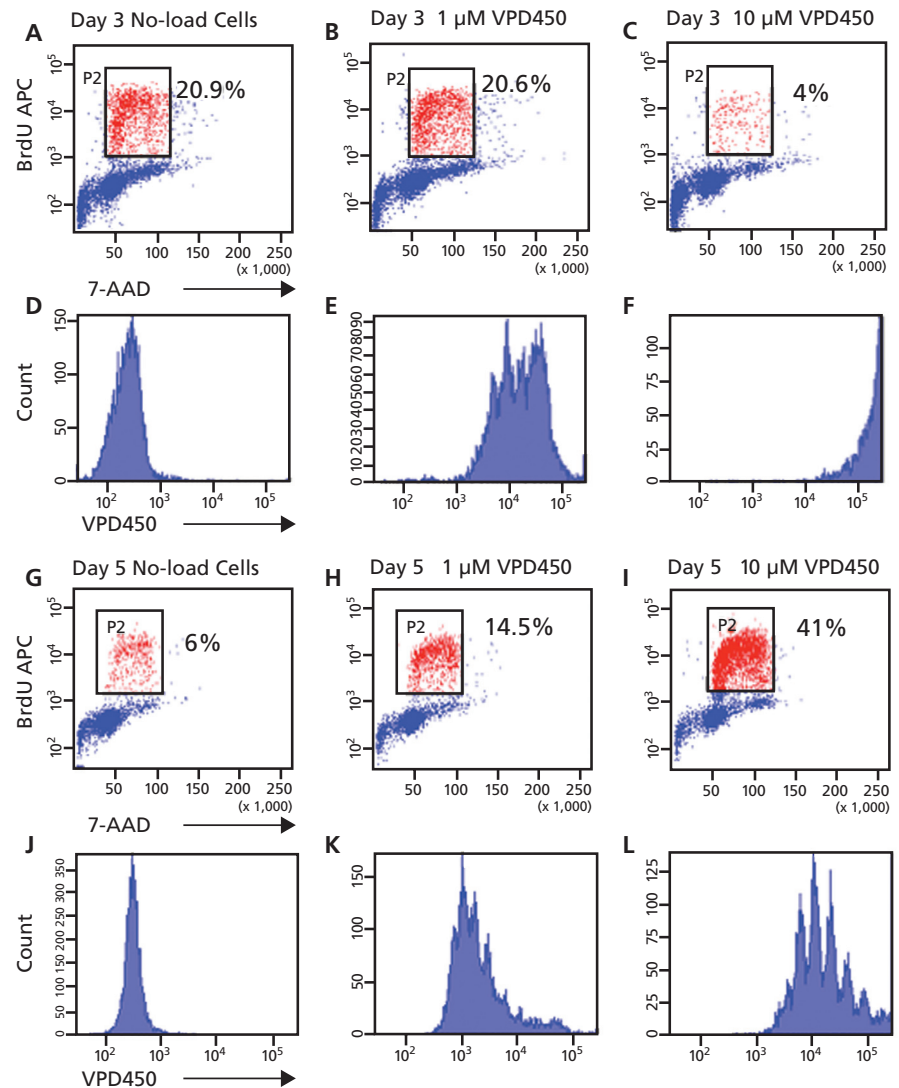
In order to find the optimal cell number for labeling with a fixed 1- $\mu$ M concentration of VPD450 dye, we set up several experiments loading three different concentrations of cells: either  $1 \times 10^6$  cells/mL,  $1 \times 10^7$  cells/mL, or  $2 \times 10^7$  cells/mL. The intensity of the initial load was compared to the ability of cells to undergo cell division (measured as number of VPD450 fluorescent peaks) after three days in culture.



**Figure 1.** Human PBMCs at different cell concentrations were either loaded with 1  $\mu$ M of VPD450 for 10 minutes at 37°C or were left as untreated controls (no-load cells).  $1 \times 10^6$  cells were plated onto a 24-well plate coated with anti-CD3 $\epsilon$  + soluble anti-CD28 antibodies, then incubated for three days. Cells were loaded with BrdU prior to harvest, then stained for incorporated BrdU (APC BrdU) and DNA levels (7-AAD). Panel A represents cells only controls (no-load cells). Cell concentrations varied from  $1 \times 10^7$  cells/mL (B),  $2 \times 10^6$  cells/mL (C), to  $1 \times 10^6$  cells/mL (D) when they were labeled with VPD450. Corresponding VPD450 histograms for the cultured cells are shown in panels E–H. The  $2 \times 10^6$  cells/mL concentration (C) displayed a decrease in BrdU incorporation of 29%, while the  $1 \times 10^6$  cells/mL concentration displayed a decrease in BrdU incorporation of 63%. This data suggests that cell concentrations greater than  $2 \times 10^6$  cells/mL should be used for VPD450 labeling to observe expected responses in cell proliferation.

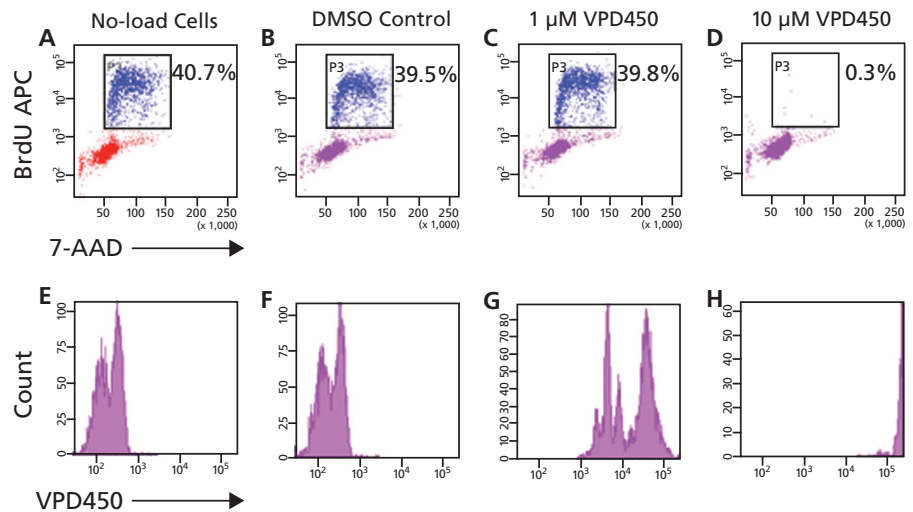
## Dye concentration

Determining the optimal concentration of the VPD450 dye is also important when setting up an experiment. It is always preferable to load with as little dye as possible while still maintaining optimal peak brightness and resolution. Higher concentrations of dye alter the ability of cells to respond to stimulation. The following example is from human PBMCs that were loaded with varying concentrations of VPD450 dye, and then stimulated for five days with PHA. Cell aliquots were pulsed for 1 hour with BrdU, harvested, and then stained at days 3 and 5.



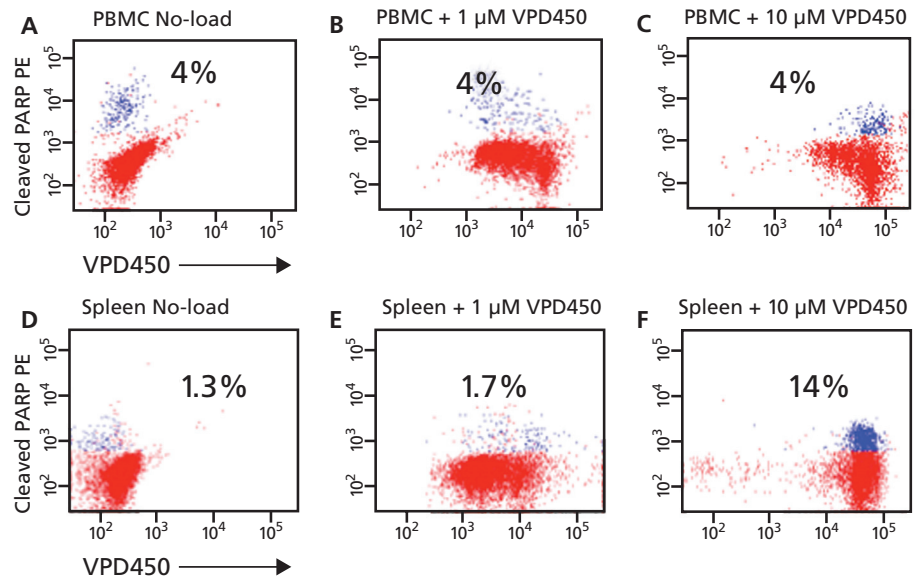
**Figure 2.** Human PBMCs were loaded with varying concentrations of VPD450 dye or left untreated as controls (no-load cells), then stimulated for five days with PHA. Cell aliquots were pulsed for 1 hour with BrdU, harvested, then stained with APC Anti-BrdU antibody (APC BrdU) and 7-AAD at days 3 (A–F) and 5 (G–L). The top panels (A–C and G–I) illustrate BrdU and 7-AAD staining, while the lower panels (D–F and J–L) illustrate the corresponding VPD450 histograms. Labeling cells with VPD450 at higher concentrations negatively affects the ability of the cells to proliferate at day 3. There was a reduction in the percentage of BrdU<sup>+</sup> cells in cells prelabeled with 10 μM of VPD450 (C) compared to both the control cell (no-load cells) (A) and the 1-μM VPD450-loaded cell populations (B). On day 5 the phenomena were reversed. The no-load cells and the 1-μM VPD450-loaded cell population had much lower percentages of BrdU<sup>+</sup> cells (G and H) compared to the 10-μM VPD450-loaded cell population (I). The 10-μM VPD450 load affected the ability of cells to proliferate in response to PHA when compared to the control cells and the 1-μM VPD450-loaded cell population.

This same phenomenon was observed in the mouse system as shown by the following data for day 2 responses of cells stimulated with anti-CD3 $\epsilon$ /anti-CD28 antibodies.



**Figure 3.** C57BL/6 mouse splenocytes were either loaded with varying concentrations of VPD450, dimethylsulfoxide (DMSO) as an excipient control, or left as untreated controls, and then stimulated with CD3 $\epsilon$  and CD28 antibodies for two days. Cells were loaded with BrdU prior to harvest, and then stained for incorporating BrdU (APC BrdU) and 7-AAD. The top panels (A–D) illustrate BrdU and 7-AAD staining. The bottom panels (E–H) illustrate the corresponding VPD450 histograms. The control cells (no-load cells) (A) and the 1- $\mu$ M VPD450-loaded cell population (C) demonstrated similar percentages of BrdU<sup>+</sup> cells (40.7% and 39.5%, respectively). The 10- $\mu$ M VPD450-loaded cell population (D) was negatively affected by the dye in its ability to incorporate BrdU (0.3% BrdU<sup>+</sup> cells). To confirm that the decrease in proliferation was not due to DMSO (which was used as a solvent for VPD450), a DMSO control was included (B). A similar percentage of DMSO-treated cells incorporated BrdU when compared to the no-load cells control group and the 1- $\mu$ M VPD450-loaded cell populations, suggesting that the concentration of DMSO used as solvent for VPD450 was not significantly toxic to the cells.

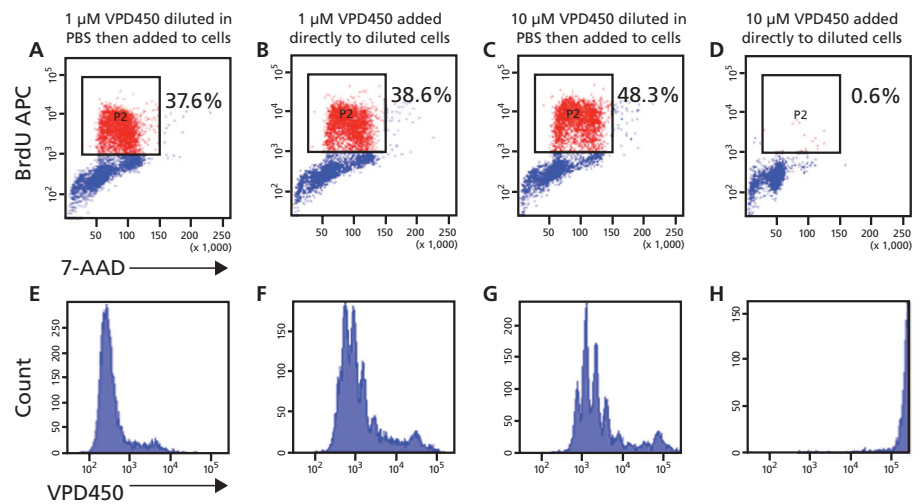
We observed that labeling cells with 10  $\mu\text{M}$  of VPD450 led to a significant decrease in cell proliferation. Therefore, we tested whether the dye also caused apoptosis by assaying for the apoptotic marker, cleaved poly (ADP-ribose) polymerase (PARP) (Figure 4). This data suggests that VPD450 does not lead to significant apoptosis in PBMCs, but may lead to an increase in apoptosis in mouse splenocyte populations when higher concentrations of dye are used.



**Figure 4.** PBMCs or BALB/c mouse spleen cells were loaded for 10 minutes with either 1  $\mu\text{M}$  of VPD450, 10  $\mu\text{M}$  of VPD450, or left untreated as controls (no-load). PBMCs were incubated for three days with anti-CD3 $\epsilon$  antibody whereas mouse splenocytes were stimulated for two days with anti-CD3 $\epsilon$  + anti-CD28 antibodies. The cells were harvested and then stained with PE-Anti-Cleaved PARP (Cleaved PARP PE) (blue events). The PBMCs do not appear to have any increased frequency of apoptosis compared to the control cells (A–C). Mouse spleen cells appear to be more sensitive to the dye. The 10- $\mu\text{M}$  concentration of VPD450 (F) led to an increase in the percentage of apoptotic cells compared to spleen no-load cells (D) and cells treated with 1  $\mu\text{M}$  of VPD450 (E).

## Cell loading

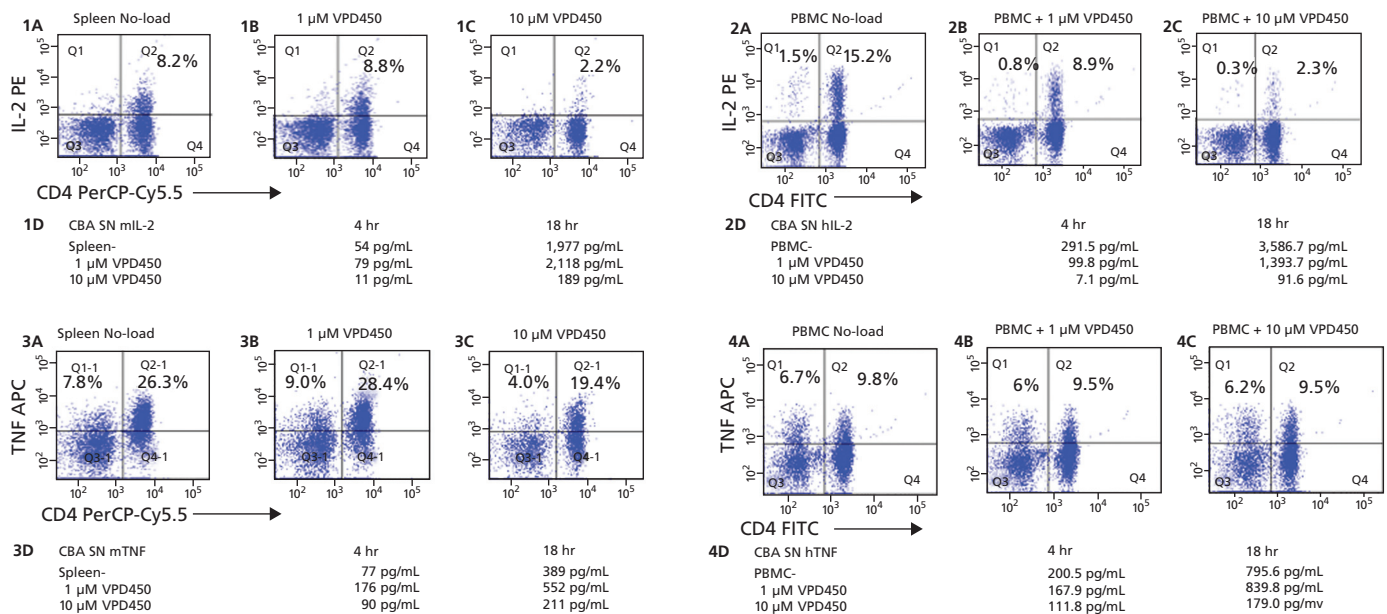
Another important detail that we chose to address was the technique of loading cells with VPD450. Loading cells with VPD450 would appear to be a simple process. However, since the dye binds to plastic, we found that it is important to add the dye directly to the diluted cell suspension rather than adding the dye to an empty tube first and then transferring the cells to that tube. The following experiment underscores the importance of adding the dye directly to the diluted cells.



**Figure 5.** Mouse splenocytes were loaded for 10 minutes at 37°C with VPD450 using two different loading techniques—either adding the dye directly to diluted cells (B, D, F, and H) or diluting the dye in PBS and then transferring the solution to a dispersed cell pellet (A, C, E, and G). The stock concentration of VPD450 is 1 mM in DMSO. To load cells with 1 μM of VPD450, we added 1 μL of VPD450 to 1 mL of cell suspension in 1X PBS. Alternatively, we diluted the VPD450 in 1X PBS first and then transferred the solution to a dispersed cell pellet. A similar protocol was followed for the higher concentration 10-μM VPD450 load. The cell concentration used in this experiment was  $1 \times 10^7$  cells/mL. When comparing the two different dye loading techniques in 1-μM loads, the group with the dye added to the resuspended cells in the 1-mL volume (B) showed discernable VPD450 peak generations whereas the 1-μM volume diluted in PBS prior to adding to cells (A) did not show any defined peaks. Alternatively, the 10-μM VPD450 load, when added to cells directly, severely inhibited the cells' responses and allowed for minimal BrdU incorporation as expected (D). However, when the 10-μM dose of VPD450 dye was first diluted in PBS and then transferred to a cell population (C), the percentage of BrdU incorporation did not decrease, and the VPD450 peaks were very well defined as seen in (G). We hypothesize that as the dye was diluted in PBS prior to addition to the cells, some VPD450 dye bound to the tube thereby lowering its concentration. The remaining dye was sufficient to label the cells and was then at a low enough concentration so that it did not interfere with cell proliferation, thereby leading to well-defined peaks as seen in (G). Based on these observations, we recommend that the DMSO-diluted dye be added directly to the cell suspension. Because the dye binds to plastic, we do not recommend diluting the dye in PBS in a separate tube prior to adding it to the cells.

## Dye effects on the cell population

Optimal cell concentrations and VPD450 dye load concentrations are critical to having a successful experiment. Therefore, we also chose to examine the potential effects that this dye can have on the ability of a cell population to produce cytokines or to transduce signals in response to exogenously added recombinant cytokines. Two techniques—intracellular cytokine (IC) staining and BD™ Cytometric Bead Array (CBA)—were used to evaluate cytokine production. IC staining determines the percentage of cells within a given population expressing a given cytokine while CBA analysis determines the amount of secreted cytokine produced by a population of cells.

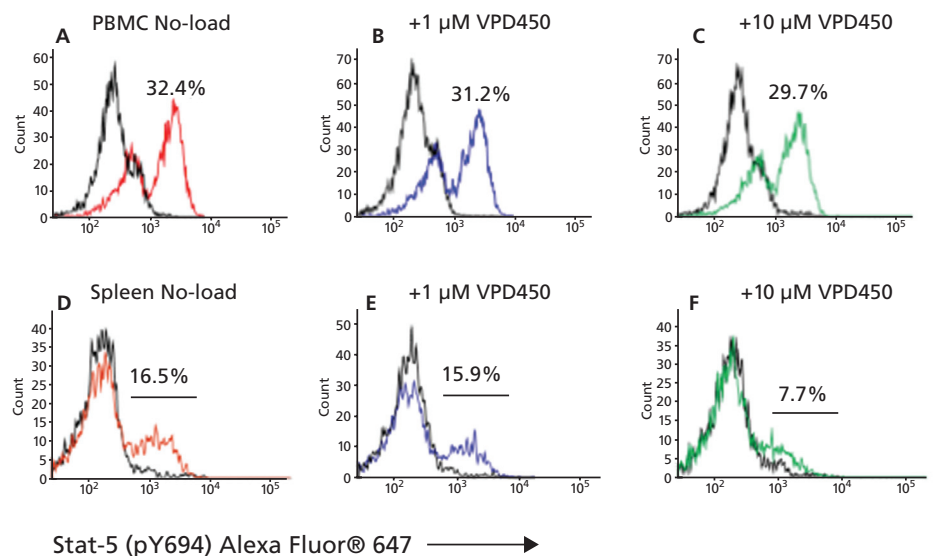


**Figure 6.** IC staining and CBA analysis of mouse splenocytes and human PBMCs stimulated with PMA + ionomycin  $\pm$  BD GolgiStop™ (monensin) protein transport inhibitor and cultured for either 4 hours or overnight. Mouse splenocytes or human PBMCs were isolated, loaded with either 1  $\mu$ M of VPD450, 10  $\mu$ M of VPD450, or left as controls (no-load). Cells were washed, then cultured at  $2 \times 10^6$  cells/mL in complete medium with PMA (50 ng/mL) and ionomycin (1  $\mu$ g/mL)  $\pm$  BD GolgiStop (monensin) for either 4 hours (for intracellular cytokine staining and CBA analysis) or cultured overnight (for CBA analysis). For IC staining, cells were harvested, fixed, and permeabilized with BD Cytotfix/Cytoperm™ fixation/permeabilization solution, then stained with fluorescent anti-cytokine antibodies: either IL-2 PE, TNF APC, and CD4 PerCP-Cy™5.5 for mouse cells or IL-2 PE or TNF APC and CD4 FITC for PBMCs. For the CBA assay, cells were set up as above but without BD GolgiStop and the culture supernatants were harvested at 4 hours and 18 hours, then analyzed with the BD CBA Mouse or Human Th1/Th2 Cytokine Kit. Panels 1A–C are mouse splenocytes stained with IL-2 PE vs CD4 PerCP-Cy5.5, panels 2A–C are human PBMCs stained with IL-2 PE vs CD4 FITC, panels 3A–C are mouse splenocytes stained with TNF APC vs CD4 PerCP-Cy5.5, and panels 4A–C are human PBMCs stained with TNF PE vs CD4 FITC. The corresponding CBA analysis data is found in Figures 1D through 4D. This data demonstrates that there was a decrease in the ability of cells to produce IL-2 when loaded with 10  $\mu$ M of VPD450 in both the mouse and the human system. Figures 1C and 2C were compared with the no-load figures 1A and 2A. The human system demonstrated a decrease in IL-2 expression in the 1- $\mu$ M VPD450 load (2B) when compared to the no-load cells (2A). The mouse system did not show any decrease in IL-2 production at the 1- $\mu$ M VPD450 load. The CBA data confirms the observations seen with the IC staining as shown in panels 1D and 2D. TNF production does not follow the same trend as IL-2. The decrease in TNF production by activated cells labeled with 10  $\mu$ M of VPD450 was much less than IL-2 production. Comparing panels 3A and 3B, the percentage of cells positive for TNF was similar, while there was a reduction in the percentage of positive cells for the 10- $\mu$ M VPD450 condition (3C). In the human system, there appeared to be no significant difference in the generation of TNF-positive cells as shown in panels 4A–4C. However, the level of soluble TNF produced by cells labeled with 10  $\mu$ M of VPD450 was significantly less when compared with the control or the 1- $\mu$ M VPD450-labeled group. The results suggest that VPD450, when loaded at higher concentrations, can impact a cell's ability to produce specific cytokines.



## Effects of dye on cell signaling events

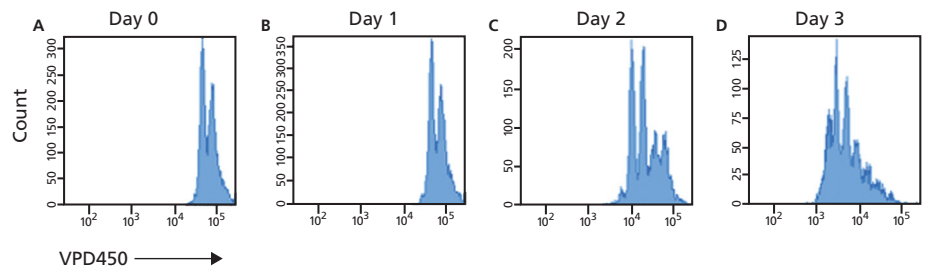
As previously observed, we know that the VPD450 dye at high concentrations decreases the ability of cells to secrete certain cytokines—especially IL-2. We chose to evaluate the cell's ability to respond to IL-2 after VPD450 labeling and compared to a no-load control. When cells are stimulated with IL-2, they signal by phosphorylating Stat-5 (pY694). Human PBMCs and mouse splenocytes were isolated, then loaded with either 10  $\mu$ M of VPD450, 1  $\mu$ M of VPD450, or left as no-load controls. Cells were plated at  $2.5 \times 10^6$  cells/well (2 mL) in a 12-well tissue culture plate, then incubated for 30 minutes at 37°C (prior to the addition of 300 ng of recombinant human IL-2) or were left untreated. Cells were incubated for 15 minutes, then fixed using BD Cytofix™ fixation buffer, followed by permeabilization with BD Phosflow™ Perm Buffer III prior to staining with anti-Stat5 (pY694) Alexa Fluor® 647 and anti-CD4 FITC. In the human system, the VPD450 dye at either concentration had no effect on the loaded cell population's ability to phosphorylate Stat5 (pY694) when compared to the no-load controls. However, mouse splenocytes were affected by the 10- $\mu$ M VPD450 dye concentration as seen by the nearly 50% decrease in the percentage of Stat5 (pY694)-positive cells.



**Figure 7.** PBMCs (A–C) or BALB/c mouse splenocytes (spleen) (panels D–F) were loaded with varying concentrations of VPD450 or left as untreated controls (no-load). Cells were plated at  $2.5 \times 10^6$  cells/well (2 mL) in a 12-well tissue culture plate and incubated for 30 minutes at 37°C (prior to the addition of 300 ng of recombinant human IL-2) or were left untreated. The cells were incubated for 15 minutes, then fixed using BD Cytofix fixation buffer, followed by permeabilization with BD Phosflow Perm Buffer III prior to staining with anti-Stat5 (pY694) Alexa Fluor® 647 and anti-CD4 FITC. The black line shows the unstimulated cells and the colored lines show the cells stimulated with IL-2. PBMCs loaded with VPD450 did not appear to show a significant difference in their ability to signal in response to IL-2 (A–C). Mouse spleen cells (gated on CD4<sup>+</sup> cells) showed a 50% decrease in their ability to signal in response to IL-2 when loaded with 10  $\mu$ M of VPD450 (F) compared to the no-load cells (D) and cells prelabeled with 1  $\mu$ M of VPD450 (E).

## Controls

We have demonstrated that the optimal concentration of VPD450 is 1  $\mu\text{M}$  and the optimal cell number is greater than or equal to  $2 \times 10^6$  cells in 1 mL. Next we determined the controls necessary for using VPD450. We observed that a VPD450-loaded day 0 control is important when setting up an experiment to account for the initial load characteristics of each cell population. The initial cell load might not be a single distinct peak, but might appear as two distinct peaks as seen in panel A of Figure 8.



**Figure 8.** Mouse splenocytes were enriched for CD4<sup>+</sup> cells using a positive panning selection technique. The enriched CD4<sup>+</sup> cells were loaded with 1  $\mu\text{M}$  of VPD450, then stimulated with anti-CD3 $\epsilon$  + CD28 (2  $\mu\text{g}/\text{mL}$ ) antibodies for three days. Cells were harvested at each day of culture including an aliquot for a day 0 time point to determine how the cell population loaded with the VPD450 dye. Panel A = day 0 load, Panel B = day 1 time point, Panel C = day 2 time point, and Panel D = day 3 time point. The cell population did not load as a single peak at day 0 (A). If this control was not present, it would be assumed that the cells had completed one round of division at day 1 (B). At day 2 (C), the cell population started to divide, which is evident by the three additional peaks, and at day 3 (D), the cell population had completed five rounds of division. The day 0 load is very important for recording the baseline VPD450 load.

## Conclusions

VPD450 can be very useful in determining the kinetics of when key events are occurring in an experimental system. The critical parameters for establishing a successful experiment are cell number, dye concentration, and load technique. Whenever a dye is added to cells, it will have some effect on the population's ability to respond to stimuli. The key is to use as little dye as possible which should minimize unwanted effects of the dye in your experimental system. It is extremely important to include a no-dye load control for comparison in your experimental setup since this control will provide the optimal response of the cell population to each treatment condition. An additional zero timepoint dye-load control is also recommended. This is useful for determining how well the cell population loaded with dye initially. Cell populations do not necessarily display as one defined peak—they may show a double peaks of VPD450 fluorescence intensities.

From experiments we determined that loading higher numbers of cells ( $1 \times 10^7$  to  $2 \times 10^7$  cells/mL) with the 1- $\mu\text{M}$  dose of VPD450 dye are recommended when compared to loading low cell numbers ( $1 \times 10^6$  cells) with the same dose based on the decrease in BrdU incorporation observed compared to the no-load cell controls. We have also shown that using high dye concentrations of 10  $\mu\text{M}$  negatively affects the ability of cells to proliferate and to secrete certain cytokines. The high concentration of VPD450 also led to decreased Stat5 (pY694) signaling by mouse cells that were stimulated with IL-2. Based upon our experiments, it has been determined that the optimal concentration for using this dye is 1  $\mu\text{M}$  and the optimal cell loading concentration is between  $1 \times 10^7$  to  $2 \times 10^7$  cells/mL.

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