ALDAGEN
TECHNOTES

# Flow Cytometer Set-up for ALDEFLUOR® and ALDECOUNT® Assay with Phenotyping: 4-color Compensation

The ALDEFLUOR (AF) and ALDECOUNT (AC) assays are compatible with immunophenotyping and cell viability methods. Both reagents show an emission spectrum similar to FITC with peak emission at 512 nm. Due to the spectral overlap of the ALDEFLUOR reagent with fluorochromes that are detected below 650 nm, we recommend using antibodies conjugated to fluorochromes that emit at higher wavelengths for antigens which typically exhibit low levels of expression. For example, to examine the coexpression of CD34 on ALDH-bright ($ALDH^{br}$) cells use the antibody combination, CD45 phycoerythrin (PE), 7-aminoactinomycin D (7-AAD) and CD34 allophycocyanin (APC). Due to the brightness of the ALDEFLUOR reagent fluorophore, adequate compensation cannot be achieved with commercially available fluorescent beads. We recommend the preparation cellular isotype-matched controls for each fluorescent parameter. Compensation control tubes should be set up as indicated in Table 1. (See Technotes TSB03 and TSB04 for more details).

Table 1: Compensation Matrix
For each compensation control tube add the antibodies as indicated by an X. See manufacturer's instructions regarding appropriate amount of antibody to use.

| | FL1 compensation control | FL2 compensation control | FL3 compensation control | FL4 compensation control |
|---|---|---|---|---|
| AF or AC reacted cells | X | X | X | X |
| FL2 Antibody | | X | | |
| FL3 Antibody | | | X | |
| FL4 Antibody | | | | X |
| FL2 Isotype matched antibody | X | | X | X |
| FL3 Isotype matched antibody | X | X | | X |
| FL4 Isotype matched antibody | X | X | X | |

## Procedure

## Setting compensation

1. Create data plots as shown in Figure 1.

2. In set-up mode, place DEAB control sample on the cytometer. On the Forward Scatter (FSC) vs. Side Scatter (SSC) plot (A), adjust FSC and SSC voltages or gains to center nucleated cell population within plot A. Adjust the R1 region to encompass the nucleated cell population based on scatter.

3. On the FL1 vs. SSC plot (B), adjust the FL1 voltage to place the right edge of the stained population at the 2nd log decade on the dot plot while retaining all nucleated cell populations on scale. **Note: all live nucleated cells will be fluorescent due to the intracellular pool of substrate**.

4. Place the corresponding AF- or AC-reacted sample on the cytometer. Create a region R2 in plot B to encompass the cell population that is ALDH-bright and side scatter low ($ALDH^{br}SSC^{lo}$). Remove the tube.

5. Place FL1 compensation control sample on the cytometer. On the FL2 vs. FL4 plot (E), adjust FL2 voltage so that all signals fall within first decade of the FL2 axis. Verify similar signal placement in plot D.

6. On the FL3 vs. FL4 plot (F), adjust FL3 voltage so that all signals fall within first decade of the FL3 axis. Verify similar signal placement in plot D.

7. On the FL2 vs. FL4 plot (E), adjust FL4 voltage so that all signals fall within first decade of the FL4 axis. Verify similar signal placement in plot F.

8. On the FL1 vs. FL2 plot (C), adjust the FL2 - FL1 compensation percentage to remove FL1 signal carryover into FL2 and to place population on FL1 axis. Verify position of positive FL1 signals and negative FL2 signals on plot G.

9. Place FL2 compensation control sample on the cytometer. On the FL2 vs. FL4 plot (E) check for spillover of signal into FL4 channel. If spillover is detected, lower FL4 voltage until all signals are within first decade of FL4 axis. On the FL2 vs. FL3 plot (D), adjust FL3 - FL2 compensation to remove FL2 signal carryover into FL3 and to place population on FL2 axis. Verify position of positive FL1 and FL2 signals on plots C and G.

10. Place FL3 compensation control sample on the cytometer. On the FL2 vs. FL3 plot (D) check for spillover of FL3 signal into FL2 channel. If spillover is detected, adjust compensation to remove FL3 signal carryover into FL2 and to place population on FL3 axis. On the FL3 vs. FL4 plot (F) check for spillover of FL3 signal into FL4 channel. If spillover detected, adjust compensation to remove FL3 signal carryover into FL4 and to place population on FL3 axis. Verify position of positive FL1 and FL3 signals on plot H.

11. Place FL4 compensation control sample on the cytometer. On the FL2 vs. FL4 plot (E) check for spillover of FL4 signal into FL2 channel. If spillover is detected, lower FL2 voltage until all signal is within 1st decade of FL2 axis. On the FL3 vs. FL4 plot (F) adjust compensation to optimize FL4 positive signal. Verify position of positive FL1 and FL4 signals on plot I.

12. Place FL1 control sample on the cytometer. Verify adequate detection of AF or AC signal in FL1 channel and appropriate placement of negative signals in FL2, FL3 and FL4 channels.

## Data acquisition

1. Remove cytometer from set-up mode and acquire a data file for each compensation tube with a minimum of 20,000 events using the same instrument settings for each tube.

2. Without adjusting instrument settings, collect a data file of 100,000 events for each ALDH and DEAB sample.

Figure 1: Compensation Template
Data plots for performing compensation. See procedure for details.