Appendix 1: 3-Color Advanced Flow Cytomtery Analyses of Sample Histograms

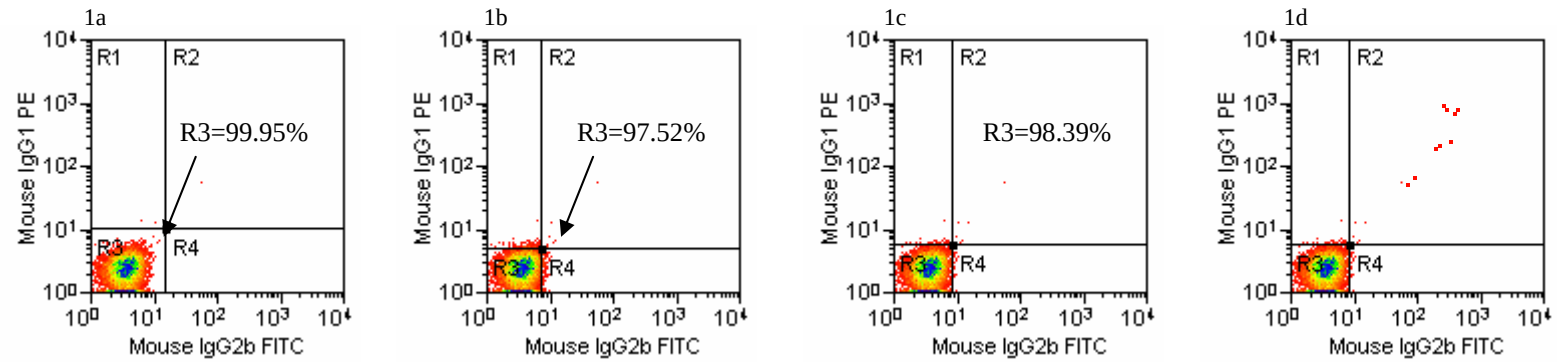
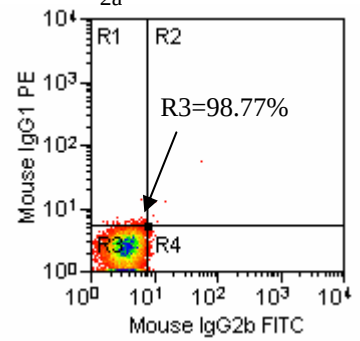


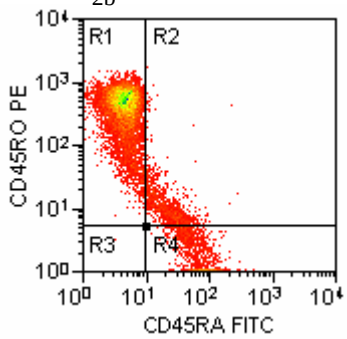
Figure 1: Isotype controls are used as a guide for quadstat cursor placements in test samples. The lower left quadrant ***must*** contain between 98% and 99% of all events. Figure 1a contains too many events in R3 and Figure 1b contains too few. Figure 1c shows correct cursor placement. Occasionally an additional population of events will be seen that forms a diagonal streak in the upper right quadrant. In this case the cursors should be placed to closely abut the negative events in R3. (Figure 1d) ***Only in this circumstance is it permissible to have less than 98% of the events in the lower left quadrant. (Figure 1d)***

Gated on CD4+(bright)

2a



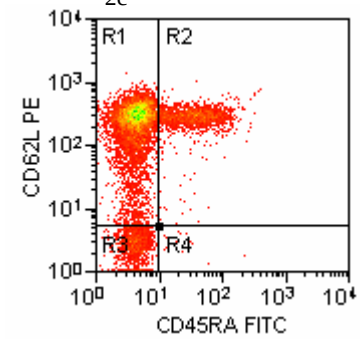
2b



R2=11.37%
R4=10.07%

Total CD45RA=21.44%

2c

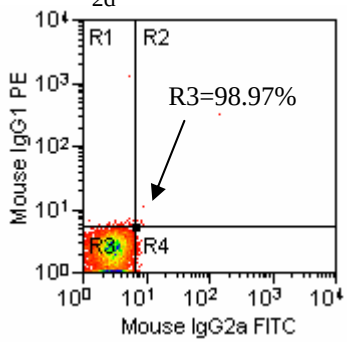


R2=19.37%
R4= 0.18%

Total CD45RA=19.55%

Gated on CD8+(bright & dim)

2d



2e

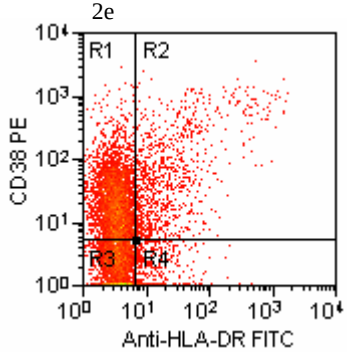


Figure 2: In this sample the cursors were moved slightly to the right on the CD45RA-FITC to delineate the obvious valley in this sample. Total CD45RA % positive was used as a quality control measure. The total CD45RA % should match within 3% for the two tubes containing CD45RA. CD38/HLA-DR samples show a wide variety of patterns and therefore the cursors ***must never*** be moved from the isotype positions for the CD38/HLA-DR tube.

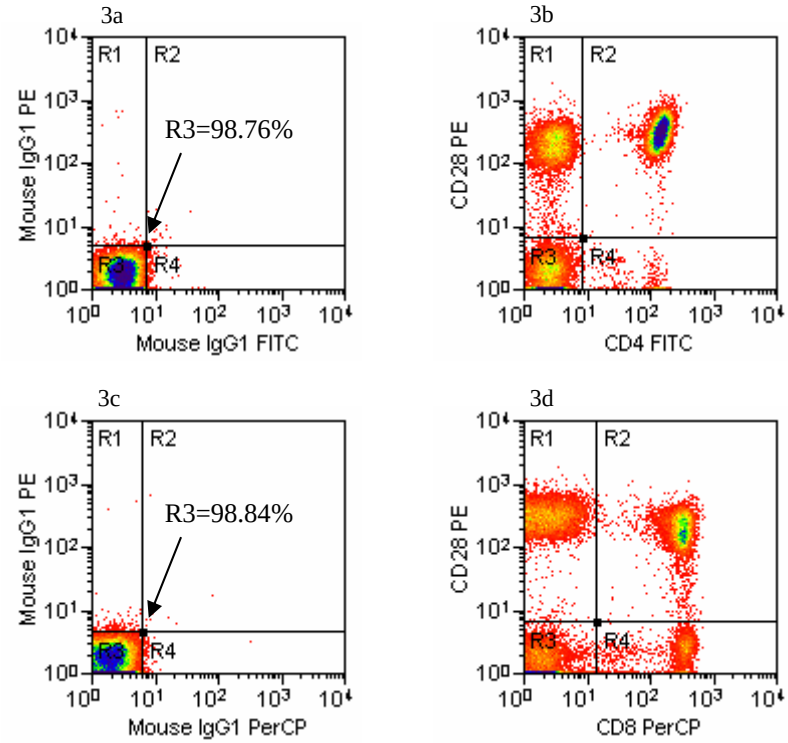
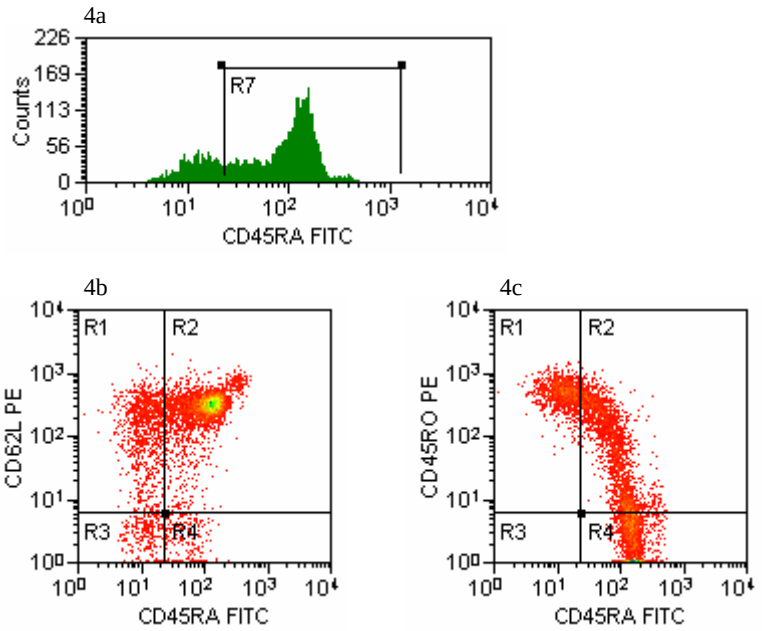


Figure 3: The same specimen that was shown in Figure 2 required only minor cursors adjustments to fit the obvious valleys in these CD4/CD8/CD28 histograms.



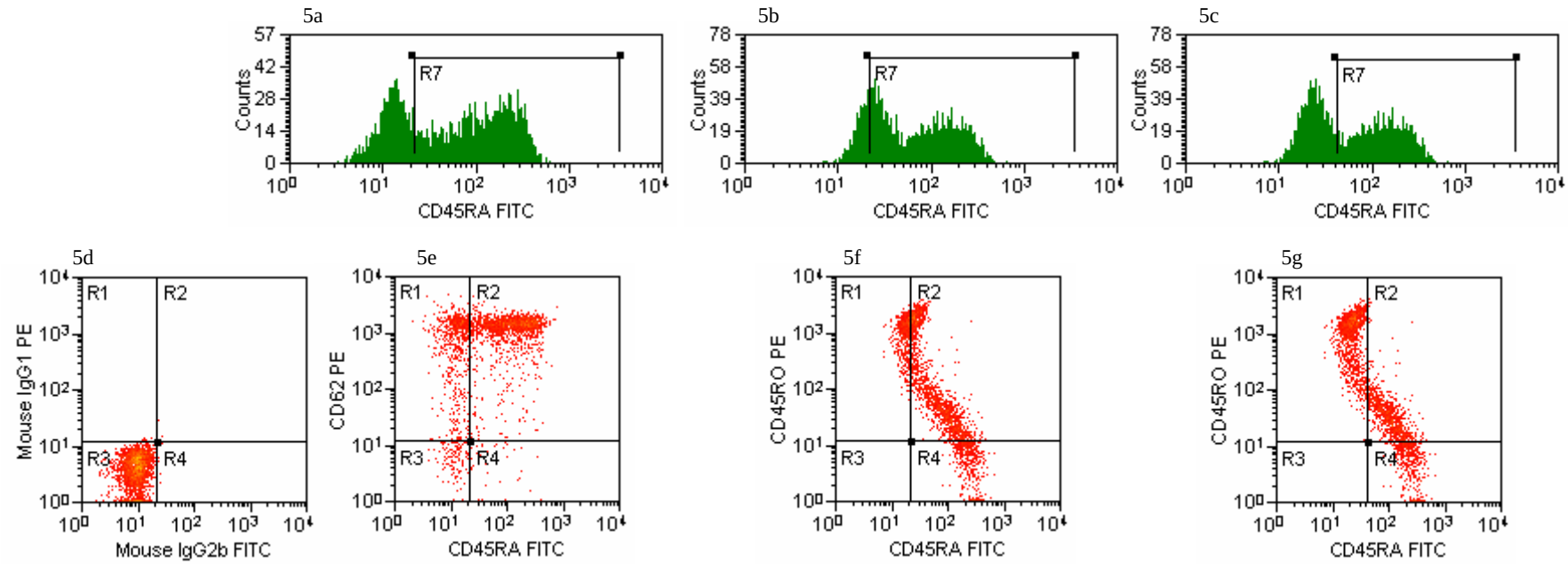
R2=70.27%
R4= 4.04%

Total CD45RA=74.31%

R2=44.94%
R4= 30.47%

Total CD45RA=75.41%

Figure 4: A single parameter histogram (4a) should be used as a tool for determining the proper placement of the CD45RA-FITC cursor. The right side of the negative peak can be seen more clearly in the single parameter histogram than in the two-parameter plots. ***CD45RA-FITC frequently requires a slight adjustment of the vertical cursor to the right of that used for the isotype controls..***



R2=63.61%
R4= 1.54%
Total CD45RA=65.15%

R2=65.12%
R4=16.28%
Total CD45RA=81.40%

R2=38.58%
R4= 16.28%
Total CD45RA=54.86%

Figure 5: Occasionally a sample will show a variation between the two samples containing CD45RA. The cursor placement shown for CD45RA/CD62L is not appropriate for the CD45RA/CD45RO sample(5b&5f). b. The cursor setting shown in 5c&5g is the appropriate setting. Note that in this case the sums due to not match in either setting.

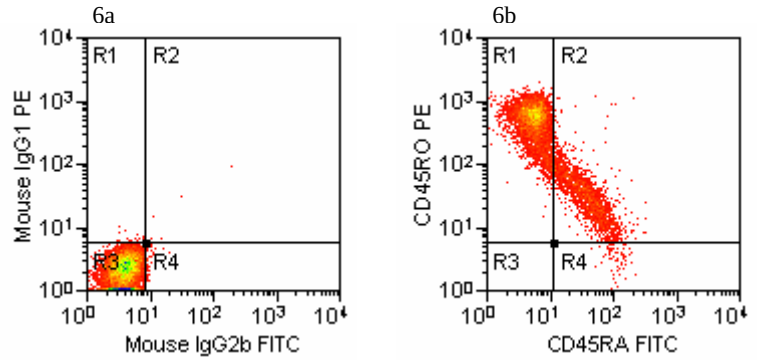
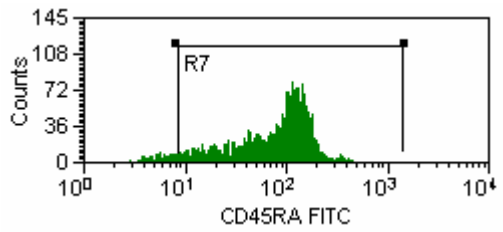
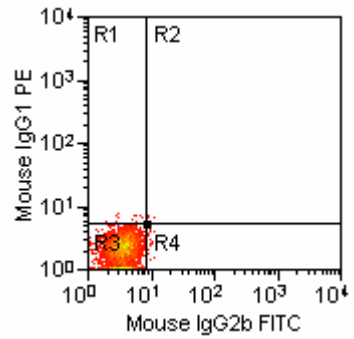


Figure 6: Patients sometimes have very low numbers of CD45RO-RA+ cells.

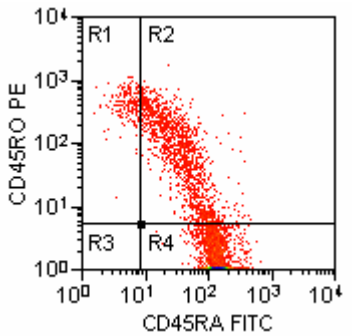
7a



7b



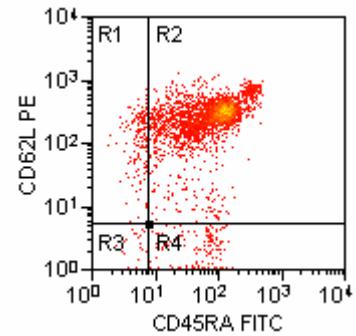
7c



R2=43.54%
R4=49.65%

Total CD45RA=93.19%

7d

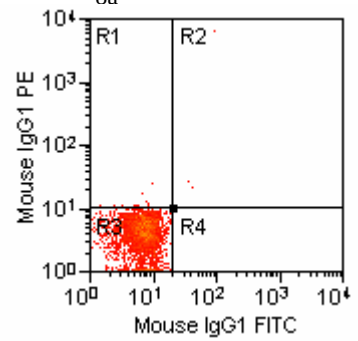


R2=88.66%
R4= 4.94%

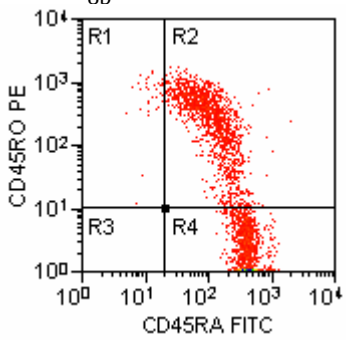
Total CD45RA=93.60%

Figure 7: Only 6-7% of the cells in this sample are CD45RA negative. A cutoff for the negative peak is not visible in the single parameter histogram(7a). ***The best available information for positioning the quadstat cursors in this circumstance is the isotype cursor setting and no adjustment should be made.***

8a

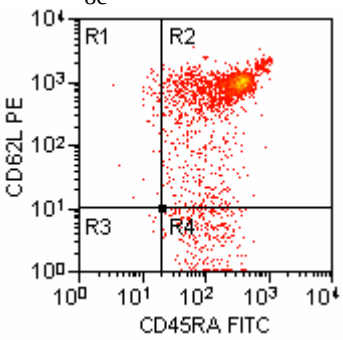


8b



R2=52.99%
R4=45.42%
Total CD45RA=98.41%

8c



R2=87.83%
R4=10.33%
Total CD45RA=97.66%

Figure 8: Occasionally samples have expression of CD45RA on all of the T-cells in the sample. This is a known mutation in which CD45RA expression is not down-regulated. ***The best available information for positioning the quadstat cursors in this circumstance is the isotype cursor setting and no adjustment should be made.***

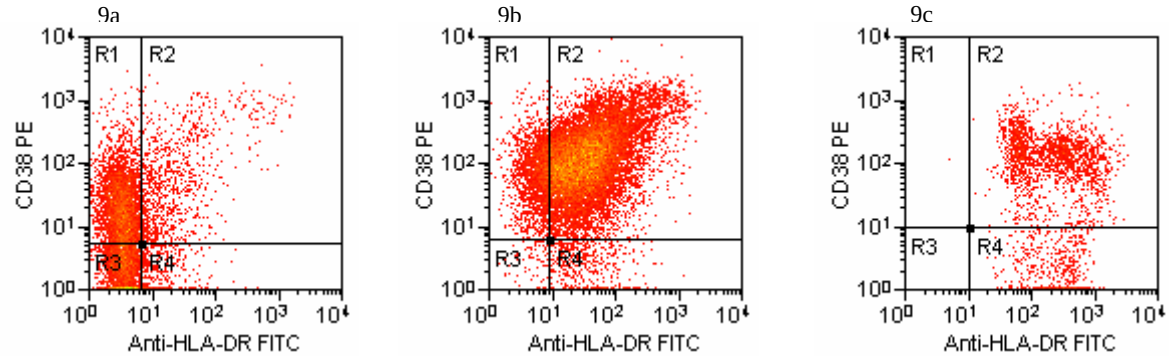


Figure 9: CD38/HLA-DR samples show a wide variety of patterns and therefore the cursors ***must never*** be moved from the isotype positions.

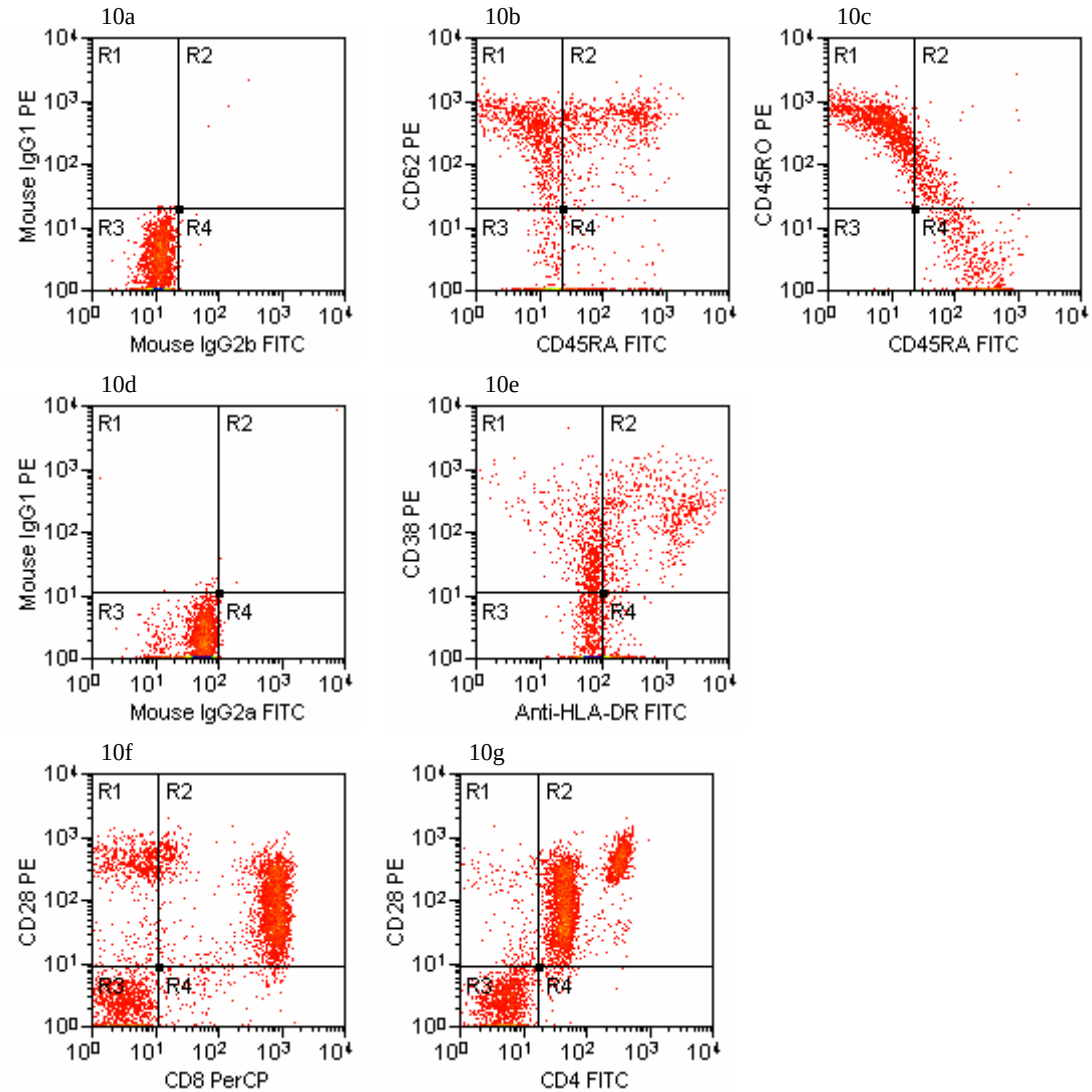


Figure 10: Occasionally samples show artifactual staining(10d,10e,10f,10g) which resembles uncompensated spectral overlap despite appropriate compens
This is an artifact that results from an immunoglobulin present in the plasma of the blood specimen. If pre-washing the sample does not remove this artifact,
results should not be reported.

Summary:

Isotype control cursor settings should contain 98% to 99% of all events in the lower left quadrant.

The CD4/CD8/CD28 cursor settings should be adjusted to match the obvious valleys.

CD45RA-FITC cursor settings frequently need to be adjusted to delineate the negative population more accurately.

CD38/HLA-DR cursor settings must match those of the isotype controls.