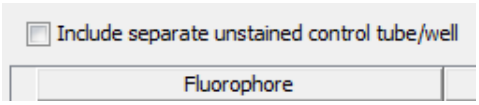
PROCEDURE A4: Performing Automated Compensation in BD FACSDiva™ Software

1. Click *Experiment --> Compensation Setup --> Create Compensation Controls*. Un-tick the box for the Unstained Compensation Control tube. Click OK.



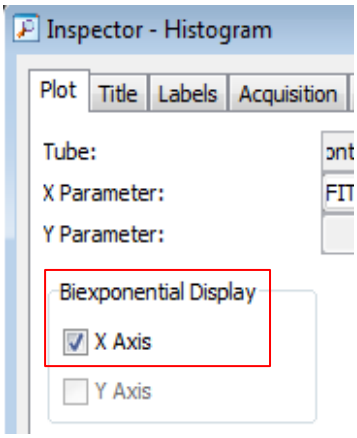
2. A new Specimen called "Compensation Controls" is created in the Browser, plus a series of Normal Worksheets (one sheet per fluorochrome). Expand the Specimen and activate the first Tube.

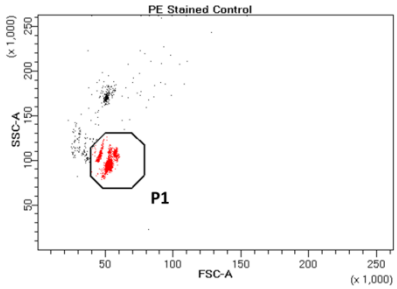
3. Acquire the first CompBead Control. If necessary, adjust FSC and SSC voltages to bring the beads on scale. Place the **P1** gate around the bead population. Right-click the **P1** gate and select "Apply to All Compensation Controls". ***Record data (5,000 events) on LOW flow rate.***

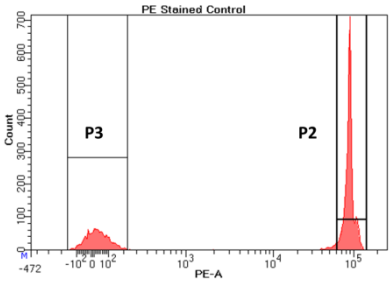
   a. Adjust the **P2** interval gate to encompass the positive population.

   b. Click on the histogram and apply *biexponential scaling* to the X axis.

   c. Add an interval gate (**P3**) to encompass the negative population.



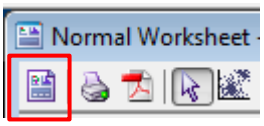




4. Record data for the other CompBead/Single Stained Cell Controls and repeat **Steps 3 a - c** for each. ***Overwrite the data with Single Stained Cells if antigen expression is higher than the CompBead signal.***

5. Click *Experiment --> Compensation Setup --> Calculate Compensation*. Select *"Apply Only"* when prompted.

6. Toggle back to the Global Worksheet by clicking the first icon on the Worksheet toolbar. Add a new Specimen and Tubes for your Full Stained and FMO samples.



7. Click *Experiment --> Experiment Layout* to apply marker names and stopping gates. On the Global Worksheet, add dot-plots and gates as shown in **Figure 1**.

Start acquiring data using FSC and SSC voltages appropriate for lymphocytes.
Record at least 30,000 events in the Lymphocyte gate using a LOW flow rate.