vFC™ Protocol 0.4 - Fluorescence Compensation using vCal™ nanoComp Beads

Objective

Determine the spillover and compensation matrices to account for spectral spillover in the relevant fluorescence channels.

Materials

- vCal™ nanoComp antibody capture nanobeads (two peaks, one positive, one negative, 800 nm diameter polystyrene, $1x10^7$/mL)
  Note: Capture beads are available for each of mouse, rat, rabbit, and hamster antibodies.
- Fluorescent antibody conjugate(s) (10x)
- vFC™ Staining Buffer
- v-bottom plate or 0.65 mL microfuge tubes

Procedure

1. Briefly vortex vCal™ nanoComp Beads. n
2. Add one drop (~50 uL) of nanoComp beads in a v-bottom plate or 0.65 mL microfuge tube for each marker to be calibrated.
3. Add 5 uL of each fluorescent antibody conjugate to be calibrated to a different well.
4. Incubate for 1 hour at RT.
5. Wash beads by in 300 uL buffer by two cycles of filtration (<0.45 um pore) or centrifugation (15,000 xg, 10 min) and resuspension. Resuspend in buffer (100-300 uL) and measure at same fluorescence detector gains as for vFC™ analysis.
6. Save data files with informative names that include the data and antibody conjugate used to stain the bead (eg 210704 nanoComp CD9 PE.fcs).

Data Analysis

1. Open the vCal™ AbCap Compensation Bead Report layout using FCS Express or FCS Express Reader
2. Load the data files from the antibody-stained nanoComp beads (plus the unstained bead control) and a Lipo100™ reference sample into the Data List.
3. On the **nanoComp Tab,** use the bivariate plot of scatter parameters (eg VSSC vs SSC) to identify and gate on the population of single beads. Load the single color bead and Lipo100 data into the appropriate individual histograms.
4. Click on the **Tools** tab→**Transformations** group→**Compensation and Unmixing** command to open the **Compensation and Unmixing** navigator (Figure 1).
5. The **Compensation and Unmixing** navigator can be moved anywhere on the screen; it is dockable and pinnable, and you can move it around the screen or close and reopen as needed.

6. Click on the blue plus button,  , to **Add a new compensation** (Figure 1, red outline).
7. A text box highlighted in blue will appear with the text **New Compensation** (Figure 1).

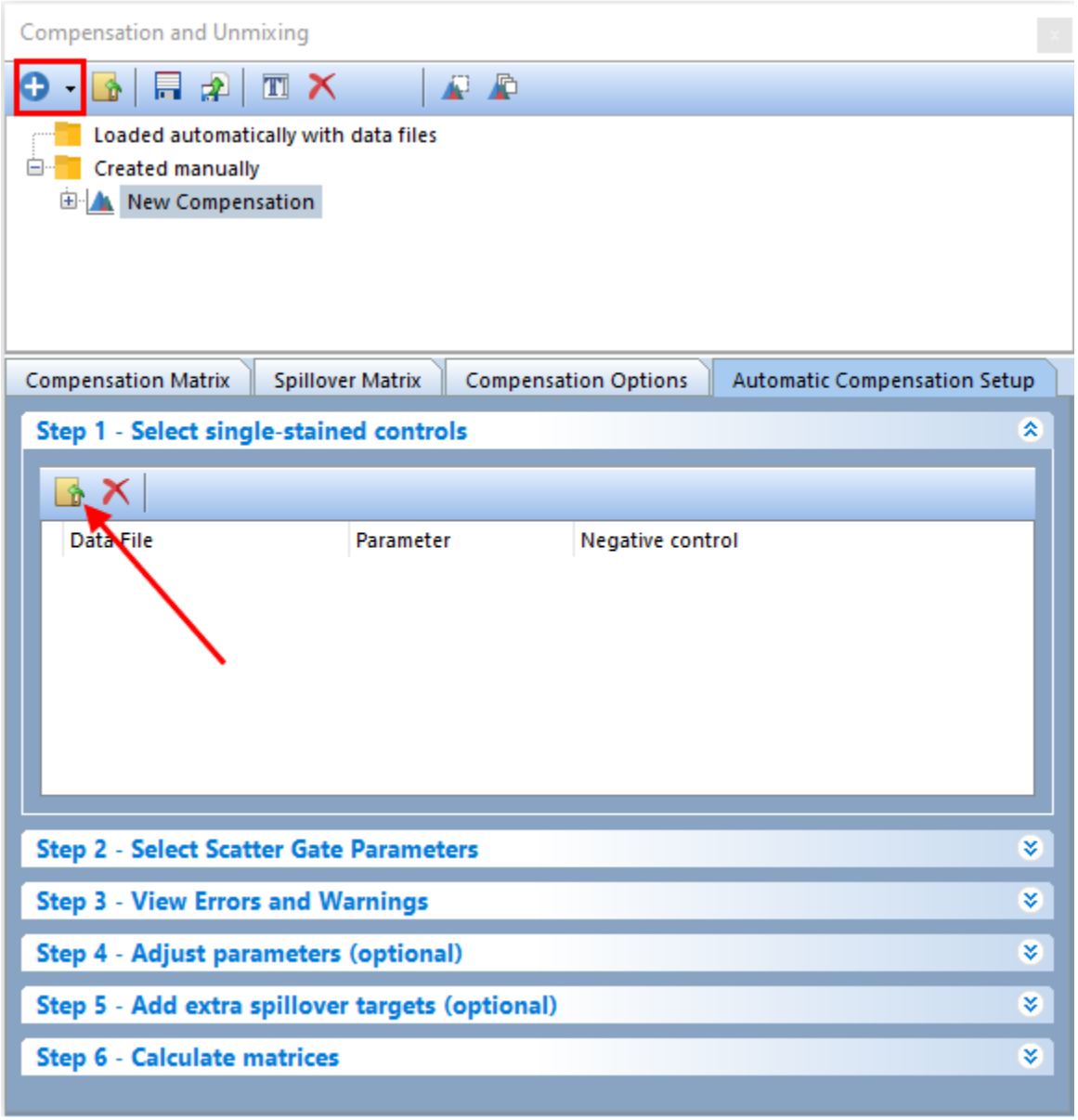
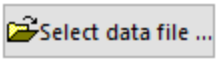


Figure 1. Compensation and Unmixing navigator: use to add new compensations, edit definitions, create Automatic Compensations, and unmix spectral signatures

8. Click on the Add data for compensation button,  , in the **Automatic Compensation Setup→Step 1 - Select single-stained controls** window (Figure 1, red arrow).
9. The **Standard Open Data Dialog** will appear (Figure 2).
10. Note: based on your User Options, the Advanced Open Data Dialog may appear. If the **Advanced Open Data dialog** appears, please select the 

button to access the **Standard Open Data Dialog**.

11. Select and load the data files from the antibody-stained nanoComp beads (plus the unstained bead control) and the Lipo100™ reference samples.
12. Click **Open file**.

Check that all of the single-stained control files and the unstained control file have been brought into the **Select single-stained control** window and have been assigned the correct parameter and assigned a negative. You may correct the **Parameters** and define the **Negative Control** used per control as necessary. When loading a universal negative, or unstained sample, it is assigned automatically as the **Negative control** for each single-stained control (Figure 2).

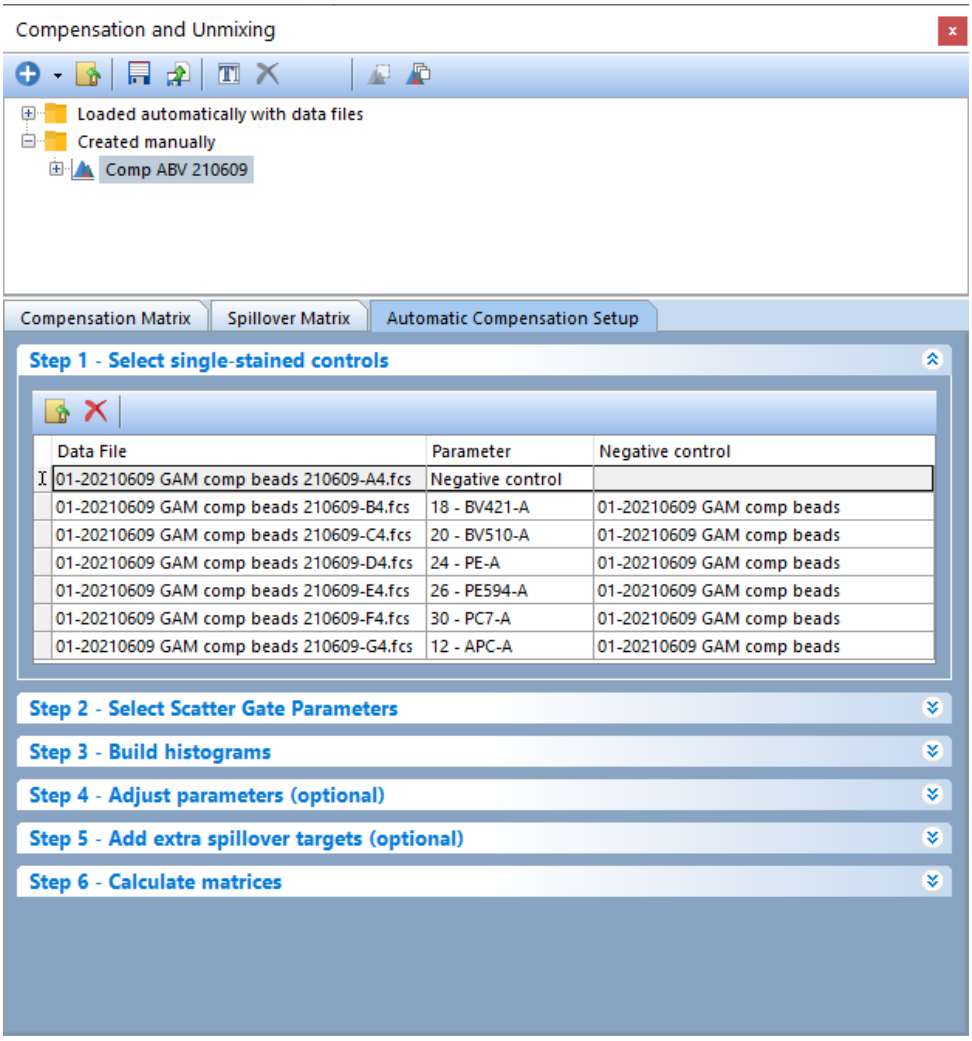


Figure 2 Files for controls have been added to Step 1 in the Automatic Compensation Setup and are matched automatically to their respective parameters.

13. Set the Scatter Gate Parameters (Step 2) to SSC-H and VSSC-H (Figure 3).

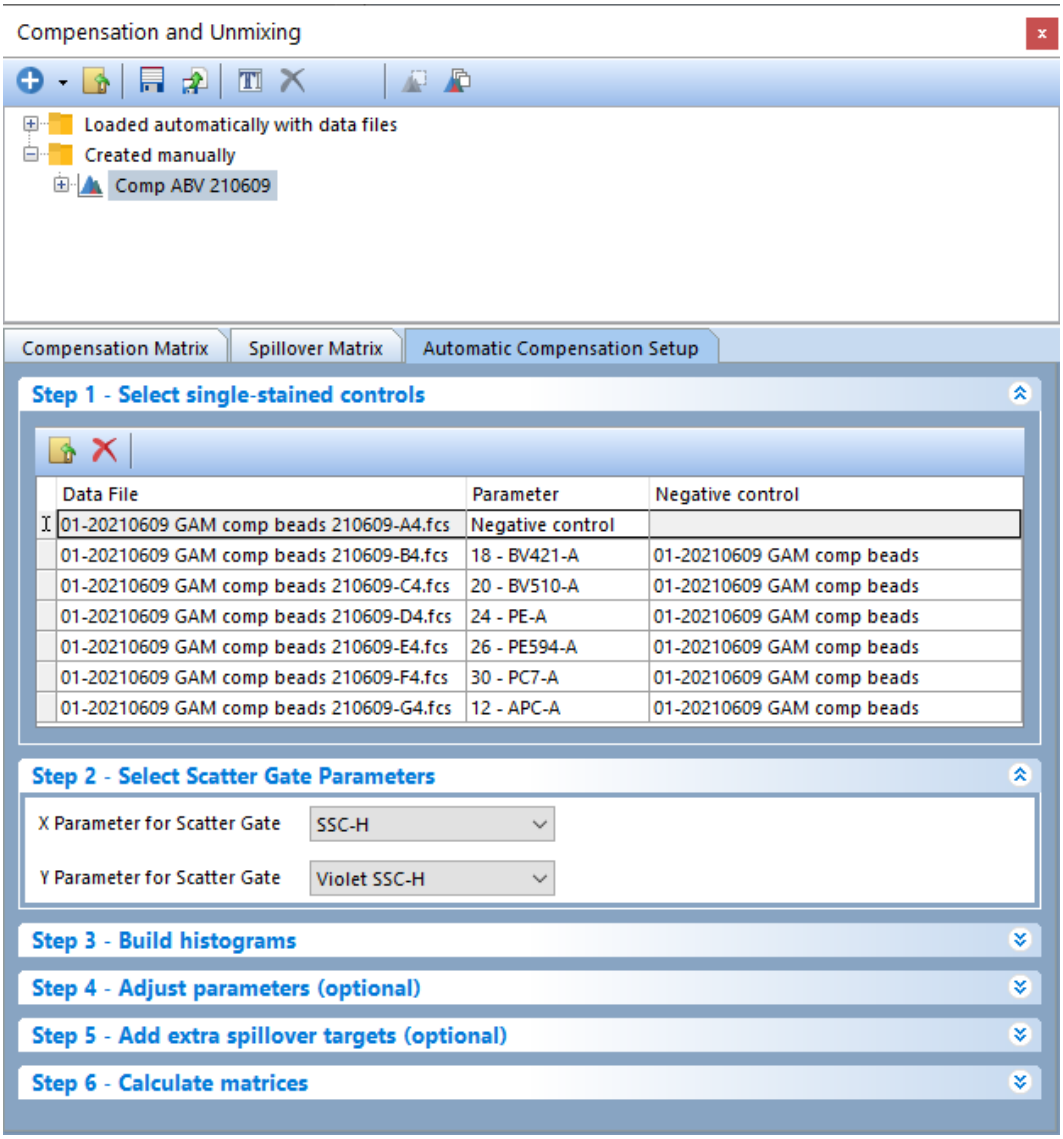


Figure 3. Select scatter gate parameters

14. Click Build Histograms (Figure 3), and FCS Express will automatically create a page for Scatter Plot and also for each individual Single Stained control.
15. Inspect the plots on the newly created pages (Figure 4). Inspect and adjust the Scatter Gate if necessary to select the population of single stained beads. For the antibody-stained beads, confirm that the positive and negative populations are correctly identified.
16. Click Calculate Matrices (Step 6) using the median fluorescence intensity values. The Spillover and Compensation matrices will be calculate (Figure 5).

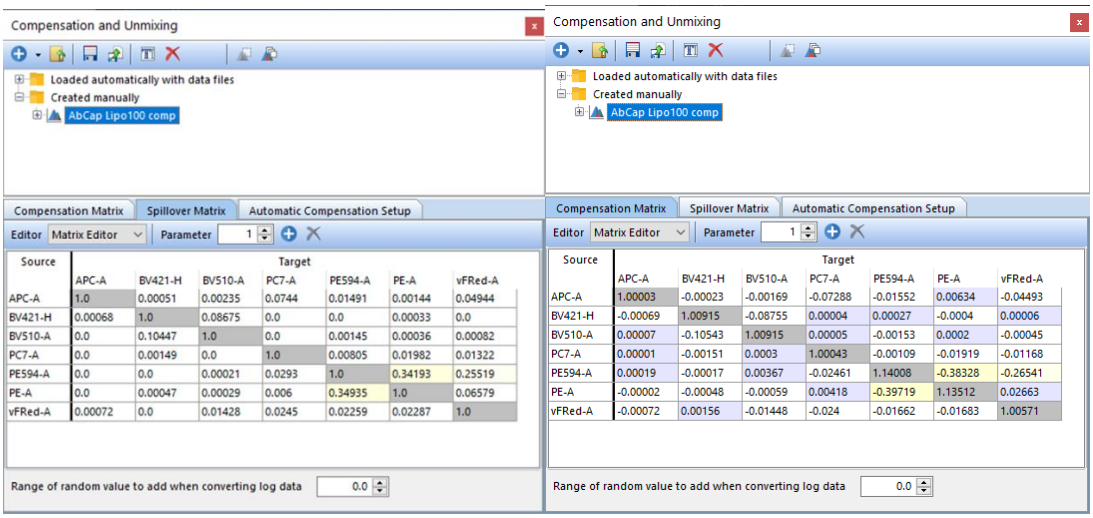


Figure 5. The calculated Spillover and Compensation matrices.

17. Expert the compensation matrices as a **.compensation* file with an informative name (eg. 210609 AbCap Lipo100.compensation).
18. Save the Layout with a similarly informative name as documentation of the calculations that produced the Spillover and Compensation matrices.

Notes

For more information about using FCS Express for Compensation and Spectral Unmixing, see the FCS Express manual, Tutorials, and videos at denovosoftware.com.

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