



Save time with the MACSQuant® Instrument

Automated compensation

Background

Instrument compensation is crucial for the optimal acquisition and display of data obtained by flow cytometry. Compensation essentially accounts for the inherent overlap in emission spectra observed between different fluorochromes. This fluorescence spectral overlap, or 'spillover', will result in the detection of individual fluorochromes in more than one fluorescence channel. Spillover must be determined and corrected. This is especially important when multicolor analyses are performed – without proper compensation, results may be misinterpreted.

Performing auto-compensation on the MACSQuant® Family of flow cytometers

Most flow cytometer users would acknowledge that instrument compensation is a rather laborious and time-consuming procedure. This is not the case when using the MACSQuant® Family of flow cytometers, which can perform fully automated compensation on all channels at one time. Using either single-stained cells or compensation beads, the MACSQuant Instruments can accurately and reliably perform automated compensation based on a 8×8 matrix (each channel compensated against the other 7 channels). In this application note, 8-color automated compensation using peripheral blood mononuclear cells (PBMC) is detailed for the MACSQuant Analyzer 10.

Materials and methods

Reagents and solutions

- Freshly prepared PBMC sample
- Suitable fluorochrome-conjugated antibodies; for a comprehensive list visit www.macsquant.com
- Sample buffer: phosphate buffered saline, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA; keep buffer cold (2–8 °C).

Note: Single-stained compensation beads can also be used for automated compensation. Please see the user software guide for more details on compensation.

Materials

- MACSQuant Analyzer 10 with MACS® MiniSampler
- Nine 5 mL (12×75 mm) polystyrene round bottom tubes
- MACS® Chill 5 Rack

Note: Automated compensation can also be performed using a 96-well plate and the Chill 96 Rack.

Laser	Channel	Filter (nm)	Dye or parameter
Violet 405 nm	V1	450/50	CFP, VioBlue
	V2	525/50	VioGreen, Pacific Orange™
Blue 488 nm	B1	525/50	GFP, FITC
	B2	585/40	PE
	B3	655-730	PI, PerCP, PE-Cy™5.5
	B4	750 LP	PE-Vio770, PE-Cy7
Red 635 nm	R1	655-730	APC
	R2	750 LP	APC-Vio770, APC-Cy7
Blue 488 nm	FSC	488/10	Size
	SSC	488/10	Granularity

Table 1: Table of the MACSQuant Analyzer 10 channels with the fluorescent protein, dye, or parameter measured in each channel.

Cell preparation and staining

1. Label nine 5 mL polystyrene round bottom tubes from 1 to 9.
2. Freshly prepare PBMCs.
3. Determine the number of cells in the PBMC sample by utilizing the count function of the MACSQuant Analyzer 10. Centrifuge cells at 300×g for 10 minutes. Aspirate supernatant and resuspend cells in sample buffer at a concentration of 1×10^6 cells per 100 μ L buffer.
4. Aliquot 100 μ L of cells into all 9 tubes.
5. Add an appropriate fluorochrome-conjugated antibody for channel V1 to tube 1 at the recommended titer, e.g., 10 μ L of the CD8-VioBlue® antibody, human (# 130-094-152). Repeat the process for tubes 2–8 using suitable fluorochrome-conjugated antibodies for the remaining fluorescence detection channels. Tubes 1–8 should now contain single-stained cells. Tube 9 should remain unstained and will serve as a blank sample.
6. Mix cells and incubate for 10 minutes in the dark at 4°C.
7. Wash cells by adding 1–2 mL of sample buffer to each tube and centrifuge at 300×g for 10 minutes. Aspirate supernatant.
8. Resuspend cell pellets in 1 mL of sample buffer for a final concentration of 1×10^6 /mL.
9. Proceed to performing automated 8-color compensation on the MACSQuant Analyzer 10.

Note: It is recommended that the selected cell surface markers are brightly expressed on the cell population of interest and represent a frequent (>10%) population of cells in the sample, e.g., CD4, CD3, or CD8.

Eight-color compensation on the MACSQuant® Analyzer 10

1. Prime the MACSQuant® Analyzer 10. After 30 minutes warm up period, perform an automated PMT calibration. See user manual for details.
2. Use tube 9 (unstained) to properly adjust the FSCvSSC to be appropriate for PBMCs. Alternatively, open a PBMC-specific bank setting. See user software guide for details.
3. Place a pre-cooled Chill 5 Rack onto the MACS MiniSampler and select **Chill 5 tube rack** from the **Rack** drop-down menu (figure 1). Make sure the large rack template opens in the center of the display. If it does not, open it by clicking on the  icon.

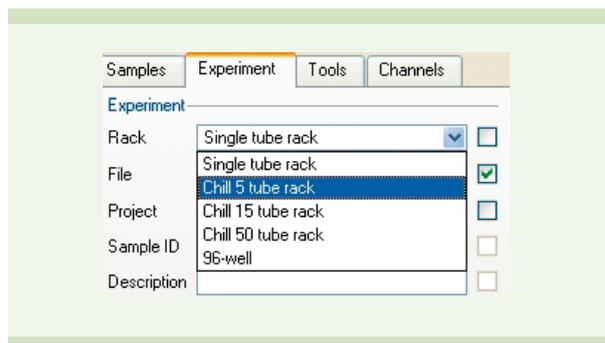


Figure 1: Selecting a Chill 5 tube rack.

4. Position each of the 9 tubes with single-stained cells in columns along the Chill 5 Rack. In this example the order is as follows: VioBlue (A1), VioGreen™ (B1), FITC (C1), PE (D1), PerCP (A2), PE-Vio770™ (B2), APC (C2), APC-Vio770™ (D2), and Blank (A3).
5. Select and activate the corresponding rack positions. Click **Group** to group the samples into a single group. All grouped samples will now have a **1** in the center (figure 2).

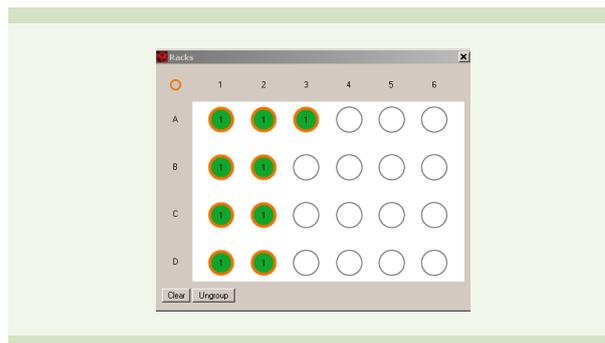


Figure 2: Activated cell samples were grouped together.

6. Check the **Express** button located under the **Settings** tab. Select **Setup** and **CompensationMultiColors** from the **Type** and **Mode** drop-down menus, respectively.

- Double-click on sample position A1 to activate it (circle with orange highlight). From the **Sample ID** drop-down menu, assign the relevant fluorochrome for compensation. In this example, cells in sample tube A1 were labeled with VioBlue (figure 3).

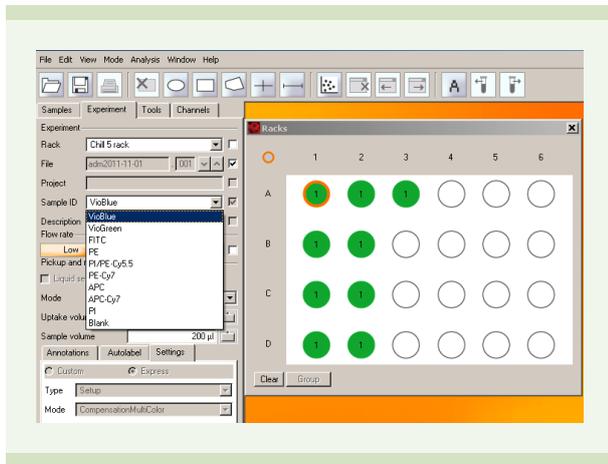


Figure 3: MACSQuantify™ Software will perform VioBlue auto-compensation on sample A1.

- Select and activate sample position **B1** and assign the relevant fluorochrome for compensation (in this example VioGreen).
- Continue this procedure for the remaining sample positions.

Note: Samples for compensation can be placed in any order. Please be sure to select the correct fluorochrome channel for each tube prior to starting the program. Additionally, the fluorochromes in this drop-down list are representative fluorochromes for each channel. As PerCP is being used in this example and it is detected in the same channel as PI/PE-Cy5.5, PI/PE-Cy5.5 should be chosen.

- Place the Chill 5 Rack with the 9 tubes on the MACS MiniSampler. Click **Start** to begin compensation.
- When prompted, draw a region around the cell population of interest, in this example, the lymphocytes. Be careful not to close the dialog box or to click **Continue** until a gate is drawn.

Note: Selecting a region of interest is highly recommended. Depending on the experiment, a particular cell type or population may be selected for compensation.

- The MACSQuant Analyzer 10 will perform 8x8-color compensation on the selected region of interest (figure 4).
- When the compensation is completed, the dialog box will open to save instrument settings. Name the instrument settings and click **Save**. Click **Cancel** to abort the process.

- The CompensationMultiColors procedure will save two data files. One filename begins with an underscore. This is the compensated file. The other filename does not begin with an underscore. This is the original grouped file prior to compensation.

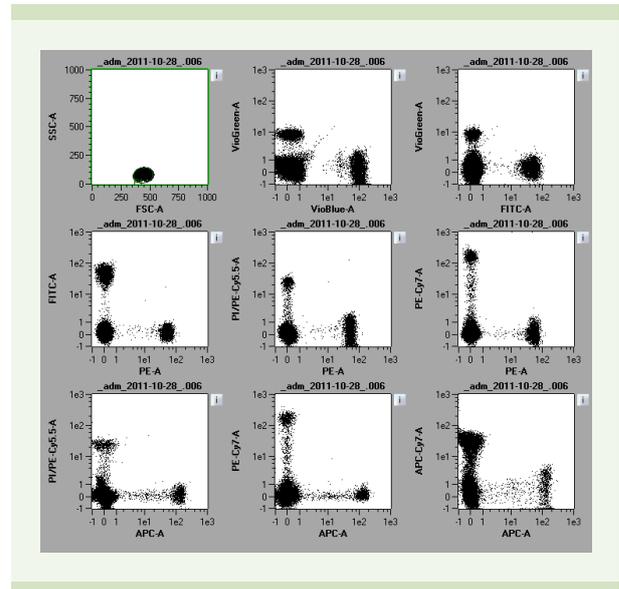


Figure 4: After 8-color compensation the results are displayed on the screen of the MACSQuant Instrument.

Conclusions

Flow cytometer compensation is often considered a tedious and time-consuming procedure that requires a significant degree of experience and expertise. The MACSQuant Instruments completely automate this procedure, making for reliable and reproducible results. Simply put: this walk-away convenience offers the researcher more time to focus on other aspects of the experiment.

For more information about additional cell analysis applications using the MACSQuant Family of flow cytometers, visit www.macsquant.com



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