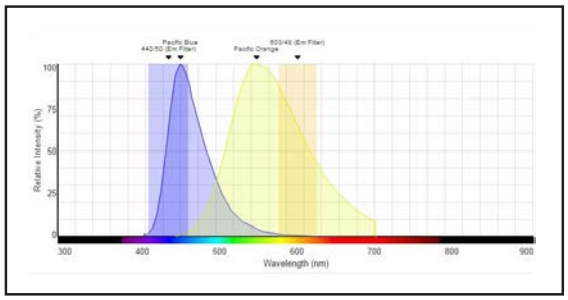
APPLICATION NOTE

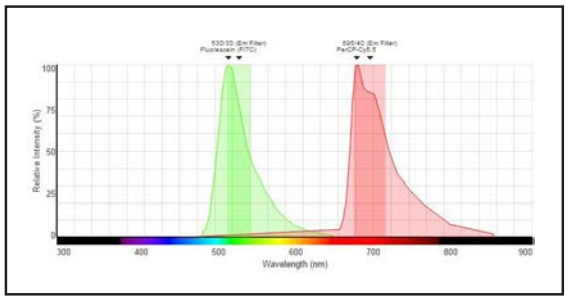
Attune NxT Flow Cytometer

Attune NxT Flow Cytometer for 6-color immunophenotyping analysis of stained human whole blood using a no-lyse, no-wash protocol, with no compensation

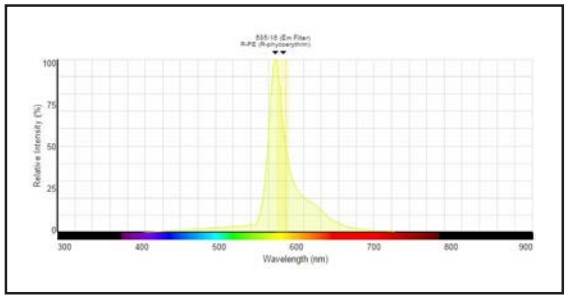
Compensation is one of the most critical, and yet poorly understood, topics in flow cytometry. Just as setting the proper voltage and knowing the range of wavelengths where fluorophores excite and emit, compensation is necessary for successful flow cytometry. Compensation is required for flow cytometry experiments because of the physics of fluorescence and the construction of flow cytometers. A fluorophore is excited and emits a photon in a range of wavelengths. Some of these photons spill into a second detector, causing single-stained samples to appear double positive. A mathematical correction is applied to flow cytometry data to address photon spillover—this is compensation.



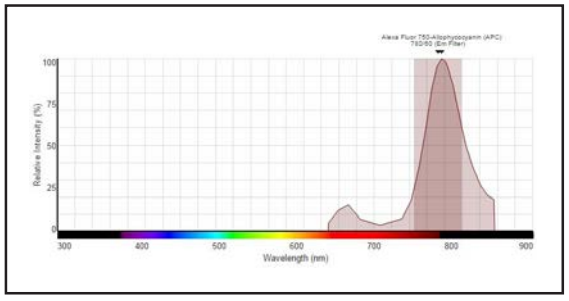
Violet laser
CD62L mAb, Pacific Blue™ conjugate
CD45 mAb, Pacific Orange™ conjugate



Blue laser
CD8 mAb, FITC conjugate
CD4 mAb, PerCP Cy®5.5 conjugate



Yellow laser
Glycophorin A mAb, PE conjugate



Red laser
CD3 mAb, APC-Alexa Fluor™ 750 conjugate

Figure 1. No-lyse, no-wash 6-color immunophenotyping panel. Data from the Fluorescence SpectraViewer, available at **thermofisher.com/spectraviewer**

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This application note describes the use of the Invitrogen™ Attune™ NxT Flow Cytometer for 6-color immunophenotyping analysis of stained human whole blood using a no-lyse, no-wash protocol. Due to optimal panel design (Figure 1), we show that our analysis does not require compensation for spectral overlap.

Compensation guidelines

- Compensation controls should be used with all multicolor experiments in flow cytometry.
- Compensation controls need to be at least as bright as the sample they apply to.
- Background fluorescence should be the same for the positive and negative control populations for any given parameter.
- The compensation color must be matched to the experimental color.
- Match fluorophores by brightness (values from the stain index) to density of the antigens—try to match brightest fluorophores with lowest-expressed antigens, and least bright fluorophores with highest-expressed antigens.
- If multiple lasers (spatially separated) are present, spread fluorophores across the lasers to minimize spillover.
- Use tandem dyes with consideration of their technical limitations.
- Know your instrument configuration—pick fluorophores that work with your instrument's filter configuration and design panels to maximize use of multilaser instruments.
- If multiple antigens are present on a cell, spread them across as many lasers as possible to minimize spillover.

Materials

- CD45 Mouse Anti-Human mAb, Pacific Orange Conjugate (Cat. No. MHCD4530TR)
- Glycophorin A (CD235A) Mouse Anti-Human mAb, PE Conjugate (Cat. No. MHGLA04)
- CD3 Mouse Anti-Human mAb, APC-Alexa Fluor 750 Conjugate (Cat. No. MHCD0327)
- CD62L Mouse Anti-Human mAb, Pacific Blue Conjugate (Cat. No. MHCD62L28)
- CD8 Mouse Anti-Human mAb, FITC Conjugate (Cat. No. MHCD0801)
- CD4 Mouse Anti-Human mAb, PerCP-Cy5.5 Conjugate (Cat. No. A15858)
- Attune NxT Flow Cytometer
- AbC™ Total Antibody Compensation Bead Kit (Cat. No. A10497)
- 96-well plates
- Flow cytometry tubes
- Gibco™ PBS, pH 7.4 (Cat. No. 10010023)
- Gibco™ RPMI 1640 Medium (Cat. No. 11875119)
- Whole blood collected into heparinized tubes

Cells from human whole blood were surface stained using the 6 antibodies listed above and analyzed on the Attune NxT Flow Cytometer. The following sample preparation protocol was used for analysis.

Antibody labeling

1. Turn on the instrument; run startup and performance test scripts as normal.
2. Compensation controls are needed to demonstrate the amount of compensation required for the experiment, in this case none was required. Create single-color compensation controls by labeling capture beads provided in the AbC Total Antibody Compensation Bead Kit (see "Compensation controls" section).
3. Create at minimum 5 samples by pipetting 100 µL of whole blood into 5 sample vessels (microfuge or flow cytometry tubes or wells within a 96-well plate). Reserve one sample as an unstained control. Additional control samples will be required if more than two antibody conjugates are used to label the sample.
4. To identify all leukocytes within the first sample, label cells in one tube using an antibody conjugate directed against the surface marker CD45 (according to the manufacturer's guidelines).
5. To identify red blood cells within the second sample, label cells in one tube using an antibody directed against the surface marker glycophorin A (according to the manufacturer's guidelines).
6. To differentiate white blood cells from red blood cells, label cells in the third sample using both the CD45 and glycophorin A antibody conjugates (according to the manufacturer's guidelines).

7. To identify the T cell subsets along with red blood cells and leukocytes, label cells using CD45, glycophorin A, CD3, CD4, CD8, and CD62L antibodies (according to the manufacturer's guidelines).
8. Incubate all labeling reactions for 20–30 minutes at room temperature, protected from light. An example of this is shown in Figure 2 in which CD45 Antibody, Pacific Orange Conjugate (Cat. No. MHCD4530TR) and Glycophorin A Antibody, FITC Conjugate (Cat. No. MHGLA014) were used to label the two cell types. Single-color controls should be included to adjust instrument voltages prior to sample acquisition.
9. Create a workspace on the Attune NxT Flow Cytometer software containing two dot plots as indicated in Figure 2:
   - Plot A: CD45 (x-axis) vs. glycophorin A (y-axis)
   - Plot B: Blue forward scatter (FSC, x-axis) vs. blue side scatter (SSC, y-axis). Additional plots may be inserted into the workspace if additional antibody conjugates were used to label cells within the sample.
10. After the 20–30 minute incubation, aliquot 1 µL of the antibody-labeled sample into 4 mL of PBS.
11. Select one of the single-color control samples and acquire the sample at a sample collection rate ≥200 µL/min (without recording) using the Attune NxT Flow Cytometer. Adjust PMT voltages such that the glycophorin A–labeled cells and CD45-labeled cells are on scale using the unstained and single-color controls prepared above.
12. While recording, acquire each of the samples using a sample collection rate ≥200 µL/minute until a total number of events that is statistically meaningful for your experiment is collected.
13. After acquiring the sample, view results of the dual-stained sample using the CD45 vs. glycophorin A plot. The $CD45^+$, glycophorin A^- cells (Figure 2) correspond to the white blood cells (WBCs) within the sample. Using this region as a parent gate, a blue FSC vs. blue SSC dot plot can be used to visualize the three standard white blood cell populations (lymphocytes, monocytes, and granulocytes) based upon their scatter properties.
14. Create a dot plot with axis for CD3 APC-Alexa Fluor 750 conjugate (RL-3) versus CD45 Pacific Orange conjugate (VL-3). Gate on CD3/CD45 double-positive cells.
15. Create 2 new dot plots to analyze the T cell subsets: (1) CD62L Pacific Blue conjugate (VL-1) versus CD8 FITC conjugate (BL-1) and use a quadrant marker to determine percentage of cell types. (2) CD62L Pacific Blue conjugate (VL-1) versus CD4 PerCP Cy5.5 conjugate (BL-3) and use a quadrant marker to determine percentage of cell types.

Compensation controls

1. Completely resuspend the AbC™ Total Compensation capture beads (Component A) and negative beads (Component B) by gently vortexing for 10 seconds before use.
2. Label a sample tube for each fluorophore-conjugated antibody you are using, and add 1 drop of AbC Total Compensation capture beads (Component A) to each tube.
3. Add a pre-titrated amount of each mouse anti-human antibody conjugate to the AbC Total Compensation capture bead suspension in the designated tube and mix well. Make sure to deposit the antibody directly to the bead suspension.
4. Incubate for 15 minutes at room temperature, protected from light.
5. Add 3 mL of PBS or other buffer to sample tubes. Centrifuge for 5 minutes at 250 x *g*.
6. Carefully remove the supernatant from tubes and resuspend the bead pellet by adding 0.5 mL of PBS or other buffer to sample tubes.
7. Add one drop of negative beads (Component B) to the tubes and mix well.
8. Vortex tubes before analyzing using flow cytometry. You may briefly sonicate to increase the percentage of singlet beads, if necessary.
9. Perform manual or automatic compensation according to the preferred procedure for the flow cytometer in use. Gate on the bead singlet population based on FCS and SSC characteristics.

Table 1. Filters used to detect each dye conjugate.

Dye	Excitation (nm)	Emission (nm)
Pacific Blue	405	440/50
Pacific Orange	405	603/48
FITC	488	530/30
PerCP-Cy5.5	488	695/40
PE	561	585/16
APC-Alexa Fluor 750	637	780/60

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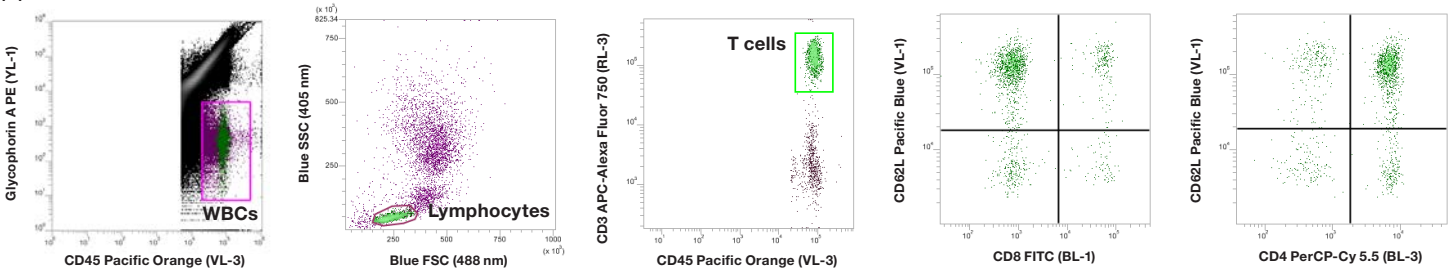
Data acquisition and gating strategy

Samples were collected on the Attune NxT Flow Cytometer using the filters shown in Table 1. The gating strategy used in our multiparameter analysis is described in Figure 2.

A



B

Compensation matrix						
	Pacific Blue (VL-1)	Pacific Orange (VL-3)	FITC (BL-1)	PerCP-Cy5.5 (BL-3)	PE (YL-1)	APC-Alexa Fluor 750 (RL-3)
Pacific Blue (VL-1)		0.0	0.0	0.0	0.0	0.0
Pacific Orange (VL-3)	0.0		0.0	0.0	0.0	0.0
FITC (BL-1)	0.0	0.0		0.0	0.0	0.0
PerCP-Cy5.5 (BL-3)	0.0	0.0	0.0		0.0	0.0
PE (YL-1)	0.0	0.0	0.0	0.0		0.0
APC-Alexa Fluor 750 (RL-3)	0.0	0.0	0.0	0.0	0.0	

Figure 2. No-lyse, no-wash 6-color immunophenotyping panel for human T cell subsets requires no compensation. (A) A fluorescence threshold was set on Pacific Orange dye and red blood cell coincident events were excluded based on glycophorin A PE positivity. The $CD45^+$, glycophorin A^- cells (boxed) correspond to WBCs in the sample. Lymphocytes were gated on based on blue FSC and blue SSC properties from which T cells were identified by CD3 expression. T cells were then analyzed for their expression of the lineage markers CD4 and CD8 and the activation marker CD62L to identify naïve/central memory T cells ($CD62L^+$) and effector memory T cells ($CD62L^-$). **(B)** Compensation matrix calculated from Attune™ NxT Software.

Conclusion

We have developed a 6-color T cell panel along with a no-wash, no-lyse strategy that requires no compensation on the Attune NxT Flow Cytometer. With configuration options of up to 4 lasers and 16 detection channels, the Attune NxT Flow Cytometer can be designed to accommodate the most common fluorophores and fluorescent proteins used in flow cytometry to match the panels you are currently running.

Find out more at **thermofisher.com/attune**

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