

Automated Preparation for 25-Color Flow Cytometric Immunophenotyping of Human PBMCs

Automated sample preparation with the Agilent NovoStation sample preparation system matches manual performance while greatly reducing hands-on time

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Abstract

Flow cytometry enables high-dimensional immunophenotyping of peripheral blood mononuclear cells (PBMCs), but manual sample preparation involving complex antibody cocktails, staining, washing, and fixation steps is labor-intensive and prone to variability. This study compared automated sample preparation using the Agilent NovoStation sample preparation system with conventional manual workflows for 25-color immunophenotyping of human PBMCs. Human PBMC samples, including unstained, single-color reference controls, and triplicate multicolor samples (30 samples total), were prepared either manually or using the NovoStation system, followed by acquisition on an Agilent NovoCyte Opteon spectral flow cytometer. Both methods achieved comparable results across 43 immune cell populations, with stain indices and cell viability showing excellent concordance ($R^2 = 0.9954$).

These findings demonstrate that automated sample preparation significantly improves operational efficiency without compromising data quality in complex immunophenotyping assays.

Introduction

Flow cytometry represents a powerful analytical technique for high-dimensional immunophenotyping of peripheral blood mononuclear cells (PBMCs), enabling comprehensive characterization of immune cell populations at the single-cell level. However, the practical implementation of complex, multicolor flow cytometric assays presents significant operational challenges that can limit their widespread adoption. Specifically, the preparation of intricate antibody cocktails, combined with the sequential steps of sample staining, incubation, washing, and fixation, remains labor-intensive and inherently prone to interassay and interoperator variability. These limitations are particularly pronounced when implementing high-dimensional panels exceeding 20 fluorescent parameters, where the complexity of reagent cocktailing and sample handling increases substantially. The standardization and reproducibility of immunophenotyping assays are critical for generating reliable, comparable data across different laboratories and time points.

Manual sample preparation workflows introduce multiple sources of variability, including inconsistent incubation times between users, variable washing efficiency, and operator-dependent pipetting accuracy. Furthermore, the substantial hands-on time required for manual processing reduces laboratory throughput and diverts skilled personnel from higher-value analytical tasks. This study addresses these challenges by systematically comparing automated sample preparation using the Agilent NovoStation sample

preparation system with conventional manual workflows for 25-color immunophenotyping of human PBMCs. The NovoStation platform integrates several features designed to enhance assay reliability and efficiency, including integrated reagent and sample traceability, onboard cooling modules to preserve antibody stability, and precise liquid-level detection for accurate pipetting. The system provides native integration with Agilent NovoCyte flow cytometers, enabling seamless automated acquisition of prepared samples. By evaluating population percentages, resolution of immunophenotypic subsets, cell recovery, and hands-on time requirements, this investigation provides empirical evidence regarding the performance equivalence and operational advantages of automated sample preparation for complex flow cytometric assays.

Experimental

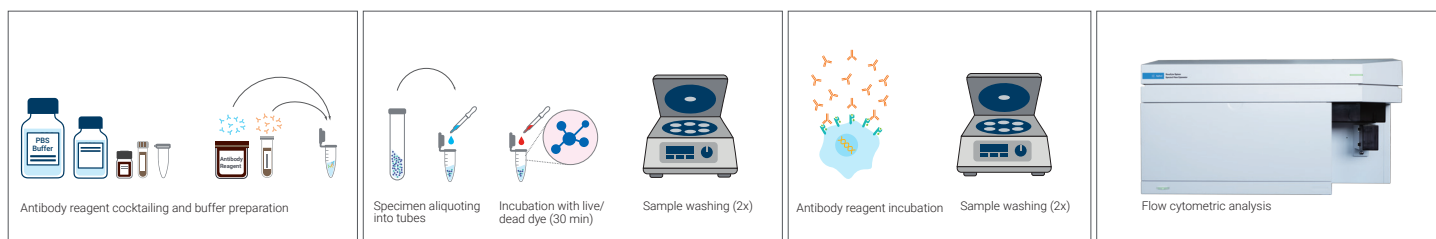
Study design

Human PBMCs were prepared for 25-color immunophenotyping using either manual or automated sample preparation workflows. A total of 30 samples were prepared, including unstained controls, single-color reference controls, and triplicate multicolor samples.

Sample preparation

Sample preparation was carried out using two different approaches: manual and automated processing with the NovoStation sample preparation system (Figure 1). For manual preparation, antibody reagent cocktails, diluted antibodies, and buffers were prepared manually, followed by

Manual sample preparation



Automated sample preparation using the Agilent NovoStation system

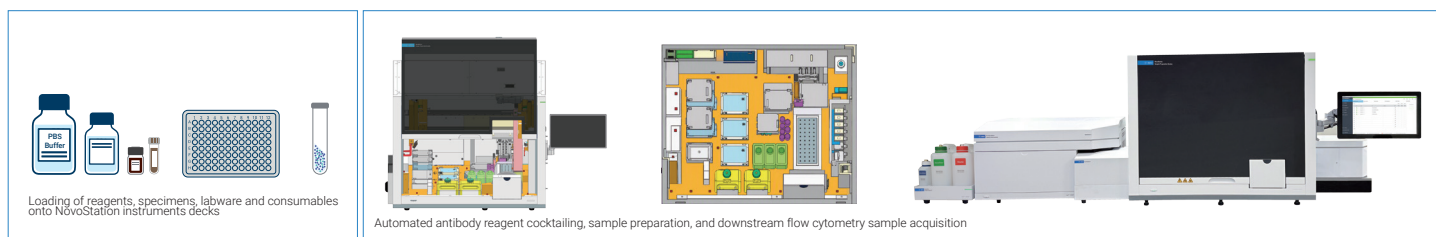


Figure 1. Workflow comparison between (top) manual sample preparation and (bottom) automated sample preparation using the Agilent NovoStation sample preparation system, demonstrating consolidated hands-on steps and reduced operator involvement.

aliquoting of PBMC specimens into tubes. The samples were then incubated with live/dead dye for 30 minutes, washed twice, and stained by adding the antibody reagent cocktail or single antibodies with an additional incubation period. After staining, the samples were washed twice more before being prepared for flow cytometric analysis.

In contrast, automated sample preparation used the NovoStation system, where reagents, specimens, labware, and consumables were loaded onto the instrument decks. The system performed antibody cocktail preparation automatically and executed the entire sample staining and washing workflow without manual intervention. Upon completion, the prepared samples were transferred downstream for flow cytometric acquisition, enabling a streamlined and hands-free preparation process.

25-color immunophenotyping panel

A 25-color antibody panel, developed based on the OMIP-024 framework, was used for comprehensive immunophenotyping of human PBMCs. The panel composition included the fluorochromes and reagents listed in Tables 1 and 2. This comprehensive panel enabled the identification and quantification of 43 distinct immune cell populations from human PBMCs, including T cell subsets, B cell subsets, NK cell subsets, monocyte populations, and dendritic cell populations.

Flow cytometric analysis

Flow cytometric analysis was performed using an Agilent NovoCyte Opteon spectral flow cytometer integrated with the Agilent NovoStation sample preparation system for automated, hands-free sample-to-result workflow. Sample acquisition and data analysis were performed using Agilent NovoExpress software, version 2.2.0. The gating strategy identified 43 immune cell populations from human PBMCs. Cell viability was assessed using live/dead blue dye, and viable CD45+ cell counts were measured in replicate samples.

Comparative analyses assessed population percentages, resolution of immunophenotypic subsets, cell recovery, and hands-on time between manual and automated sample preparation methods. Stain index values were calculated for 24 fluorochromes from the panel to evaluate resolution in lymphocyte and monocyte populations. Linear regression analysis was performed to compare the percentage parent frequencies of gated immune cell populations between manual and automated methods, with results expressed as R² values.

Table 1. Fluorescent antibody reagents used for the 25-color immunophenotyping panel.

Specificity	Fluorochrome	Clone	Vendor	Part number
Vβ2 TCR	BUV395	B6	BD	743754
CD27	BUV496	M-T271	BD	741145
CD33	BUV563	WM53	BD	741369
CD56	BUV615	NCAM16.2	BD	613001
CD11c	BUV661	B-ly6	BD	612967
IgD	BUV737	IA6-2	BD	612798
CD8	BUV805	SK1	BD	612889
CD25	BV421	M-A251	Biologend	356114
CD45	BV510	HI30	Biologend	304036
CD3	BV570	UCHT1	Biologend	300436
CD16	BV605	3G8	Biologend	302040
HLA-DR	BV650	L243	Biologend	307650
CD14	BV711	MφP9	BD	563373
CCR7	BV785	G043H7	Biologend	353230
CD123	FITC	6H6	Biologend	306014
CD19	PerCP- eF710	J3-129	ThermoFisher	46-0197-42
CD24	PE	ML5	Biologend	311106
CXCR3	PE-Dazzle594	G025H7	Biologend	353736
CD38	PE-Cy5	HIT2	BD	555461
CD4	PE-AF700	S3.6	ThermoFisher	MHCD0424
γδ TCR	PE-Cy7	B1	Biologend	331222
CD127	APC	A019D5	Biologend	351316
CCR6	APC-R700	11A9	BD	565173
CD45RA	APC-Cy7	HI100	Biologend	304128

Table 2. Additional reagents used for the 25-color immunophenotyping panel.

Item	Vendor	Part number
Live/Dead Fixable Blue (450) Viability kit	Invitrogen	L23105
Phosphate buffer saline (PBS)	Genom Biomedical Technology	GNM-14190
Fetal bovine serum (FBS)	Gibco	10099-141C
Brilliant Stain Buffer Plus	BD	566385
FC Block	Biologend	422302
True-Stain Monocyte Block	Biologend	426103

To evaluate the performance of automated sample preparation for high-dimensional immunophenotyping, we compared the Agilent NovoStation automated system with conventional manual workflows using a 25-color antibody panel for human PBMC analysis. Both methods were assessed for their ability to resolve immune cell populations, maintain cell viability, and generate quantitatively concordant results.

Flow cytometry plots showing the isolation of CD4⁺ T cells from human peripheral blood mononuclear cells (PBMCs). The process starts with PBMCs and proceeds through several gates to isolate CD4⁺ T cells. The final gate is CD4⁺ T cells, which are then analyzed for various markers. The plots show the percentage of cells in each gate and the percentage of cells in each marker-positive population.

Stable Inflow

Plot 1: FSC-A (10⁴) vs. SSC-A (10³). Gate: Single (93.7%).

Cells

Plot 2: CD45-APC (10⁴) vs. CD45-PE (10³). Gate: CD45+ Live (99.41%).

CD4+ Live

Plot 3: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (29.3%).

B cells

Plot 4: CD19-APC (10⁴) vs. CD19-PE (10³). Gate: B cells (11.48%).

CD4+ Live

Plot 5: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (79.93%).

CD4+ Live

Plot 6: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 7: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 8: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 9: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 10: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 11: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 12: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 13: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 14: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 15: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 16: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 17: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 18: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 19: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 20: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 21: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 22: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 23: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 24: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 25: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 26: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 27: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 28: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 29: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 30: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 31: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 32: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 33: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 34: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 35: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 36: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 37: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 38: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 39: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 40: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 41: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 42: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 43: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 44: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 45: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 46: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 47: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 48: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 49: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 50: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 51: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 52: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 53: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 54: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 55: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 56: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 57: CD4-APC (10⁴) vs. CD4-PE (10³). Gate: CD4+ Live (3.32%).

CD4+ Live

Plot 58: CD4-APC (10⁴) vs. CD4-

Both manual and automated sample preparation methods successfully resolved 43 distinct immune cell populations from human PBMCs using the 25-color antibody panel. Scatter and dot plots revealed comparable gating resolution and population identification between the two approaches (Figure 2).

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Quantitative analysis of population frequencies demonstrated excellent concordance between methods, with a linear regression analysis of the 43 gated populations yielding $R^2 = 0.9954$ and a linear fit of $y = 1.0042x - 0.0016$ (Figure 3). This high degree of correlation indicates that automated sample preparation via the NovoStation system produces results equivalent to manual processing across diverse cell populations.

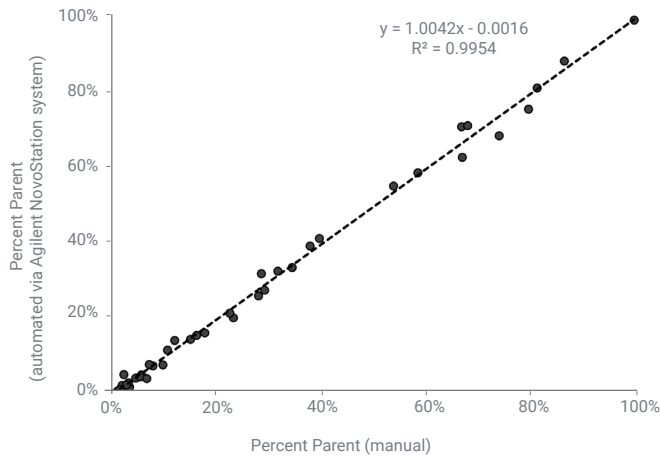


Figure 3. Quantitative concordance of population frequencies between manual and automated sample preparation methods, with highly linear correlation.

Representative population comparisons demonstrated consistent quantitative agreement across major immune cell subsets (Table 3). CD4⁺ T cells were measured at 58.41% $\pm$ 0.18% by manual preparation versus 59.08% $\pm$ 0.08% by NovoStation automation. CD8⁺ T cells showed similar concordance, with manual preparation yielding 34.61% $\pm$ 0.12% compared to 33.99% $\pm$ 0.11% for automated preparation. B cell subsets also demonstrated excellent agreement, with naive B cells measured at 37.99% $\pm$ 1.37% manually versus 39.40% $\pm$ 0.46% using NovoStation. Monocyte populations, including classical monocytes, were quantified at 66.70% $\pm$ 0.97% by manual methods compared to 71.19% $\pm$ 0.03% by automation. Dendritic cell populations, specifically myeloid dendritic cells, showed 66.83% $\pm$ 3.32% for manual preparation versus 63.37% $\pm$ 1.21% for automated preparation.

These results demonstrate that the NovoStation automation maintains quantitative accuracy across the full spectrum of immune cell populations, from abundant T cell subsets to rare dendritic cell populations, without introducing systematic bias or increased variability.

Table 3. Population comparisons show consistent quantitative agreement across immune cell subsets.

Percent Parent	Manual		Agilent NovoStation System	
	Average	SD	Average	SD
CD45 ⁺ Live	99.39%	0.03%	99.48%	0.01%
CD14 ⁺	79.36%	0.64%	75.89%	0.45%
HLA-DR ⁺	28.74%	0.63%	32.36%	0.51%
Nonclassical Mo	3.29%	0.11%	2.46%	0.07%
Intermediate Mo	3.78%	0.17%	2.27%	0.19%
Classical Mo	66.70%	0.97%	71.19%	0.03%
NKT	8.21%	0.04%	7.98%	0.03%
CD3-CD19 ⁺	15.29%	0.17%	14.96%	0.15%
B cells	3.60%	0.10%	3.45%	0.03%
T cells	81.04%	0.20%	81.47%	0.16%
V δ 2 ⁺ $\gamma\delta$ T	2.29%	0.01%	2.31%	0.04%
V δ 2 ⁺ $\gamma\delta$ T	3.08%	0.09%	2.88%	0.07%
CD4 ⁺ T	58.41%	0.18%	59.08%	0.08%
CD8 ⁺ T	34.61%	0.12%	33.99%	0.11%
Activated Th	2.34%	0.12%	2.73%	0.22%
Th-CM	29.35%	0.30%	27.95%	0.18%
Th-EM	16.46%	0.31%	16.02%	0.26%
Th-EMRA	0.39%	0.04%	0.51%	0.03%
Th-Naive	53.79%	0.20%	55.52%	0.31%
Activated Tc	7.50%	0.27%	8.28%	0.32%
Tc-CM	4.94%	0.03%	4.70%	0.18%
Tc-EM	23.47%	0.29%	20.65%	0.30%
Tc-EMRA	31.87%	0.38%	32.71%	0.48%
Tc-Naive	39.72%	0.64%	41.94%	0.35%
Th1	18.06%	0.15%	16.64%	0.17%
Th2	67.88%	0.21%	71.49%	0.55%
Th17	6.04%	0.17%	5.57%	0.16%
Treg	5.68%	0.27%	5.23%	0.07%
Transitional B	1.57%	0.34%	1.42%	0.23%
Plasmablast	1.81%	0.10%	1.59%	0.33%
IgD-CD27 ⁺ B	22.81%	0.44%	22.43%	0.10%
Switched memory B	28.20%	0.78%	26.22%	0.12%
Naive B	37.99%	1.37%	39.40%	0.46%
Unswitched memory B	11.01%	0.48%	11.96%	0.55%
CD16 ⁺ CD56dim	2.68%	0.06%	5.67%	0.20%
CD16 ⁺ CD56hi	3.19%	0.13%	2.90%	0.06%
CD16 ⁺ CD56 ⁺	7.04%	0.10%	4.40%	0.22%
CD16 ⁺ CD56dim	73.83%	0.61%	69.10%	0.77%
CD16 ⁺ CD56hi	1.95%	0.08%	1.65%	0.11%
Basophils	10.07%	0.69%	8.17%	0.48%
DC	86.20%	0.46%	88.61%	0.39%
mDC	66.83%	3.32%	63.37%	1.21%
pDC	12.36%	0.83%	14.54%	1.26%

Cell viability and recovery

Cell viability was assessed using live/dead blue dye, with viable CD45+ cells measured across three replicate samples from the same donor (Figure 4).

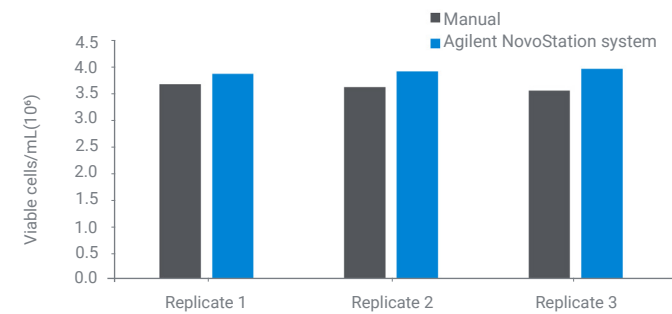


Figure 4. Viable CD45+ cell counts across three replicate samples prepared by manual and Agilent NovoStation system-automated methods, measured using live/dead blue dye.

The comparable cell viability and recovery between manual and automated methods confirms that the NovoStation system does not introduce additional cell stress or loss during the automated sample preparation steps. This preservation of sample quality is critical for accurate immunophenotyping, particularly when working with limited specimens.

Stain index and fluorochrome resolution

Stain index analysis across 24 fluorochromes from the 25-color panel (CD45 BV510 excluded) revealed comparable signal resolution between manual and automated methods (Figure 5). Both approaches achieved similar values for each fluorochrome, demonstrating that NovoStation automation did not compromise antibody binding efficiency or fluorescence signal quality compared to manual staining protocols.

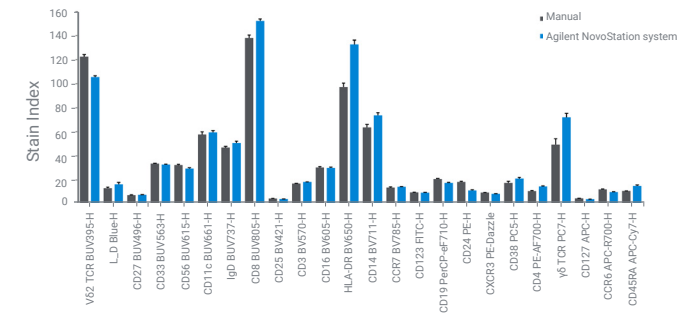


Figure 5. Stain index comparison across 24 fluorochromes from the 25-color immunophenotyping panel, comparing manual and Agilent NovoStation system-automated methods.

The maintenance of high stain indices across the full fluorochrome panel indicates that automated pipetting, mixing, and incubation steps performed by the NovoStation system achieve equivalent antibody-antigen binding kinetics to manual processing. This finding is particularly important for complex multicolor panels where optimal staining of all markers is essential for accurate population identification and quantification.

Workflow efficiency and hands-on time reduction

The NovoStation system significantly enhances workflow efficiency by reducing hands-on time for 30 samples from 85.5 minutes manually to just 7.9 minutes, a 90.8% reduction in direct operator involvement (Table 4). While the total assay time slightly increased due to automated processing steps (170.5 minutes manual versus 190.8 minutes automated), the lengthy hands-off period (182.9 minutes) allows operators to perform other tasks concurrently, improving overall laboratory throughput.

Table 4. Hands-on time comparison for manual versus Agilent NovoStation system automated sample preparation.

Step	Manual		Agilent NovoStation System	
		Time (min)		Time (min)
Prepare cocktail	Hands-on	17.8	Hands-on	2.5
			Hands-off	16.9
Aliquot PBMC samples and add Live/Dead Blue for incubation, then wash 2x	Hands-on	17.9	Hands-on	5.4
	Hands-off	51.0	Hands-off	166
Add antibody for incubation and wash 2x	Hands-on	49.8		
	Hands-off	34.0		
Total sample preparation	Total hands-on	85.5	Total hands-on	7.9
	Total hands-off	85.0	Total hands-off	182.9
	Total	170.5	Total	190.8

Furthermore, seamless integration with the NovoCyte Opteon spectral flow cytometer enables automated sample acquisition immediately after preparation, creating a hands-free sample-to-result workflow. This integration minimizes manual handling, reduces error risk, and standardizes timing between preparation and analysis, offering substantial operational advantages for complex immunophenotyping assays.

Conclusion

This study demonstrates that automated sample preparation using the Agilent NovoStation sample preparation system achieves comparable results to manual methods for 25-color flow cytometric immunophenotyping of human PBMCs. Both approaches yielded equivalent stain indices across 24 fluorochromes, with resolution of 43 immune cell populations showing strong quantitative concordance ($R^2 = 0.9954$). Cell viability and recovery were also comparable between methods. Notably, the NovoStation automation reduced hands-on time from 85.5 minutes to 7.9 minutes for 30 samples, while maintaining assay quality. Integration with the Agilent NovoCyte Opteon spectral flow cytometer enabled automated sample acquisition, establishing a true hands-free sample-to-answer workflow. These results support the NovoStation sample preparation system as an effective platform for high-dimensional immunophenotyping while improving laboratory efficiency.

References

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2. Maecker, H. T.; McCoy, J. P.; Nussenblatt, R. Standardizing immunophenotyping for the Human Immunology Project. *Nat. Rev. Immunol.* **2012**, 12 (3), 191–200.

Products used in this application

Agilent products

[Agilent NovoCyte Opteon spectral flow cytometer](#) 

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