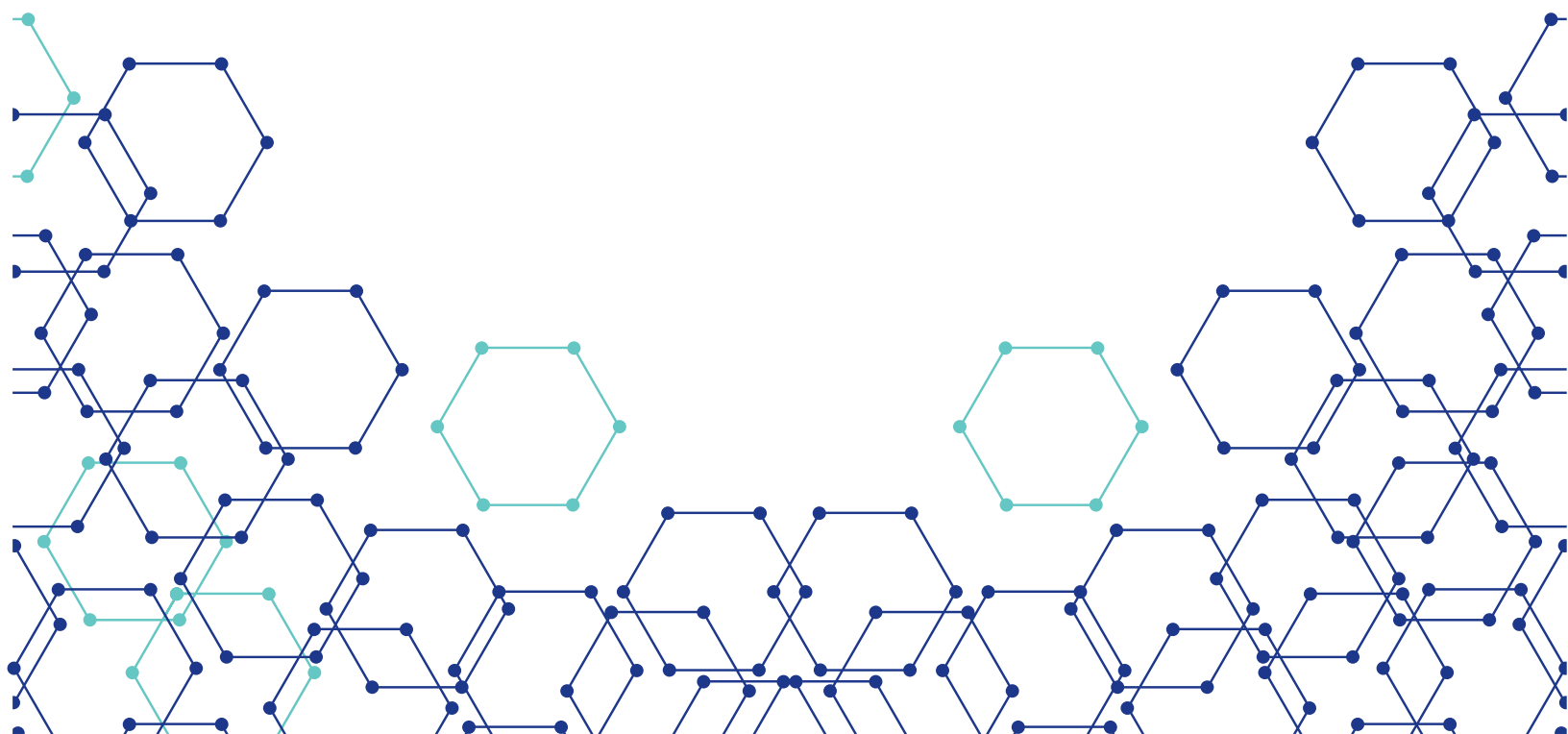


Validation of C-FREE™ Pluto Wash at 384-Well Scale

Technical and biological validation of automated,
high-throughput cell washing



Contents

1. Introduction	1
2. Study Overview & Validation Strategy	2
3. Technical Validation at the 384-Well Format (Bead Model)	4
4. Biological Validation Using Surface Staining	5
a. Automation Equivalence at the 96-Well Format	5
b. Biological Validation at the 384-Well Format	7
5. Conclusions and Appropriate Use Cases	10

1. Introduction

High-throughput cell staining workflows increasingly require automation-compatible washing to support higher assay density, walkaway workflows, and improved reproducibility. Centrifugation-based washing remains a limiting step due to constraints on throughput, integration with liquid handlers, and susceptibility to operator- and timing-related variability.

The use of 384-well plate formats enables increased experimental throughput and reduced reagent consumption, but introduces technical challenges for washing. Reduced working volumes and shallow liquid columns increase sensitivity to aspiration height, residual volume, and flow rate, creating higher risk for sample loss and inconsistent wash performance. Reliable adoption of 384-well workflows therefore requires wash strategies that are explicitly validated for retention, wash efficiency, and assay performance at this scale.

This application note evaluates C-FREE™ Pluto Wash for use in 384-well flow cytometry sample preparation workflows. Performance is assessed through a structured validation approach that links technical fluidic controls to biologically relevant endpoints, allowing results at the 384-well format to be interpreted relative to established manual centrifuge and automated 96-well workflows.

2. Study Design and Validation Strategy

Purpose and Overall Rationale

This study was designed to evaluate the feasibility, robustness, and biological relevance of washing workflows performed in a 384-well plate format on the Pluto platform. The validation strategy combines platform-level technical controls with biologically relevant assays to ensure that performance is assessed both mechanically and functionally.

Technical Challenges of 384-Well Washing

Washing in a 384-well format presents inherent technical challenges compared to 96-well formats, including reduced working volumes, shallower liquid columns, increased susceptibility to evaporation, and tighter tolerances for aspiration height and residual volume. At this format, small deviations in fluid handling can result in disproportionately large effects on sample retention and wash efficiency. Demonstrating reliable washing performance therefore requires validation of uniformity and robustness across all wells within the plate.

Relevance of C-FREE™ Washing

The Pluto platform employs a C-FREE™ (centrifuge-free) washing approach, eliminating the need for centrifugation steps commonly used in manual workflows. By relying on controlled fluid exchange and gravity-assisted settling, C-FREETM washing simplifies automation and enables compatibility with higher-density plate formats such as 384-well plates, while reducing workflow complexity and manual handling.

Rationale for Dual Validation Models

Two complementary validation models were used:

- Fluorescent beads were selected as a platform-level control to assess fluidic performance independent of biological variability. Beads provide a consistent, non-deformable model for quantifying retention and wash efficiency.
- Cryopreserved PBMCs were used as a biologically relevant stress test. PBMCs represent a heterogeneous and fragile cell population commonly used in immunophenotyping assays, making them suitable for evaluating the impact of washing on cell recovery, staining quality, and population integrity.

Together, these models enable separation of fluidic performance from biological effects, strengthening interpretation of results.

Rationale for the Comparison Chain

Performance was evaluated across a structured comparison chain:

- Manual centrifuge washing, following a standard SOP, as the baseline reference
- Pluto Wash at the 96-well format, to establish automation equivalence
- Pluto Wash at the 384-well format, to evaluate performance at increased well density

This stepwise approach ensures that results observed at the 384-well format can be interpreted in the context of both manual and automated 96-well workflows.

3. Technical Validation at the 384-Well Format (Bead Model)

Purpose

The bead-based validation was designed to demonstrate **fluidic robustness and wash efficiency** in a 384-well plate format, independent of biological variability.

Bead Retention Across a 384-Well Plate

Fluorescent beads were distributed uniformly across wells of a 384-well plate and subjected to defined Pluto wash protocols. Bead retention was quantified on a per-well basis following washing. Overall retention across the plate was measured at **94.3%**, demonstrating consistent recovery under optimized wash conditions (Figure 1).

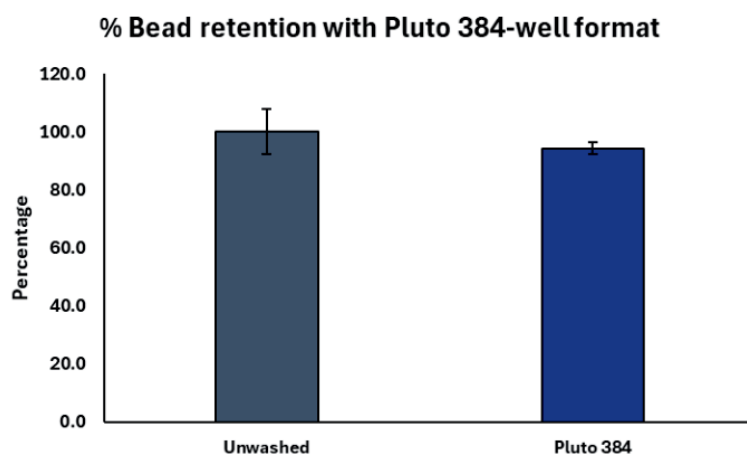


Figure 1. Bead retention across a 384-well plate following Pluto washing.

Wash Efficiency and Dye Dilution Performance

Wash efficiency was evaluated using dye dilution as a proxy for effective fluid exchange. Residual dye signal following successive wash cycles was compared against standards, showing progressive reduction consistent with efficient washing (Figure 2). These results confirm that Pluto washing effectively removes soluble components while maintaining bead retention.

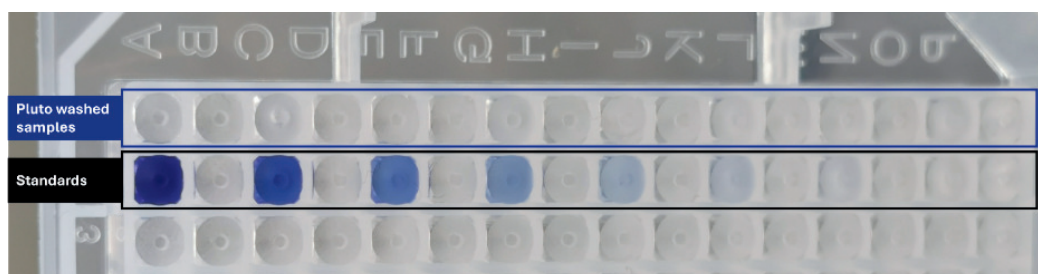


Figure 2. Wash efficiency assessed by dye dilution in a bead-based model.

Effects of Flow Rate, Wash Volume, and Residual Volume

Flow rate, wash volume, and residual volume were systematically varied to assess their impact on bead retention and wash efficiency. These parameters were optimized to balance efficient dye removal with minimal bead loss at the 384-well format.

Interpretation of Bead Model Results

By using beads to remove biological sources of variation, the model isolates fluidic performance and demonstrates uniform, reproducible washing across a 384-well plate. These results establish a technical foundation upon which biological performance can be evaluated in follow-up studies.

4. Biological Validation Using Surface Staining

Purpose

Biological validation was performed to assess whether washing at the 384-well format maintains **assay performance and biological integrity** in a user-relevant flow cytometry application.

4A. Automation Equivalence at the 96-Well Format

Comparison of Manual Centrifuge and Pluto 96-Well Format

Cryopreserved PBMCs were stained using a standard surface antibody panel and processed using either manual centrifuge washing or automated washing on Pluto at the 96-well format.

Assay Endpoints

Performance was evaluated using the following endpoints:

- **Cell recovery** showed comparable performance between manual and automated workflows (Figure 3).
- **Stain index** for key markers demonstrated equivalent staining quality between methods (Figure 4).
- **Population frequencies** for major immune subsets were consistent across workflows, indicating preserved biological representation (Figure 5).

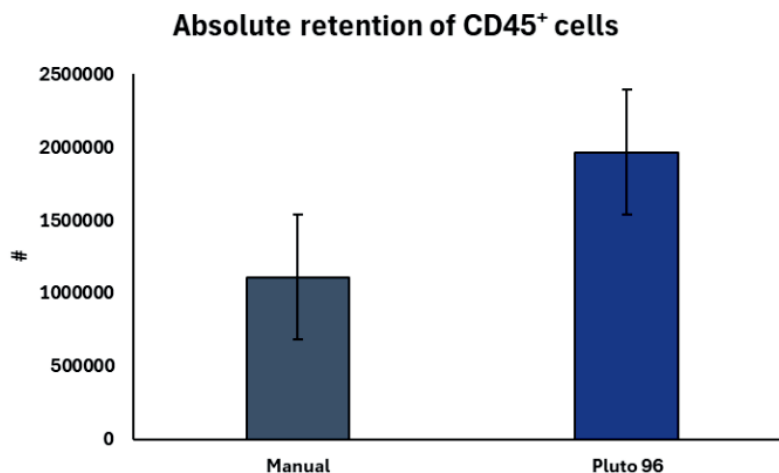


Figure 3. Cell recovery following manual centrifuge washing and automated Pluto washing at the 96-well format. Cryopreserved PBMC recovery measured after surface staining, demonstrating comparable cell retention between manual and automated workflows.

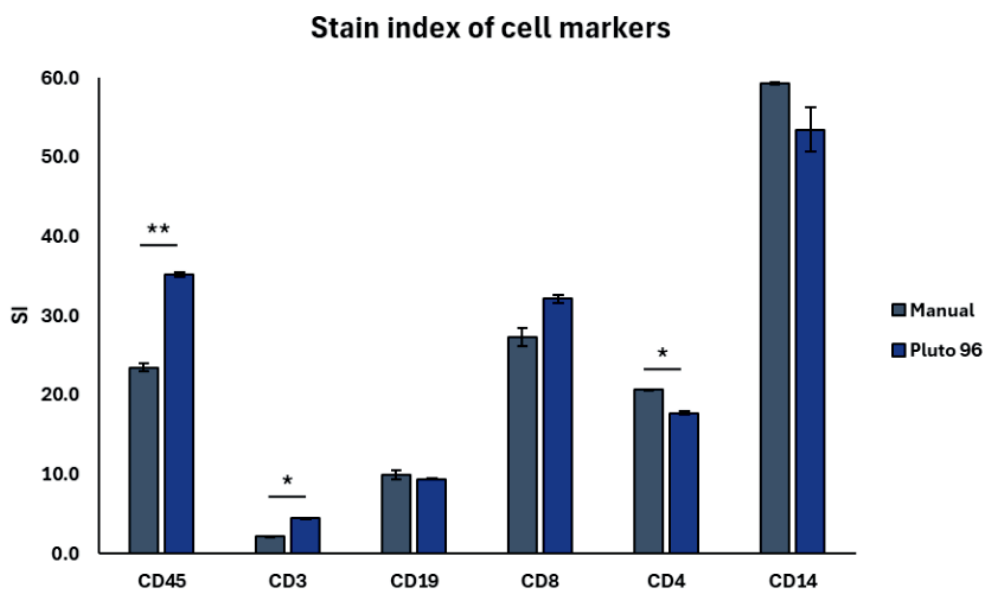


Figure 4. Stain index comparison for representative surface markers following manual centrifuge washing and Pluto washing at the 96-well format. Quantitative assessment of staining quality showing equivalent stain index values between workflows.

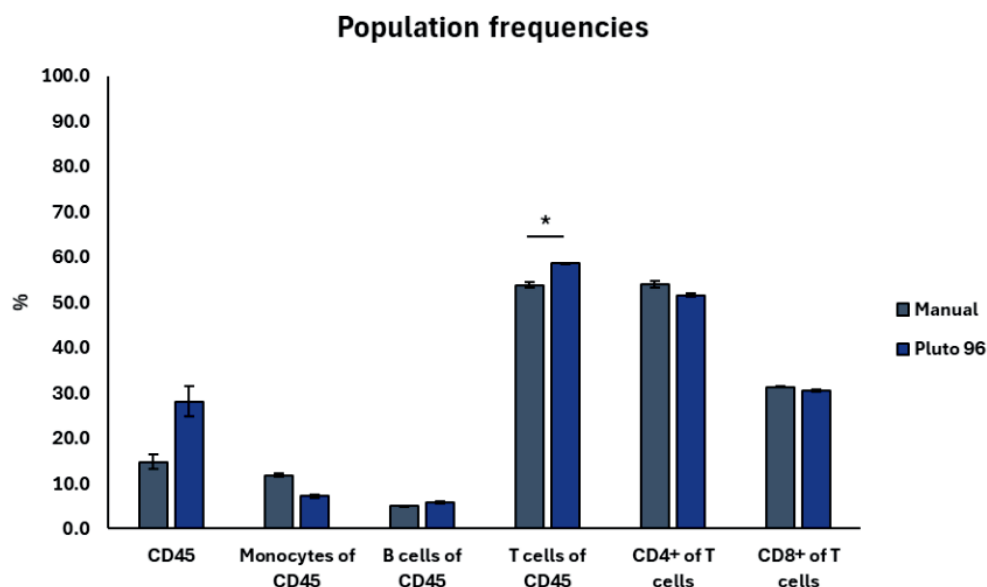


Figure 5. Population frequencies for major immune subsets following manual centrifuge washing and Pluto washing at the 96-well format. Frequencies of T cells, B cells, monocytes, and defined subpopulations identified by surface staining.

Conclusion

Automated washing on Pluto at the 96-well format maintains equivalence to conventional centrifuge-based SOPs across key biological and analytical endpoints, supporting its use as a replacement for manual washing prior to evaluation at higher well density.

4B. Biological Validation at the 384-Well Format

Purpose

The 384-well biological validation was designed to assess whether increasing assay density preserves cell recovery, staining quality, population integrity, and consistency across wells.

Comparison of 96-Well and 384-Well Automated Workflows

Cryopreserved PBMCs were stained using a TBNK antibody panel and processed using Pluto 96- and 384-well workflows. Key parameters, including wash buffer composition, wash intensity, incubation conditions, and staining volumes, were harmonized where possible.

Cell Recovery at the 384-Well Format

Cell recovery following automated washing at the 384-well format remained comparable

to the 96-well workflow (Figure 6), despite differences in working volume and surface-area-to-volume ratio. These results indicate that Pluto washing supports efficient cell handling under the tighter volume constraints of 384-well plates.

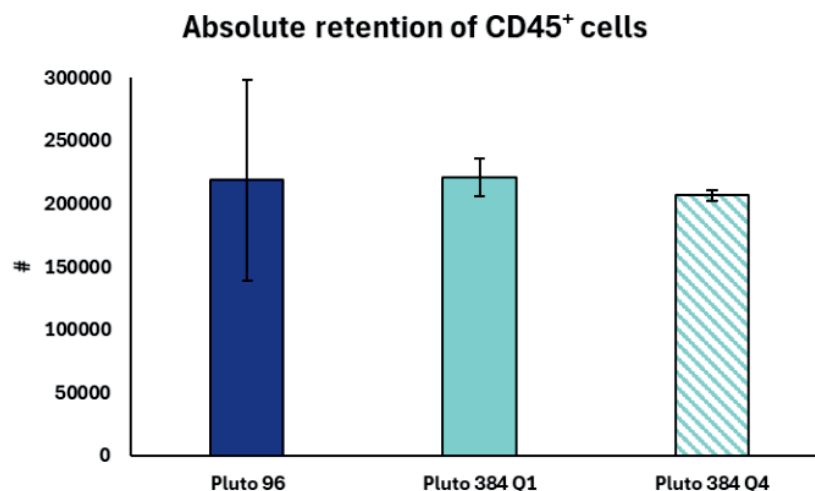


Figure 6. Cell recovery following automated washing at 96- and 384-well formats. Comparison of PBMC recovery following Pluto washing under harmonized conditions.

Staining Quality and Marker Resolution

Staining performance was evaluated by comparing stain index values (Figure 7) and marker resolution (Figure 8) for representative TBNK markers across formats. Marker resolution at the 384-well format was comparable to that observed at the 96-well format, with no systematic increase in background. Notably, 384-well Pluto samples showed a slightly stronger positive shift for CD3 and viability markers.

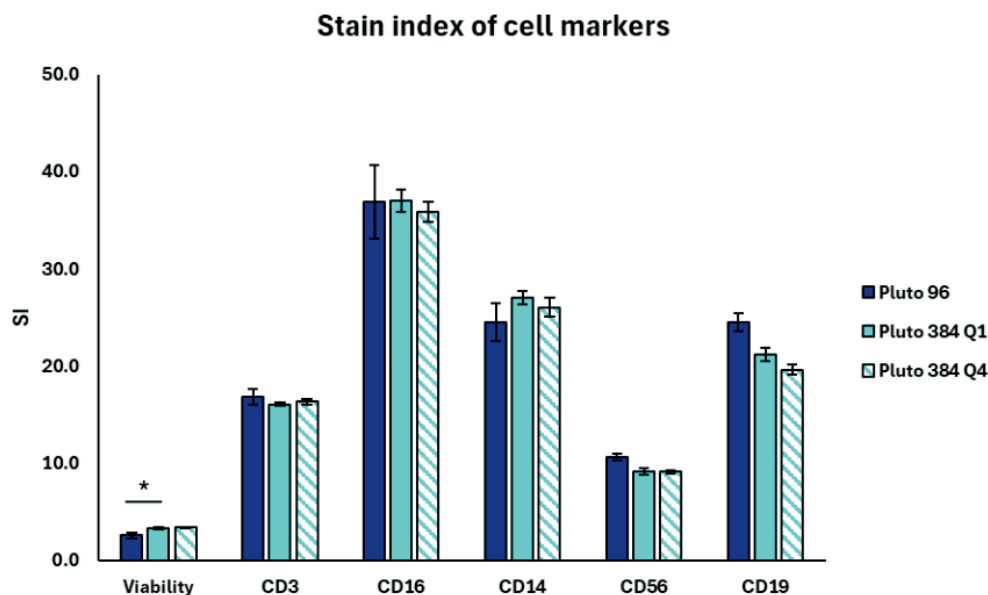


Figure 7. Stain index comparison between 96- and 384-well formats. Quantitative assessment of staining performance for representative TBNK markers.

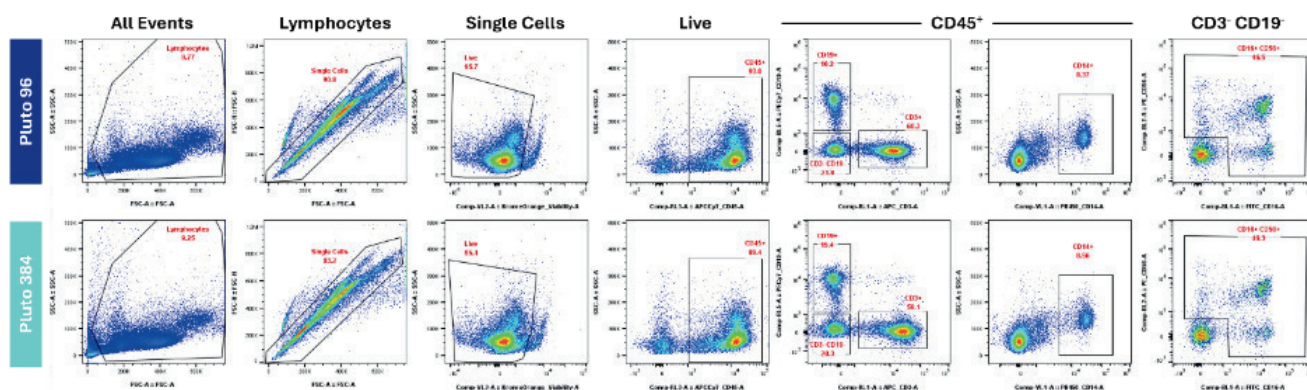


Figure 8. Representative marker resolution for TBNK staining on 96- and 384-well formats. Representative plots illustrating marker separation and background signal.

Preservation of Population Frequencies

Frequencies of major immune populations (T cells, B cells, NK cells, and defined subpopulations) were consistent between 96- and 384-well workflows, indicating that higher cell packing density associated with smaller well formats did not introduce selective cell loss or population bias.

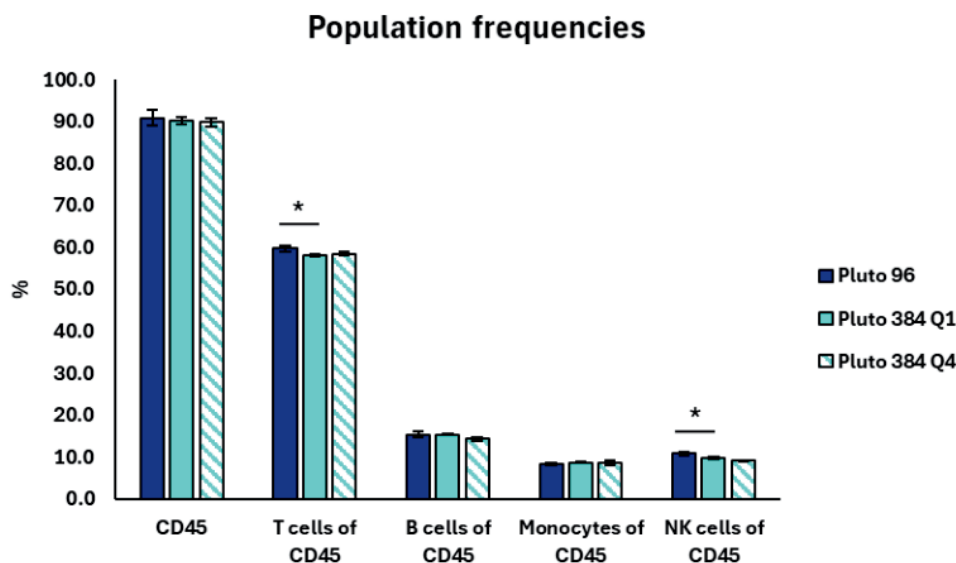


Figure 9. Comparison of population frequencies between 96- and 384-well Pluto workflows.

Well-to-Well and Quadrant Consistency

Well-to-well and quadrant-level consistency were evaluated across the 384-well plate using biological readouts, including cell recovery (Figure 6), population frequencies (Figure 9) and staining metrics such as stain index (Figure 7) and marker resolution (Figure 10).

Variability across wells and quadrants remained low, demonstrating uniform biological performance across the plate.

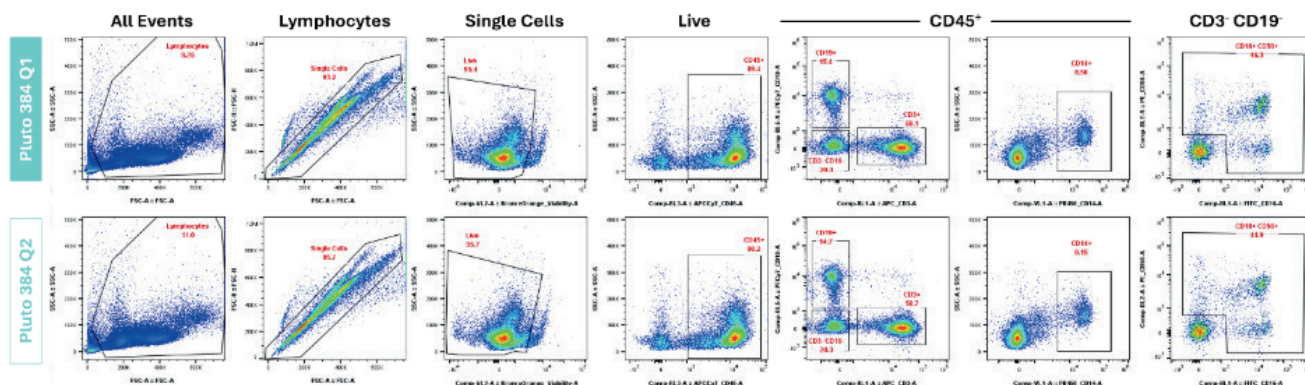


Figure 10. Representative marker resolution for TBNK staining across quadrants of a 384-well plate. Representative plots demonstrating consistent marker resolution across plate quadrants following automated washing.

Interpretation of 384-Well Biological Validation

Together, these results demonstrate that Pluto washing can be applied at the 384-well format while preserving key biological and analytical endpoints. The combination of acceptable cell recovery, maintained staining quality, consistent population frequencies, and uniform performance across wells supports the suitability of Pluto for high-density flow cytometry workflows without reliance on centrifugation.

5. Conclusion

This study demonstrates that C-FREE™ Pluto Wash enables reliable washing at the 384-well format for surface marker flow cytometry workflows. Bead-based validation confirms robust technical performance, with high retention, effective wash efficiency, and uniform behavior across wells and quadrants, demonstrating controlled fluid exchange under reduced volumes and tighter tolerances.

Biological validation using cryopreserved PBMCs shows equivalence across a structured comparison chain from manual centrifuge SOPs to automated 96-well and 384-well workflows. At the 384-well format, cell recovery, staining quality, marker resolution, and population frequencies are comparable to lower-density workflows, with low well-to-well and quadrant-level variability.

Together, these results indicate that increased assay density does not introduce systematic loss, bias, or degradation of surface staining performance, enabling higher-throughput, reagent-efficient, and automation-compatible workflows.

6. C-FREE™ Pluto Platforms for 384-Well Workflow Implementation

The workflows described in this study can be implemented using either [Pluto Code](#) or the [Pluto HT Workstation](#).^{*} Pluto Code enables integration of [C-FREE washing](#) into existing liquid handling platforms via software, supporting incorporation of validated 384-well wash steps into broader automated workflows. The Pluto HT Workstation provides a standalone benchtop workstation, combining the same washing principles with other liquid handling functions for cell sample preparation.

**Full 384-well product compatibility for these platforms is expected to be available in the second half of 2026.*

Contact Us:

Please visit at curiox.com/contact or scan the QR code below to speak with an expert about our 384-well platform availability.

