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WHITEPAPER

TECHNICAL EVALUATION

Automating Antibody Cocktail Preparation for High-Dimensional Flow Cytometry

Closing the gap between automated acquisition and manual sample prep

KEY HIGHLIGHTS

VALIDATED

24-color
Validated PBMC, Stanford HIMC

PRECISION

<4%
Coefficient of variation at 5 µL dispense

EFFICIENCY

70%
Reduction in hands-on prep time

INSIDE THIS PAPER

- Automated cocktail preparation for panels up to **72 antibody parameters** per run
- Stanford HIMC evaluation: stain index, CV%, and reproducibility data for a 24-color PBMC panel
- Automated FMO and Single Color Control generation from panel definition
- Parameter capability matrix across **Pluto LT, MT, and HT** instrument configurations
- Audit trail, electronic signatures, and **21 CFR Part 11**–aligned compliance capabilities

SECTION I

INTRODUCTION

The Challenge of Consistency in Antibody Cocktail Preparation

Antibody cocktails are the backbone of modern flow cytometry assays, enabling researchers to resolve complex cell populations with precision. Preparing these cocktails manually is time-intensive, error-prone, and highly operator-dependent. This challenge is most acute in high-dimensional immunophenotyping, where each additional marker increases the risk of pipetting inconsistencies, reagent waste, and missed controls.

A 24-color spectral panel requires more than 50 pipetting steps per batch. At volumes of 1–5 µL per antibody, small errors compound across parameters and propagate to every downstream sample. Fluorescence Minus One (FMO) and Single Color Control (SCC) preparations add further manual burden—this often causes essential controls to be omitted or prepared inconsistently.

Multi-site and multi-operator studies amplify the problem. Cocktails prepared by different individuals, at different sites, or across different shifts introduce systematic variability that is difficult to distinguish from biological signals.

Despite widespread adoption of automated cytometers and digital analysis platforms, most laboratories continue to rely on manual pipetting for upstream cocktail preparation. The gap between automated acquisition and manual preparation is widening as panel complexity increases.

The Solution: Integrated Automation with Pluto and Antibody Cocktailing Wizard

The C-FREE™ Pluto Workstation family, combined with the Antibody Cocktailing Wizard (ACW) software, addresses this gap directly. ACW runs by automating cocktail preparation on the same Pluto instrument used for sample prep. A single compact workstation handles cocktail preparation, staining, and centrifuge-free washing on one deck, driven by SOP-style protocols rather than operator-written scripts. ACW eliminates the need for a separate automation platform for cocktail preparation, removing a handoff that is often the largest remaining source of variability in high-dimensional workflows.

All Pluto Workstations (LT, MT, HT) support up to 72 antibody parameters per run, automated generation of FMO and SCC controls, full reagent traceability, and compliance-ready audit logging—all without scripting or re-optimization.

Researchers at the Stanford Human Immune Monitoring Center (HIMC) evaluated the platform for 24-color immunophenotyping. Staining quality matched or exceeded manual preparation, hands-on cocktail preparation time was reduced by approximately 70%, and performance was consistent across operators and runs.

What follows is the workflow in detail, the Stanford evaluation results, and the parameter capabilities across the Pluto LT, MT, and HT configurations.

SECTION II

WORKFLOW OVERVIEW - From Antibody Vial to Flow-Ready Sample

The C-FREE™ Pluto Workstation Platform

The Pluto Workstation family (LT, MT, HT) automates the complete sample preparation workflow on a single instrument: antibody cocktail preparation, staining, and centrifuge-free washing, from reagent vials to flow-ready cells. It is purpose-built for flow cytometry — not a general-purpose liquid handler adapted to the task. The workflow this paper focuses on begins with cocktail preparation.

Key platform characteristics:

- Dedicated antibody source racks compatible with U-bottom, V-bottom, and flat-bottom vials from all major suppliers (BD, BioLegend, Cytek, Thermo Fisher, and others). No proprietary consumables required.
- Pressure-driven pipette-based liquid level detection (pLLD) for accurate volume verification before aspiration.
- Direct USB connection between ACW laptop software and the Pluto Runner App on the Workstation tablet. No separate scripting environment required.
- Output to 96-well deep-well plate (DWP) enables seamless transition into the Workflow Wizard (WW) for end-to-end automated staining, or can create a large-volume mastermix in a 5 mL vial or 25 mL conical tube for later use.

Source Rack Types

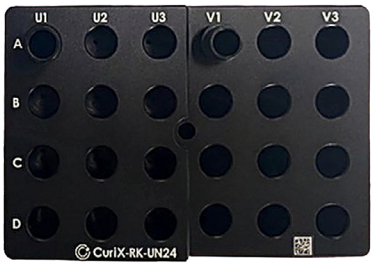
CuriX-RK-MX6

Large Volume Rack (6 pos, LT/MT)

CuriX-RK-FX12

Flex Rack (12 pos × 2)

CuriX-RK-UN24



UV Rack (24 pos × 2 on LT/MT)

Figure 1. Pluto source rack types. Total source capacity: 72 positions on all Pluto Workstations (LT, MT, HT).

Instrument configurations:

- Pluto LT: Up to 32-well throughput. Suited for assay development and individual researcher workflows.
- Pluto MT: Up to 72-well throughput. Suited for core labs and multi-operator immunophenotyping.
- Pluto HT: Up to 96-well throughput with 4-channel Vari-span pipette. Suited for biopharma QC, CROs, and multi-site harmonization.

Antibody Cocktailing Wizard (ACW) Software

ACW is a Windows laptop application that guides the user from antibody registration through panel design, validation, and run report. It communicates with the Pluto Workstation over USB; no internet connection or scripting environment is required.

The software is organized into six sequential tabs. Table 1 summarizes the workflow.

Step	ACW Tab	Action
1	**MasterList**	Import or manually enter antibodies: target, fluorochrome, clone, supplier, catalog, lot number, volume, expiry. CSV import supported.
2	**Define Panel**	Select antibodies from the MasterList . Assign source rack positions. Set µL/test values. Save panel as JSON file for reuse across operators and sites.

Step	ACW Tab	Action
3	**Preview & Validate**	Select configuration mode (Mastermix, FMO, SCC, or combination). Set number of tests and dead volume compensation. Run Simulation: validates Required Volume, expiry dates, and source/output position conflicts.
4	**Protocol Export**	Review color-coded Rack Map for vial placement. Confirm Pre-Export Validation. Generate protocol. Multiple modes (MM + FMO + SCC) combined in one protocol. Connect laptop to Pluto Workstation via USB. Transfer protocol file. Protocol executes on Workstation.
5	**Report**	After run completes: retrieve execution report via USB. Generate PDF with panel version, lot numbers, volumes dispensed, timestamps, operator ID. MasterList volumes updated automatically.
6	**Audit & Logs**	Review timestamped record of all user and system actions. Filter by user, action type, or date range. Export audit report for compliance or quality review.

Table 1. Six-step ACW workflow from antibody registration to run report. Steps 1–4 are performed on the laptop. Steps 5–6 require USB connection to the Pluto Workstation.

Multiple configuration modes (Mastermix, FMO, SCC) can be combined in a single protocol. After completing Preview & Validate for one mode, the user returns to that tab, selects the next mode, and appends it using the 'Add' option at export. All modes execute sequentially in a single Pluto run.

Cocktail to Flow-ready samples: End-to-end automation on Pluto Workstation with Antibody Cocktailing Wizard and Workflow Wizard

When ACW outputs directly to the Workflow Wizard (WW) reagent plate (96-well DWP), the complete sequence—cocktail preparation through sample staining and washing—executes on a single Pluto Workstation without manual handoff. Parameter ceilings in this end-to-end mode reflect the combined constraints of both ACW and the WW (see Section IV).

SECTION III
METHODS

Stanford Human Immune Monitoring Center (HIMC) Evaluation Design

Human peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation, cryopreserved, and later thawed using a standard protocol (Gupta *et al*., 2018). Cells were split into two workflows: automated (Pluto LT) and manual. The Pluto LT system performed all steps—reagent addition, mixing, incubation, and washing—automatically after deck setup. The manual workflow used pipetting and centrifugation.

Cells were stained with LIVE/DEAD® Fixable Red viability dye (15 min, RT), followed by a 24-color surface antibody cocktail (30 min, RT). Washing steps used PBS/FBS with centrifugation (8 min at 638 × g, 787 × g post-fixation). Viability was assessed via Vi-Cell (Beckman Coulter), and samples were acquired on a BD Symphony A5 and analyzed in FlowJo.

Both methods used identical antibody panels, sample inputs, and downstream staining and acquisition protocols. Two variables were compared: cocktail preparation method (manual vs. Pluto + ACW), shown between charts; and wash method (Pluto C-FREE™ vs. centrifuge-based), shown within each chart. Where sample volume allowed, each donor was processed across all four combinations in triplicate; one of the six donors had insufficient volume for the ACW cocktail preparation arm, so that arm includes five donors (Figure 3b) while the manual arm includes all six (Figure 3a). Key performance metrics included cell recovery, staining quality, and assay precision. The evaluation used six cryopreserved PBMC donors, performed in triplicates by a single operator. The 24-color antibody panel comprised the following markers: PD1 (CD279), CD20, CD25, CD127, CD14, CD57, IgD, CD16, CD27, CD11c, CD3, CD45RA, CD8, CD19, CD4, CD28, CD123, CD56, HLA-DR, CD11b, CD33+CD66b, CD38, and CCR7 (CD197); Brilliant Stain Buffer was included in the cocktail formulation.

Evaluation and Reagent Setup

Before initiating a run, the Pluto Workstation was configured with antibody source vials loaded into the appropriate source racks and output labware placed in the designated output position. For Mastermix: Flex Rack or Large Volume Rack. For FMO and SCC with >12 parameters: 96-well deep-well plate (CuriX-DWP). Tip racks were placed in default deck positions per instrument type.

Required Volume Verification was enabled in ACW Settings, and the Pluto used pLLD to measure liquid level in each source vial before dispensing began. Insufficient volume would trigger a top-up prompt before the run proceeded.

Panel Configuration in ACW

Antibodies were registered in the ACW MasterList with target, fluorochrome, clone, supplier, catalog number, lot number, vial type, volume, and µL/test. Registration was performed via CSV import using the ACW template; volume data updated automatically after each completed run report.

Each panel entry required a source rack position, output position, and µL/test value. Panels were saved as JSON files for reuse across operators, sites, and future runs. Antibody order determined dispensing sequence (top to bottom). For Mastermix mode, antibodies were ordered in descending µL/test to ensure smaller volumes were not dispensed first into empty wells. The Volume-Ordered Precision Dispensing setting in ACW Advanced Run Settings automated this ordering.

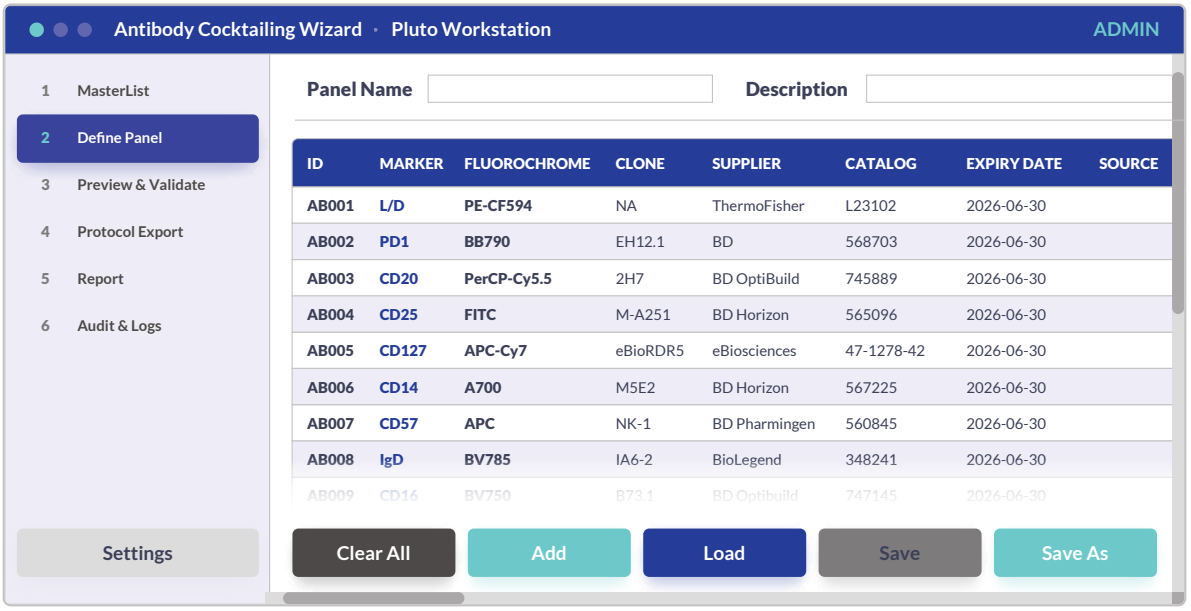


Figure 2. A completed 24-color panel in ACW. Source positions, output assignments, and µL/test values are assigned per antibody. The panel is saved as a JSON file for reuse.

Pre-Run Validation

Before protocol export was unlocked, the user ran Simulation in the Preview & Validate tab. Simulation performed four checks. Table 2 summarizes each check and its resolution path. Simulation did not evaluate spectral overlap or reagent compatibility — panel spectral design was verified in the cytometer's panel design tool before importing into ACW.

Validation Check	Condition Flagged	Resolution
Required Volume	Remaining vial volume < volume required for protocol	Update volume in MasterList , replace vial, reduce tests, or adjust dead volume compensation
Expiry Status	Expiry date at or past today's date	Replace with non-expired reagent; warning does not block export but flags result reliability risk
Source Position Conflict	Two or more vials assigned to same source well	Return to Define Panel; reassign duplicate source positions to distinct wells
Output Position Conflict	Selected output well overlaps with a well used in a previous protocol within the same batch session	Select a different output starting well, or use Clear All to reset the batch session

Table 2. ACW Simulation validation checks. Volume and position errors block protocol export; expiry warnings flag the issue without blocking export.

Protocol Export, Run Execution, and Reporting

On passing Simulation, the Protocol Export tab unlocked. The user reviewed the color-coded Rack Map and confirmed the Pre-Export Validation popup before generating the protocol and transferring it via USB to the Pluto Runner App. The protocol executed with a single tap — no scripting, no USB flash drive transfer, and no filename matching required.

Following each run, ACW generated a PDF report containing the panel version, antibody lot numbers, volumes dispensed, run timestamps, and operator ID. MasterList volumes were decremented automatically based on actual dispensed volumes. These records provided per-run traceability for the evaluation and were used to confirm dispensing accuracy independently of staining results.

SECTION IV

RESULTS

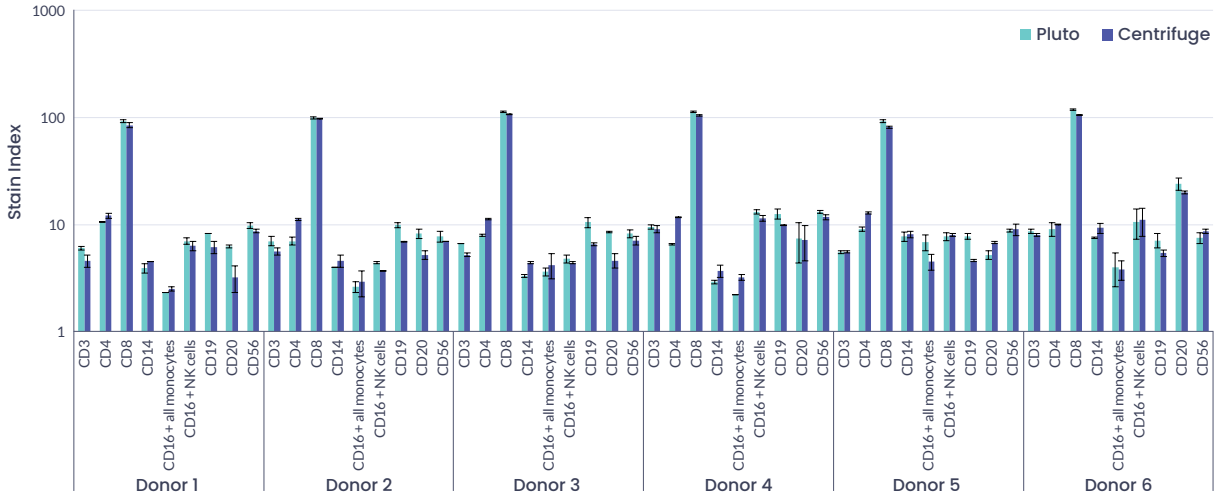
Stanford HIMC Evaluation and Parameter Capability

Evaluations conducted at the Stanford HIMC assessed the C-FREE™ Pluto LT Workstation for 24-color immunophenotyping using PBMC panels. Cocktails prepared on Pluto were compared against manually prepared cocktails from experienced operators across stain index, coefficient of variation, and hands-on preparation time.

Stain Index and Population Separation

Pluto-prepared cocktails achieved stain indices equivalent to or exceeding those of manually prepared panels across the 24-color panel. Fluorescence intensity distributions were comparable across all major immune subsets (CD3, CD4, CD8, CD19, CD56, and others). No degradation in population resolution was observed in automated preparations.

3a. Stain Index by Automation vs Manual Handling: Manually Prepared Antibody Cocktails (6 Donors)



3b. Stain Index by Automation vs Manual Handling: ACW-Prepared Antibody Cocktail (5 Donors)

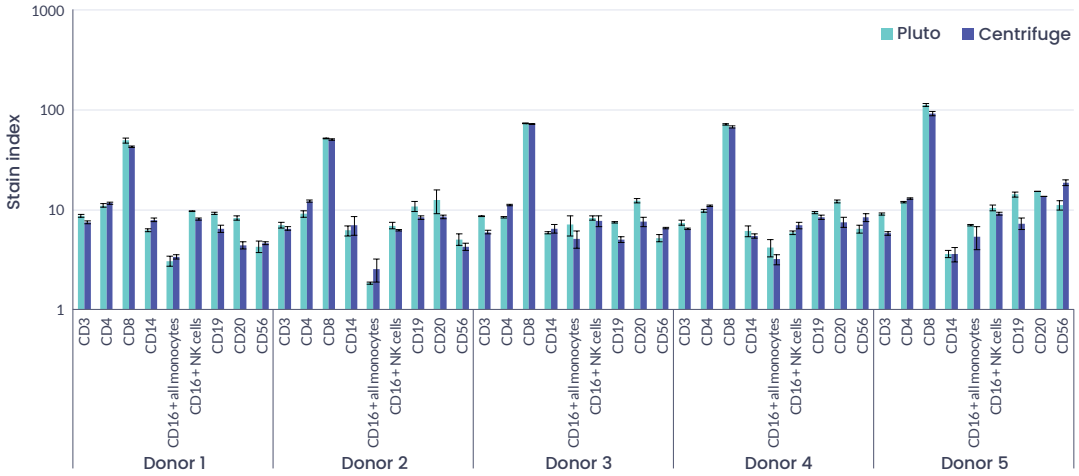


Figure 3. Stain index across multiple markers for manually prepared (3a) and ACW-prepared (3b) 24-antibody cocktails, each tested with Pluto C-FREE™ wash and centrifuge wash. Comparison between charts shows major immune markers' stain index equivalence across cocktail preparation methods. Comparison within each chart shows wash method has no material effect on staining quality regardless of how the cocktail was prepared.

Coefficient of Variation

Automated workflows produced lower intra- and inter-assay variability relative to manual preparation. CV% at 5 µL dispense volume was confirmed below 4% across replicate preparations. CD3 and CD4 markers showed reduced CV% across replicate runs, consistent with elimination of operator-dependent pipetting error at small volumes.

Time Savings

Manual cocktail preparation for the 24-color panel required approximately 90 minutes of hands-on time, including volume calculations, pipetting, and mixing. The Pluto workflow reduced hands-on time to approximately 15 minutes (deck setup and protocol configuration), with total run time of 60–70 minutes. Hands-on time across the full workflow — encompassing cocktail preparation, staining, and washing — was reduced by approximately 90%.

Hands-on time step alone for the cocktail preparation was reduced by approximately 70%, consistent with the operator experience reported by Stanford HIMC. This reduction in hands-on time translates directly to available analyst time for data review, panel optimization, and downstream work.

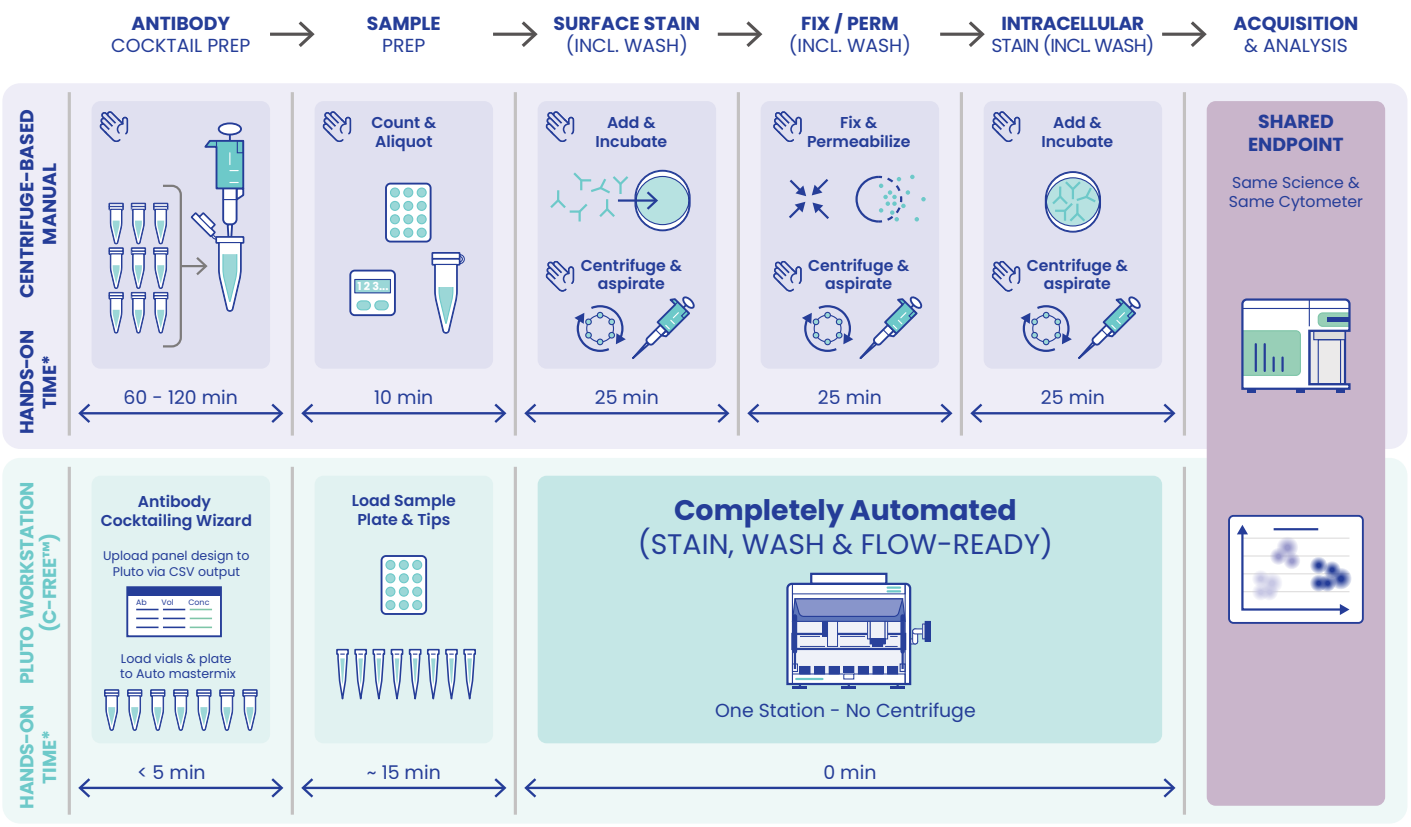


Figure 4. End-to-end workflow time — manual vs. Pluto, from cocktail preparation to flow-ready samples. * Hands-on time per phase. Pluto runs unattended after setup.

MANUAL — vs — PLUTO
~2.5–3.5 hrs / ~20 min

REDUCTION OF
~90%
in hands-on time

ELIMINATED
Centrifuge
at every wash

Metric	Manual Workflow	Pluto Workstation
Hands-on prep time (24-color panel)	~90 minutes	~15 minutes
Total run time	~90 minutes	~60–70 minutes
CV% at 5 µL dispense	Operator-dependent	< 4%
Stain index vs. manual	—	Equivalent or superior
FMO/SCC generation	Manual, frequently omitted	Automated from panel definition
Lot and operator traceability	Manual logs (if kept)	Tamper-proof PDF per run

Table 3. Performance comparison for 24-color PBMC panel preparation, Stanford HIMC. Hands-on time, CV%, and stain index data from the evaluation.

> *"The antibody cocktail automation with Curiox completely changed our lab efficiency. We cut prep time by 70% and achieved unparalleled consistency."*
>
> **Dr. Holden Maecker**
> Stanford Human Immune Monitoring Center

Liquid Level Detection and Reagent Volume Tracking

ACW's Required Volume Verification feature uses pLLD to confirm that each source vial contains sufficient volume before the run begins. Insufficient volume triggers a top-up prompt; the run does not proceed until volume is confirmed adequate. Following each completed run, ACW automatically decrements MasterList volumes based on actual dispensed volumes, enabling proactive inventory management and audit-ready records of reagent consumption.

Enabling Required Volume Verification increases total run time modestly (approximately 10–15% for a 24-parameter run). It is disabled by default for speed in routine use; most laboratories with high-cost reagents or regulated workflows find the tradeoff acceptable.

Parameter Capability: Maximum Antibodies per Run

The maximum number of antibody parameters in a single uninterrupted run depends on instrument type (LT, MT, HT), control configuration (Mastermix only, or with SCC and/or FMO controls), and workflow type — cocktailing only or cocktail → stain → wash (Table 4).

	Mastermix Only	MM + SCC or FMO	MM + SCC and FMO
Cocktailing only	72	72	48
Cocktail → Stain → Wash	72	LT: **31** MT: **71** HT: **72**	LT: **15** MT: **35** HT: **45**

Table 4. Maximum antibody parameters per single run vs. instrument type, control configuration and workflow type. Numbers apply to all three instruments (Pluto LT, MT, HT) unless an instrument is specified individually.

45-Color End-to-End Feasibility

For laboratories running high-plex panels at or near 45 colors — the current high end of routine OMIP-benchmark use — instrument selection has a direct impact on what is achievable in a single end-to-end run.

For laboratories running high-plex panels at or near 45 colors, Pluto HT is the only instrument supporting a full end-to-end run with complete SCC and FMO controls (max 45 parameters, bound by reagent plate at 91/96 wells). Pluto MT reaches 45 colors under Mastermix-only and MM + one control type configurations but not with full SCC and FMO. Pluto LT supports 45-color Mastermix-only runs but does not reach 45 colors in end-to-end mode with controls. For requirements beyond 45 colors, configuration-specific assessment by a Field Application Scientist is recommended.

SECTION V

DISCUSSION - Automated Preparation Does Not Compromise Data Quality

Interpretation and Implications

A persistent concern about automating antibody cocktail preparation is that mechanical handling might introduce new error sources or require extensive re-optimization. The Stanford HIMC evaluation addresses this concern directly: Pluto-prepared cocktails produced stain indices and population resolution equivalent to or exceeding manually prepared controls across a 24-color PBMC panel, with lower CV% across replicates. These findings are consistent with results from NIST, ETH Zürich, and AbbVie, where reproducibility was confirmed across independent sites and operators.

The likely mechanism is straightforward: automated dispensing eliminates operator-to-operator volume variation at the 1–5 µL range, where manual pipetting is most prone to error. For panels above 20 parameters, the cumulative effect of per-parameter volume accuracy is substantial.

Controls as a Scaling Problem

FMO and SCC controls are not optional in high-dimensional cytometry; they are required for accurate gating and compensation matrix calculation. In manual workflows, the additional pipetting burden of generating one FMO tube per parameter and one SCC tube per parameter is frequently cited as a reason controls are omitted or abbreviated, particularly in busy core labs.

ACW generates controls automatically from the panel definition. No separate pipetting plan is required. This removes the practical barrier to complete control generation in routine workflows—a meaningful shift for assay standardization.

Traceability and Compliance in Regulated Research

As flow cytometry workflows increasingly support regulated research (biopharma drug development, QC, and multi-site clinical studies), the absence of traceable preparation records introduces audit risk. Manual logs, when kept, are not tamper-proof and do not capture operator-level attribution at the action level.

ACW's audit trail records every user action with timestamp and electronic signature. Every run produces a PDF report containing lot numbers, operator ID, panel version, and dispensed volumes. The system is designed to support 21 CFR Part 11–aligned workflows for electronic records and electronic signatures, subject to the user organization's procedural controls (see footnote 2).

Capability	Detail
Audit trail	Tamper-proof encrypted storage. Hash-chain integrity. All actions timestamped and user-attributed.
Electronic signatures	Password re-entry required for critical operations: panel save, protocol export, data modification.
Role-based access control	Four roles: Admin, Specialist, Operator, Viewer. Only Admin can create or modify accounts.
Session security	15-minute inactivity timeout. Account lockout after 5 consecutive failed login attempts.
Traceability per run	Every PDF report includes lot numbers, operator ID, panel version, volumes dispensed, and timestamps.
LIMS connectivity	CSV data exchange for integration with existing laboratory information management systems.

Table 5. ACW compliance capabilities. 21 CFR Part 11 compliance requires both ACW software controls and organizational procedural controls (SOPs, password policies, audit review). For Research Use Only.

Operational Considerations

Several practical considerations apply when implementing ACW in routine laboratory workflows:

- Required Volume Verification — enabling pLLD-based verification increases total run time by approximately 10–15% for a 24-parameter run. Disabled by default; most laboratories with high-cost reagents or regulated workflows find the tradeoff acceptable.
- Dead volume compensation — configure using Extra Tests or Extra Volume methods based on known dead volume for the output labware. Curiox-validated values are available in ACW Settings for supported vial types.
- Output labware for high-control panels — for panels with >12 FMO or SCC outputs, the 96-well DWP must be used as output labware; the 12-position Flex Rack is insufficient.
- Mastermix well volume cap — MM well volume is capped at 1,550 µL per well. At high antibody counts or high µL/test values, the mastermix may exceed this and require more than one reagent plate well, reducing the effective parameter ceiling.
- Technical replicates — parameter ceilings in Table 4 assume a single full-stain sample with no technical replicates. Additional MM destination wells can trigger multi-8 transfer mode on MT and HT.
- Spectral panel design — ACW Simulation does not check spectral overlap or reagent compatibility. Panel spectral design must be completed in the cytometer's panel design tool before importing into ACW.

Limitations of the Current Evaluation

The Stanford HIMC data presented here is from a 24-color PBMC panel. Generalizability to panels above 30 colors, intracellular staining protocols (which use two mastermix outputs), or non-PBMC sample types has not been independently published. Laboratories operating at 40–45 colors or with non-standard protocols should conduct their own bridging evaluation before fully replacing manual preparation.

The 70% hands-on time reduction reflects a specific comparison (single-operator, 24-color panel). Time savings will vary with panel size, number of technical replicates, and the degree of existing manual workflow optimization in the receiving laboratory.

SECTION VI

CONCLUSION

Automated antibody cocktail preparation using the C-FREE™ Pluto Workstation and ACW software delivers equivalent or superior staining performance relative to manual preparation, with measurable reductions in hands-on time and intra-assay variability. The Stanford HIMC evaluation of a 24-color PBMC panel confirms these outcomes under real laboratory conditions.

Beyond reproducibility, ACW addresses operational gaps that manual preparation cannot resolve at scale: complete and consistent FMO and SCC control generation, lot-level traceability per run, and audit-defensible records for regulated research environments. These capabilities are delivered within the same compact Pluto Workstation that handles staining and centrifuge-free washing, eliminating the need for a separate automation platform.

For laboratories running panels up to 25 parameters with Mastermix-only or single control type preparation, Pluto LT provides a fully capable end-to-end path. For panels at 45 colors with complete SCC and FMO controls, Pluto HT is the instrument of record. Pluto MT covers the range between these two configurations.

As panel complexity continues to increase and multi-site standardization requirements tighten, automated cocktail preparation transitions into a prerequisite for reproducible, audit-ready immunophenotyping. Standardizing the irreversible upstream step — sample preparation — is what allows data generated on Pluto today to remain comparable across sites tomorrow, and usable as input to AI- and ML-driven analysis pipelines as those tools enter routine flow cytometry practice.

SCHEDULE A DEMO

Schedule a 30-minute virtual demonstration

with a Curiox application scientist, please contact your local representative or visit **www.curiox.com/pluto** or email **sales@curiox.com**.

REFERENCES

[1] 70% hands-on time reduction: Dr. Holden Maecker, Stanford Human Immune Monitoring Center. Results may vary by lab context and panel complexity.
[2] 21 CFR Part 11 compliance requires both ACW software controls and organizational procedural controls. For Research Use Only. Not intended for diagnostic procedures.
[3] Gupta R, Puleo A, Maecker H. PBMC Processing and Cryopreservation Standard Operating Procedure. Stanford Human Immune Monitoring Center, Stanford University School of Medicine. Available at: iti.stanford.edu/himc/protocols.html

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