Analytical Validation of a 29-Color Spectral Flow Cytometry Panel for Broad Immunophenotyping and Functional Activity Assessment in Clinical Trials

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Introduction

We developed and validated a fit-for-purpose 29-color spectral flow cytometry panel on the Cytek Aurora platform to enable comprehensive immune profiling in clinical immunotherapy studies. The assay captures a broad range of immune populations—including T cells, NK cells, B cells, and myeloid subsets—while allowing deeper characterization of conventional and unconventional T cell subsets and their functional states through activation and cytokine markers. Using stimulated, cryopreserved peripheral blood mononuclear cells (PBMCs) to model clinical samples and incorporating strategies such as iNKT enrichment and advanced spectral unmixing with autofluorescence correction, the panel demonstrated robust performance across >2000 reportable parameters. Approximately 60% of reportables met predefined precision criteria, with most variability driven by rare cell populations or low-expression of functional markers. Overall, this platform supports high-dimensional immune monitoring, enabling improved biomarker discovery, response assessment, and safety evaluation in cell therapy and immunotherapy trials

Panel Configuration

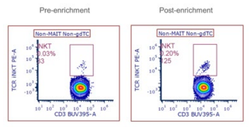
Tube ID	UltraViolet		Violet		Blue		Yellow green		Red	
	Specificity	Fluorochrome	Specificity	Fluorochrome	Specificity	Fluorochrome	Specificity	Fluorochrome	Specificity	Fluorochrome
Tube 1 FMx	CD3	BUV 395	CXCR6	BV421		AF488	Vα24Jα18 TCR	PE	CXCR3	APC
	CD8	BUV496	CD4	cFluor V450	CD15	cFluor B548		RY586		AF647
	CD19	BUV 563	Viability	LIVE/DEAD Aqua	CD56	RB613	Siglec-8	PE-Dazzle 594	CD169 (Siglec-1)	VioBright R720
		BUV615	Vα7.2 TCR	BV605	CCR4	BB700		PE-Cy5		APC-eFluor780
	CD45	BUV737	TCR γδ	BV711	CTLA-4	RB705	CD244	PE-Cy5.5	HLA-DR	APC-Fire 810
	CD11b	BUV805	CD206	BV785		RB780		PE-Cy7		
Tube 2 Full Stain	CD3	BUV395	CXCR6	BV421	RORγT	AF488	Vα24Jα18 TCR	PE	CXCR3	APC
	CD8	BUV496	CD4	cFluor V450	CD15	cFluor B548	IL-13	RY586	TGFβ1	AF647
	CD19	BUV563	Viability	LIVE/DEAD Aqua	CD56	RB613	Siglec-8	PE-Dazzle 594	CD169 (Siglec-1)	VioBright R720
	IFNγ	BUV615	Vα7.2 TCR	BV605	CCR4	BB700	T-bet	PE-Cy5	IL-17A	APC-eFluor780
	CD45	BUV737	TCR γδ	BV711	CTLA-4	RB705	CD244	PE-Cy5.5	HLA-DR	APC-Fire 810
	CD11b	BUV805	CD206	BV785	IL-4	RB780	IL-22	PE-Cy7		
Tube 3 Unstained control										

Tube 1: FMx: intracellular staining and used for gating control; *Tube 2: Full stain: intracellular staining;* Tube 3: unstained for unmixing control.

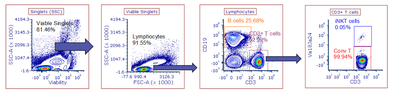
Feasibility Testing and Panel Optimization

iNKT Enrichment

- iNKT cell enrichment was performed with anti-iNKT microbeads (Miltenyi biotec) per manufacturer's protocol.
- Data shown is a comparison of pre- and post-iNKT enrichment samples from a single donor.
- Post-enrichment sample had approximately 7x higher concentration of iNKT cells than pre-enrichment sample by flow cytometry.
- Enriched iNKT cells were spiked into PBMCs for precision experiments.

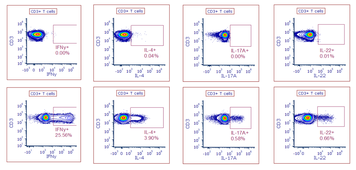


Identification of T cells and iNKT cells (PMA+ionomycin stimulated PBMCs)



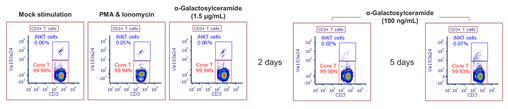
After removal of time abnormalities and doublets (not shown), viability dye-negative/SSC^{low} cells were gated, followed by gating of PBMCs using FSC/SSC. T cells and B cells were distinguished by expression of CD3 and CD19, respectively, then iNKT cells were identified from conventional T cells by expression of TCR Vα24Jα18. Staining patterns shown are representative of other stimulation conditions.

PMA+Ionomycin stimulation induce cytokine production in PBMCs



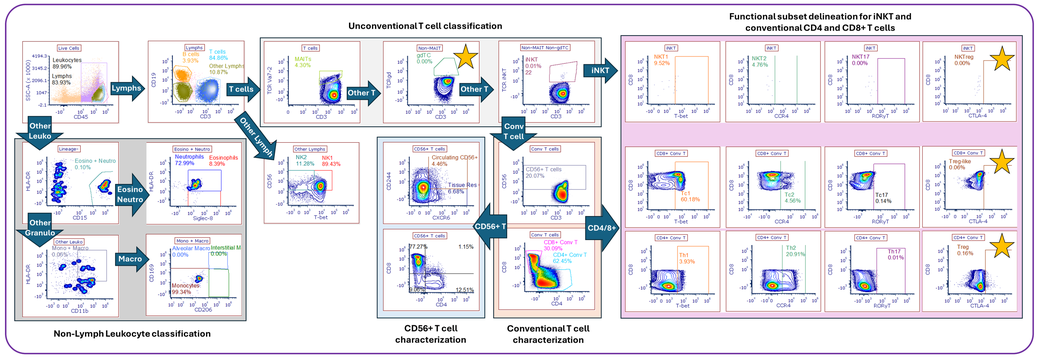
PBMCs were stimulated with PMA+iono for 6 hours at 37ºC in the presence of GolgiStop (monensin) and GolgiPlug (brefeldin A).
T cells were gated for the expression of IFNγ, IL-4, IL-17A, and IL-22 based on FMO control (not shown).
Significant production of cytokines were observed with PMA+iono.

iNKT cell numbers with stimulation



Healthy donor PBMCs were cultured for 6 hours in one of three stimulation conditions (medium only, medium with PMA and ionomycin, or medium with α-galactosylseramide; top row of plots). For the α-galactosylseramide condition, two additional longer timepoints (2 days and 5 days) were also performed to test whether longer stimulation would enhance the compound's effect. Cells were collected from each culture condition and assessed for iNKT cells using an identical sample processing and analysis procedure. Representative plots are shown for each condition.
iNKT cell abundance remains constant across different stimulation conditions and timepoints.

Gating strategy



Method

Sample processing:
Antibody cocktail was prepared using titrated antibodies volume.

Whole blood from four apparently healthy donors was collected in NaHep CPT tubes, shipped at ambient temperature and PBMCs were isolated according to the manufacturer's protocol (BD Biosciences). The PBMCs were rested overnight in RPMI/10%FBS media containing penicillin/streptomycin + GlutaMAX + HEPES, in a 37ºC/5% CO_2 incubator. These cells were stimulated in vitro with PMA and ionomycin for 6 hours (GolgiStop and GolgiPlug for the last 5 hours) followed by cryopreservation (in liquid nitrogen). On the day of experiment, vial(s) was thawed in RPMI/10%FBS media, counted and stained according to the panel configuration.

Staining protocol (extra- and intra-cellular):

1. 100 µL of PBMCs suspension in Pharmingen BSA stain buffer (stain buffer) (~2 million cells) was added to Tubes 1 and 2 (FMx and Full Stain) and 50 µL (~1 million cells) to Tube 3 (Unstained).
2. Appropriate volume of extracellular antibody cocktail mix was added to the FMX and FS tubes only.
3. Vortexed gently to mix cells and antibodies and incubated for 30 minutes in the dark at room temperature (RT).
4. 2 mL of stain buffer was added to all three tubes, centrifuged (5 min at 400 *x g* at RT) and aspirated the supernatant.
5. 2 mL of stain buffer was added to all three tubes, centrifuged (5 min at 400 *x g* at RT) and aspirated the supernatant.
6. 1 mL of FOXP3 fixation/permeabilization solution was added to all tubes, resuspended the cells and incubated for 30 minutes in the dark at RT.
7. 2 mL of permeabilization buffer (1x) was added, centrifuged (5 min at 400 *x g* at RT) and aspirated the supernatant.
8. 2 mL of permeabilization buffer (1x) was added, centrifuged (5 min at 400 *x g* at RT) and aspirated the supernatant.
9. 100 µL of permeabilization Buffer was added and mixed well.
10. Intracellular antibody cocktail mix was added to Tube 2 (FS tube) only (stain buffer was added to Tubes 1 and 3).
11. Gently vortexed and incubated for 30 minutes in the dark at RT.
12. 1 mL of permeabilization buffer (1x) was added, centrifuged (5 min at 400 *x g* at RT) and aspirated the supernatant.
13. 1 mL of permeabilization buffer (1x) was added, centrifuged (5 min at 400 *x g* at RT) and aspirated the supernatant.
14. 250 µL of stain buffer was added, mixed well and acquired on the instrument.

Reference controls & Spectral unmixing:

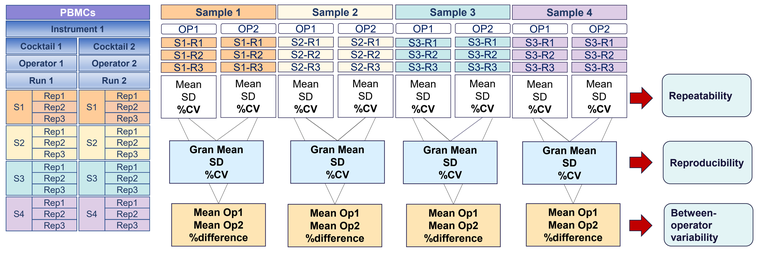
1. Stimulated PBMCs stained with CD45,CD244, IFNg and Live/Dead AQUA.
2. Ultracomp beads stained with CD169.
3. SpectraComp beads stained with all remaining markers.

All controls were stained following the extra- and intra-cellular staining protocol. Stimulated PBMCs were used for sample-specific autofluorescence signatures. Unmixing was done using multiple AF signatures for autofluorescence extraction (AF).

Reportable Populations (Summary)

- The assay quantitatively captures immune composition and function across major hematopoietic lineages, including lymphoid (T cells, B cells, NK cells) and myeloid populations (monocytes, macrophages, neutrophils, eosinophils).
- Both conventional and unconventional T cell subsets (CD4$^+$, CD8$^+$, γδ T cells, MAIT, iNKT, and NKT subtypes).
- Th1, Th2, Th17, Treg populations.
- Tc1, Tc2, Tc17 populations.
- Specialized subsets:
  - ✓ Circulating and tissue-resident, CD56$^+$, and regulatory phenotypes - defined by transcription factors (T-bet, RORγt, CTLA-4);
  - ✓ Activation markers (HLA-DR, CD244), and
  - ✓ Chemokine receptors (CXCR3, CXCR6, CCR4).
  - ✓ Functional readouts include pro- and anti-inflammatory cytokine production (IFNγ, IL-4, IL-13, IL-17A, IL-22, TGFβ).
- Reportables include frequencies (% of parent populations), and selected MFI measurements.
- Cell count (cells/µL) were reported based on Cytek Aurora volumetric calculation.
- Primary reportables are determined by their relevance and importance to a specific clinical trial.

Validation Set-up and Results



Categorization and Interpretation of Precision Testing Results

Cell Population Abundance	Reportable Evaluation Results (number of samples with given result)			Interpretation	Recommendation
	Pass	Fail	Fail – Rare/No EC		
> 100 events in all samples	4	0	-	Precise	Accepted
	3	1	-	Possibly Imprecise	Accepted with Comment
	≤2	≥2	-	Imprecision Noted	Interpret with Caution
≤100 events in one or more samples	4	0	0	Precise	Accepted
	3	0	1	Very likely precise (occasionally rare)	
	2	0	2	Likely precise (somewhat rare)	
	1	0	3	Hard to evaluate (often rare)	Inconclusive
	0	0	4	Cannot evaluate (rare/absent)	
	3	1	-	Possibly Imprecise	Accepted with Comment
	≤3	≥1	≤3	Imprecision Noted	Interpret with Caution

Acceptance Criteria:

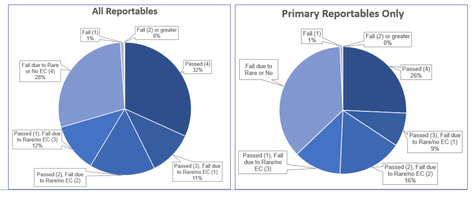
Precision (repeatability and reproducibility:
Non-rare populations: ≤25%CV
Rare populations: ≤35%CV

Inter-operator: ≤20% difference

Repeatability

Reportable Evaluation Result	All Reportables	Primary Reportables	Interpretation
Pass (4)	326	36	Precise
Pass (3), Fail due to Rare/no EC (1)	112	12	Very likely precise (occasionally rare)
Pass (2), Fail due to Rare/no EC (2)	164	23	Likely precise (somewhat rare)
Pass (1), Fail due to Rare/no EC (3)	122	17	Hard to evaluate (often rare)
Fail due to Rare or No EC (4)	292	51	Cannot evaluate (rare/absent)
Pass (3), Fail (1)	9	1	Possibly Imprecise
Fail (2) or greater	2	0	Imprecision Noted
Total	1027	140	

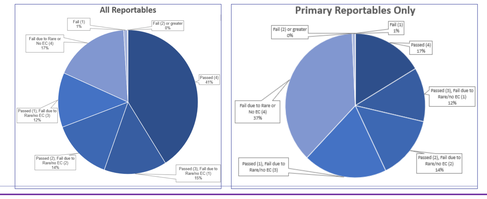
Accepted
Inconclusive
Accepted with Comment
Interpret with Caution



Reproducibility

Reportable Evaluation Result	All Reportables	Primary Reportables	Interpretation
Pass (4)	368	23	Precise
Pass (3), Fail due to Rare/no EC (1)	130	17	Very likely precise
Pass (2), Fail due to Rare/no EC (2)	124	20	Likely precise
Pass (1), Fail due to Rare/no EC (3)	110	27	Hard to evaluate
Fail due to Rare or No EC (4)	156	52	Cannot evaluate
Fail (1)	8	1	Possibly Imprecise
Fail (2) or greater	1	0	Imprecision Noted
Total	897	140	

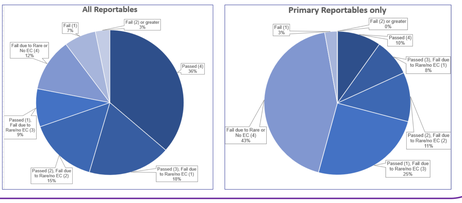
Accepted
Inconclusive
Accepted with Comment
Interpret with Caution



Between-operator variability assessment

Reportable Evaluation Result	All Reportables	Primary Reportables	Interpretation
Pass (4)	219	7	Precise
Pass (3), Fail due to Rare/no EC (1)	109	6	Very likely precise
Pass (2), Fail due to Rare/no EC (2)	91	8	Likely precise
Pass (1), Fail due to Rare/no EC (3)	51	18	Hard to evaluate
Fail due to Rare or No EC (4)	71	31	Cannot evaluate
Fail (1)	42	2	Possibly Imprecise
Fail (2) or greater	19	0	Imprecision Noted
Total	602	72	

Accepted
Inconclusive
Accepted with Comment
Interpret with Caution



Conclusion

- Rare cell detection challenges were addressed through iNKT enrichment, activated PBMCs spiking, and spectral unmixing, enabling robust characterization of diverse immune populations.
- 2000 reportables required a PASS/FAIL categorization framework, expressed as % of allowable results, to evaluate overall assay performance.
- Of 43 immune cell populations assessed, most inconclusive results were associated with rare cell types (e.g., iNKT cells).
- Similarly, among 19 functional markers (activation and cytokines), inconclusive outcomes were predominantly linked to rare populations.
- Provides simultaneous measurement of 19 functional markers, generating a high-dimensional dataset (>2000 reportables) from a single assay.
- Demonstrates strong precision and reliability, with most adequately represented populations meeting acceptance criteria for repeatability and reproducibility.
- ~40% of reportables could not be fully validated due to rarity/scarcity of certain populations and markers, particularly iNKT-related outputs.
- Overall, the assay meets validation standards for exploratory use and is suitable for clinical testing applications.

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