T cell phenotypes, differentiation states, and functional markers remain stable for two years using a blood collection kit for longitudinal, high parameter cytometry

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Background

More than 1.2 million cytometry specimens are processed annually across clinical trials. Of those, more than 20% are rendered unusable because they fall outside the 24 to 72 hour live processing window. Fresh samples processed at 72 hours show a 48% drop in total cell count (Fig. 1), an 88% decrease in monocyte frequency, and a 48% decrease in neutrophil frequency compared to same-day processing within hours of the blood draw.

TokuKit is a three-step, centrifuge-free whole blood preservation method supporting storage at -80°C for up to 24 months. Across 36 immune cell populations, frequencies show R > 0.99 at 24 months compared with same-week fixed samples, and R = 0.96 for functional marker-defined subsets (Fig. 2). Using a 41-marker mass cytometry panel supporting analysis of up to 735 immune cell subsets and states, samples from three donors were analyzed fresh and following 1-, 3-, 9-, 15-, 18- and 24-month storage (Fig. 3).

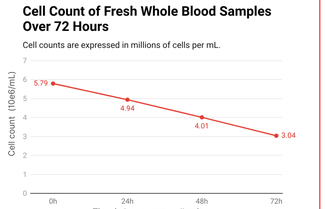


Figure 1. Line graph of cell counts (10e6) per milliliter(mL) of blood live samples over time.

Methods

Whole blood from three healthy donors was collected into sodium heparin or EDTA vacutainers. For the longitudinal stability assessment, EDTA vacutainers were used. For the fresh versus fixed comparison, both collection types were evaluated.

Live samples
Whole blood was diluted in ACK lysis buffer to lyse red blood cells and stained with a 33-marker antibody cocktail.

Fixed samples
Whole blood was fixed within 2 hours of collection using TokuKit (Stable-Lyse/Stable-Store, Smart Tube®) and frozen at -80°C in 2 mL aliquots. Samples were evaluated at 1, 3, 9, 15, 18, and 24 months post-fixation. Upon thawing, cells were labeled with a 20-plex palladium barcode, pooled, and stained with the 41-marker antibody cocktail. Cells were labeled with iridium intercalator and acquired on a Helios® mass cytometer (Standard BioTools).

Panel
The 41-marker panel profiles major immune cell lineages and functional states across up to 735 subsets. Populations and subsets with event counts below 100 at any donor-timepoint combination were excluded. No viability dye was included.

Analysis
FCS files were normalized using EQ bead-based batch normalization and debarcoded prior to analysis in CellEngine (CellCarta). Cell populations were identified by manual gating. Phenotypic population frequencies were calculated as a percentage of non-granulocyte populations. Functional marker frequencies were calculated as a percentage of the parent population. Approximately 500,000 to 700,000 total lymphocytes were analyzed per donor per timepoint. Pearson correlation coefficients were calculated between T=0 and T=24 month frequencies across all donor-marker combinations meeting the 100-event threshold.

Fresh v. Fixed Comparison

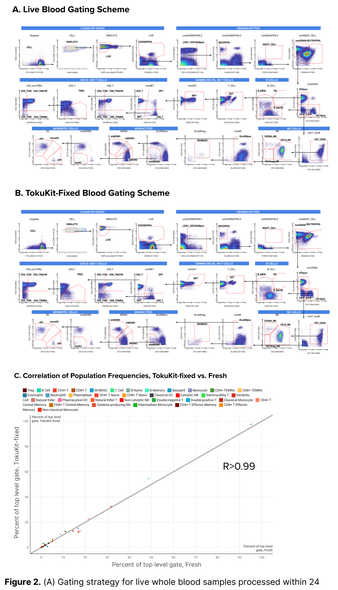


Figure 2. (A) Gating strategy for live whole blood samples processed within 24 hours of collection. (B) Gating strategy for TokuKit-fixed whole blood samples processed on the day of staining following frozen storage. (C) Correlation of immune cell population frequencies between fresh and TokuKit-fixed samples stored for 9 days at -80°C across 3 donors (R > 0.99, n=3, 33-marker mass cytometry panel).

TokuKit-Fixed Samples Preserve Immune Cell Populations

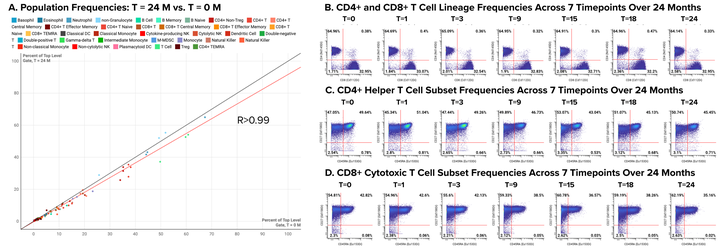
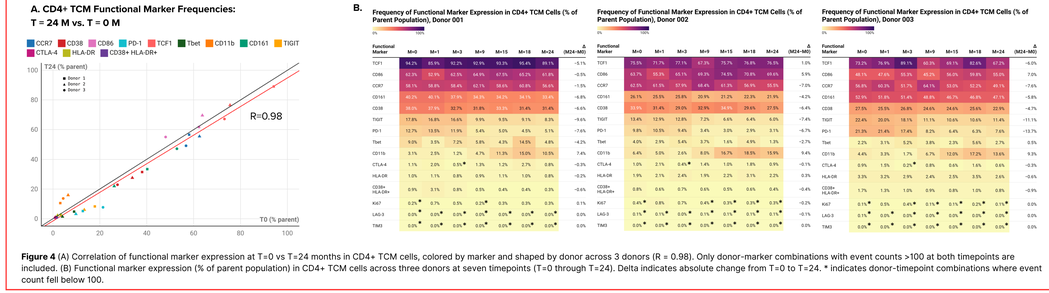


Figure 3. TokuKit-fixed whole blood preserves T cell population frequencies and differentiation states across 24 months of frozen storage (n=3 donors, 41-marker mass cytometry panel). (A) Correlation of immune cell population frequencies between TokuKit-fixed specimens processed within one week (T=0) and 24 months (T=24) post-fixation. Each point represents one immune cell population per donor, as a percentage of the top-level gate (R > 0.99). (B) CD4 vs CD8 density plots from CD3+ T cells at T=0 through T=24 confirm stable CD4+ helper and CD8+ cytotoxic T cell lineage frequencies across 7 timepoints. (C) CD45RA vs CD27 density plots from CD4+ T cells at T=0 through T=24 resolve naive, central memory, effector memory, and TEMRA subsets with stable proportions across 24 months. (D) CD45RA vs CD27 density plots from CD8+ T cells at T=0 through T=24 resolve the same four subsets with stable proportions across 24 months.

CD4+ Central Memory T Cell Functional Marker Expression is Preserved Across 24 Months of Frozen Storage



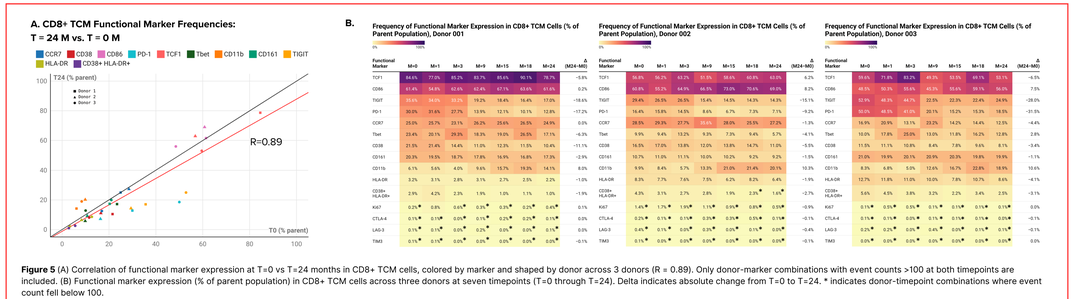
Functional Marker	M=0	M=1	M=3	M=9	M=15	M=18	M=24	Δ (M24-M0)
TCF1	94.2%	85.9%	92.2%	92.9%	93.3%	95.4%	89.1%	-5.1%
CD86	62.3%	52.5%	62.6%	66.5%	61.5%	62.5%	61.8%	-0.5%
CCR7	66.1%	56.8%	68.4%	63.1%	58.6%	62.0%	56.6%	-9.5%
CD161	40.2%	40.1%	37.9%	34.3%	34.2%	34.1%	33.4%	-6.8%
CD38	38.0%	37.9%	32.7%	31.8%	33.3%	31.4%	31.4%	-6.6%
TIGIT	17.8%	16.8%	16.6%	9.9%	9.3%	9.1%	8.3%	-9.6%
PD-1	12.7%	13.5%	11.9%	5.4%	5.0%	4.5%	5.1%	-7.6%
Tbet	5.0%	3.5%	7.2%	5.8%	4.3%	14.5%	4.8%	-0.2%
CD11b	3.1%	2.5%	1.2%	4.7%	11.3%	15.0%	10.5%	7.4%
CTLA-4	1.1%	2.0%	0.5%	1.3%	1.2%	2.7%	0.8%	-0.3%
HLA-DR	1.0%	1.1%	0.8%	0.9%	1.1%	1.0%	0.8%	-0.2%
CD38+ HLA-DR+	0.9%	3.1%	0.8%	0.5%	0.4%	0.4%	0.3%	-0.6%
Ki67	0.2%	0.7%	0.5%	0.2%	0.3%	0.3%	0.3%	0.1%
LAG-3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
TIM3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Functional Marker	M=0	M=1	M=3	M=9	M=15	M=18	M=24	Δ (M24-M0)
TCF1	75.5%	71.7%	77.1%	67.2%	75.7%	74.4%	76.5%	1.0%
CD86	53.7%	55.3%	61.1%	69.3%	74.5%	70.8%	69.4%	15.6%
CCR7	62.5%	61.5%	57.9%	68.4%	61.3%	56.9%	55.5%	-7.0%
CD161	26.1%	25.5%	25.8%	20.9%	21.2%	22.3%	21.9%	-4.2%
CD38	30.9%	31.4%	29.0%	32.9%	34.9%	29.6%	27.5%	-3.4%
TIGIT	13.4%	12.9%	12.8%	7.2%	6.6%	6.4%	6.0%	-7.4%
PD-1	9.8%	10.5%	9.4%	3.4%	3.0%	2.9%	3.1%	-6.7%
Tbet	4.0%	2.9%	5.4%	3.7%	1.6%	4.9%	1.3%	-2.7%
CD11b	6.4%	5.0%	2.6%	8.0%	16.7%	19.5%	15.9%	9.4%
CTLA-4	1.0%	2.1%	0.4%	1.4%	1.0%	1.8%	0.9%	-0.1%
HLA-DR	1.9%	2.1%	2.4%	1.9%	2.2%	3.1%	2.2%	0.3%
CD38+ HLA-DR+	0.8%	0.6%	0.7%	0.6%	0.5%	0.6%	0.4%	-0.4%
Ki67	0.4%	0.8%	0.7%	0.4%	0.3%	0.3%	0.3%	-0.2%
LAG-3	0.1%	0.1%	0.0%	0.1%	0.1%	0.1%	0.1%	-0.1%
TIM3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Functional Marker	M=0	M=1	M=3	M=9	M=15	M=18	M=24	Δ (M24-M0)
TCF1	73.2%	76.9%	89.1%	68.3%	69.1%	82.6%	67.2%	-6.0%
CD86	58.5%	47.8%	66.3%	60.3%	56.0%	59.6%	53.3%	-5.2%
CCR7	56.8%	60.3%	51.7%	64.1%	53.0%	52.2%	49.1%	-7.6%
CD161	32.9%	31.8%	31.4%	48.8%	46.7%	46.9%	47.1%	+5.8%
CD38	27.5%	25.5%	26.8%	24.6%	24.6%	25.6%	22.9%	-4.7%
TIGIT	22.4%	20.0%	18.1%	11.1%	10.6%	10.6%	11.4%	-11.1%
PD-1	23.3%	21.4%	17.4%	8.2%	6.4%	6.3%	7.6%	-13.7%
Tbet	2.2%	3.1%	5.2%	3.8%	2.3%	5.6%	2.7%	0.5%
CD11b	4.4%	3.3%	1.7%	6.7%	12.0%	17.2%	13.6%	9.3%
CTLA-4	0.9%	1.5%	0.2%	0.8%	0.6%	1.6%	0.6%	-0.3%
HLA-DR	3.3%	3.2%	2.9%	2.4%	2.5%	3.5%	2.6%	-0.6%
CD38+ HLA-DR+	1.7%	1.3%	1.0%	0.9%	0.8%	1.0%	0.8%	-0.9%
Ki67	0.1%	0.5%	0.4%	0.1%	0.1%	0.2%	0.1%	0.0%
LAG-3	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
TIM3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Figure 4 (A) Correlation of functional marker expression at T=0 vs T=24 months in CD4+ TCM cells, colored by marker and shaped by donor across 3 donors (R = 0.98). Only donor-marker combinations with event counts >100 at both timepoints are included. (B) Functional marker expression (% of parent population) in CD4+ TCM cells across three donors at seven timepoints (T=0 through T=24). Delta indicates absolute change from T=0 to T=24. * indicates donor-timepoint combinations where event count fell below 100.

CD8+ Central Memory T Cell Functional Marker Expression is Preserved Across 24 Months of Frozen Storage



Functional Marker	M=0	M=1	M=3	M=9	M=15	M=18	M=24	Δ (M24-M0)
TCF1	84.6%	77.0%	85.2%	83.7%	85.6%	90.1%	78.7%	-5.8%
CD86	61.4%	54.9%	62.6%	62.4%	67.1%	63.6%	61.6%	0.2%
TIGIT	33.5%	34.9%	32.3%	19.2%	18.4%	16.4%	17.0%	-18.6%
PD-1	35.0%	31.6%	27.7%	13.9%	12.1%	10.1%	12.8%	-17.2%
CCR7	28.9%	25.7%	23.1%	26.3%	25.6%	26.5%	24.4%	0.0%
Tbet	23.4%	20.1%	29.3%	18.3%	19.0%	26.5%	17.1%	-6.3%
CD38	21.5%	21.4%	14.4%	11.0%	12.3%	11.5%	10.4%	-11.1%
CD161	20.3%	19.5%	18.7%	17.8%	16.9%	16.8%	17.3%	-2.9%
CD11b	6.1%	5.6%	4.0%	9.6%	15.7%	19.3%	14.1%	8.0%
HLA-DR	3.2%	3.1%	2.8%	3.1%	2.7%	2.5%	2.2%	-1.0%
CD38+ HLA-DR+	2.9%	4.2%	2.3%	1.9%	1.0%	1.1%	1.0%	-1.9%
Ki67	0.2%	0.8%	0.6%	0.3%	0.3%	0.4%	0.1%	0.0%
CTLA-4	0.1%	0.1%	0.0%	0.1%	0.2%	0.2%	0.1%	0.0%
LAG-3	0.1%	0.1%	0.0%	0.2%	0.0%	0.0%	0.1%	0.0%
TIM3	0.1%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	-0.1%

Functional Marker	M=0	M=1	M=3	M=9	M=15	M=18	M=24	Δ (M24-M0)
TCF1	56.8%	56.2%	63.2%	51.5%	58.6%	60.8%	63.0%	6.2%
CD86	60.8%	55.2%	64.5%	66.5%	73.0%	70.6%	69.2%	8.3%
TIGIT	29.4%	26.5%	26.5%	15.4%	14.5%	14.3%	14.3%	-15.1%
PD-1	16.4%	15.8%	14.5%	8.6%	6.7%	7.3%	7.1%	-9.3%
CCR7	28.5%	29.3%	27.7%	31.4%	28.0%	25.5%	27.2%	-1.3%
Tbet	9.9%	9.4%	13.2%	9.3%	7.3%	9.4%	5.7%	-4.1%
CD38	16.5%	17.0%	13.8%	12.0%	13.8%	14.7%	11.0%	-5.5%
CD161	10.7%	11.0%	11.1%	10.0%	10.2%	9.2%	9.2%	-1.5%
CD11b	9.9%	8.4%	5.7%	13.3%	21.0%	21.4%	20.1%	10.3%
HLA-DR	8.3%	7.7%	7.6%	7.5%	6.2%	8.2%	6.4%	-1.9%
CD38+ HLA-DR+	4.9%	3.1%	2.7%	2.8%	1.9%	2.3%	1.6%	-2.7%
Ki67	1.4%	1.3%	1.9%	1.1%	0.9%	0.8%	0.5%	-0.9%
CTLA-4	0.2%	0.1%	0.1%	0.3%	0.3%	0.5%	0.1%	-0.1%
LAG-3	0.4%	0.1%	0.0%	0.3%	0.1%	0.0%	0.0%	-0.4%
TIM3	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	-0.1%

Functional Marker	M=0	M=1	M=3	M=9	M=15	M=18	M=24	Δ (M24-M0)
TCF1	59.6%	71.8%	83.2%	49.0%	53.5%	69.1%	53.1%	-6.5%
CD86	49.5%	50.3%	55.6%	49.3%	55.6%	59.1%	56.0%	7.5%
TIGIT	12.9%	48.3%	44.7%	22.5%	22.3%	22.4%	24.9%	+28.0%
PD-1	30.0%	46.3%	41.0%	20.1%	15.2%	15.2%	19.5%	-10.5%
CCR7	16.9%	20.9%	13.1%	23.2%	14.2%	14.4%	12.5%	-4.4%
Tbet	10.0%	17.8%	25.0%	13.0%	11.8%	16.2%	12.8%	2.8%
CD38	11.5%	11.1%	10.8%	8.4%	7.8%	9.6%	8.1%	-3.4%
CD161	21.0%	19.9%	20.1%	20.9%	20.3%	19.8%	19.9%	-1.1%
CD11b	8.3%	6.8%	5.0%	12.6%	16.7%	22.8%	19.9%	10.6%
HLA-DR	12.7%	11.8%	11.0%	10.0%	7.8%	10.7%	8.6%	-4.1%
CD38+ HLA-DR+	5.6%	4.5%	3.8%	3.2%	2.2%	3.4%	2.5%	-3.1%
Ki67	0.1%	0.5%	0.5%	0.1%	0.1%	0.1%	0.1%	0.0%
CTLA-4	0.1%	0.1%	0.0%	0.1%	0.1%	0.1%	0.0%	-0.1%
LAG-3	0.2%	0.2%	0.0%	0.4%	0.0%	0.1%	0.1%	-0.1%
TIM3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

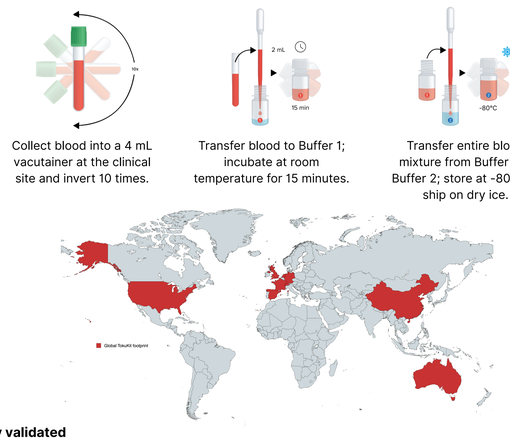
Figure 5 (A) Correlation of functional marker expression at T=0 vs T=24 months in CD8+ TCM cells, colored by marker and shaped by donor across 3 donors (R = 0.89). Only donor-marker combinations with event counts >100 at both timepoints are included. (B) Functional marker expression (% of parent population) in CD8+ TCM cells across three donors at seven timepoints (T=0 through T=24). Delta indicates absolute change from T=0 to T=24. * indicates donor-timepoint combinations where event count fell below 100.

Key Findings and Conclusion

TokuKit preserves immune cell population frequencies and functional marker expression in whole blood stored at -80°C for up to 24 months. Sponsors running CAR-T and T cell engager trials across multiple sites can collect, fix, and store whole blood at the point of collection and batch-process samples at a central laboratory, eliminating the 24 to 72-hour processing window that renders at least 20% of clinical samples unusable.

- Immune cell frequencies: R = 0.99 vs fresh, R = 0.99 at 24 months across 36 immune cell populations
- CD4+ and CD8+ T cell lineage and subset proportions stable across all 7 stability timepoints
- CD4+ TCM functional markers: R = 0.98 at 24 months
- CD8+ TCM functional markers: R = 0.89 at 24 months

TokuKit at the Clinical Site



Clinically validated
Deployed across 80+ global clinical sites, 5,000+ kits shipped, less than 1% failure rate.

Flexible collection window
EDTA vacutainers hold for 9 hours and heparin for 5 hours pre-fixation.

No -80°C freezer at the collection site required
Fixed samples remain stable at 4°C for 14 days, allowing standard refrigerated shipping to a central laboratory.

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