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Beyond PBMCs: Polymer-Based Cell Mimics for Robust TBNK Immunophenotyping Assay Validation

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ABSTRACT

Flow cytometry-based TBNK immunophenotyping is widely used to assess immune status in research, clinical diagnostics, and cell therapy development. Biological control materials such as PBMCs often serve as physiologically relevant controls, but suffer from considerable variability due to donor differences, limited stability, and fluctuations in antigen expression levels between lots. These factors make it challenging to achieve consistent assay performance, especially in longitudinal or multi-site environments. TBNK Cell Mimic (synthetic controls in PhenoCyte product line, developed by Slingshot Biosciences; henceforth referred to as TBNK Cell Mimic in this manuscript) are polymer-based cell mimics engineered to provide defined scatter properties, controlled antigen density, and stable subset ratios. Their scatter profiles are designed to be biologically relevant and comparable to those of native leukocyte populations. In this study, we performed a comprehensive analytical validation of a TBNK immunophenotyping assay using these TBNK Cell Mimics as standardized reference controls. Validation parameters included repeatability, intermediate precision, accuracy, linearity, specificity, robustness, stability, and carryover. The TBNK Cell Mimic met all predefined acceptance criteria, demonstrating $\leq 5\%$ CV for intra- and inter-assay precision and R^2 values > 0.998 across the linearity range. Accelerated stability studies performed at 25°C and 37°C showed $< 5\%$ variation in population frequencies, supporting the material's suitability for extended quality monitoring. These results indicate that TBNK Cell Mimic provides a consistent and reproducible reference material that can support assay validation and routine performance assessment. While not a replacement for biological samples when evaluating donor-specific or viability-dependent biology, their stability and lot-to-lot consistency offer a practical tool for reducing technical variability and improving harmonization across instruments, operators, and testing sites.

1 | Introduction

The TBNK (T, B, and NK) immunophenotyping assay is a widely used method for identifying and quantifying major lymphocyte subsets and is foundational in immunology research, clinical diagnostics, and cell therapy development [1, 2]. Achieving reproducible and comparable results across laboratories requires comprehensive analytical validation that evaluates precision, accuracy, linearity, specificity, stability, robustness, and reproducibility, as recommended by CLSI, ICCS, and FDA guidance [3–5].

Traditional biological control materials, particularly peripheral blood mononuclear cells (PBMCs), complicate validation efforts due to substantial donor-to-donor variability, limited stability, and fluctuations in antigen expression [6, 7]. These sources of variability affect gating reproducibility, contribute to lot-to-lot inconsistency, and hinder longitudinal or multisite standardization efforts.

Flow cytometry platforms differ in optics, detector sensitivity, and fluorochrome response, making inter-instrument comparison challenging [8, 9]. Even with bead-based calibration,

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fluorescence intensities and background levels are not fully harmonized, especially for MFI-based metrics [6]. Biological controls add another layer of variability, which can mask true instrument-to-instrument differences [7, 10].

In addition, antibody-related factors, including clone, fluorochrome, vendor, and lot-to-lot variation, introduce another layer of variability, making harmonized comparisons even more challenging. A stable, well-defined reference material can help separate biological variation from instrument effects and support more consistent performance baselines across sites [11].

To address these limitations, polymer-based synthetic reference materials such as TBNK Cell Mimic have been developed by Slingshot Biosciences. These engineered cell mimics exhibit biologically relevant forward and side scatter properties and defined levels of stable surface biomarkers, enabling lot-to-lot uniformity and long-term stability. By minimizing biological variability, Slingshot Biosciences' synthetic controls offer a stable and reproducible alternative to PBMCs for assay development, validation, and ongoing quality control.

In this study, we performed a comprehensive analytical validation of a TBNK immunophenotyping assay using TBNK Cell Mimic as standardized control materials. Validation parameters included repeatability, intermediate precision, accuracy, linearity, specificity, stability, robustness, and carryover. Our objective was to validate that synthetic cell mimics can serve as reliable and reproducible alternatives to PBMC-based controls for TBNK assay standardization.

2 | Materials and Methods

2.1 | Sample Sources

PBMCs: Cryopreserved PBMCs (AllCells, CA, USA).

Synthetic controls: Lyophilized TBNK Cell Mimic (Slingshot Biosciences).

2.2 | Reagents and Antibodies

Additional details on the antibody dilutions are provided in Table S1 (Table 1).

2.3 | Instruments and Software

Flow cytometers: Cytex Aurora, Cytex Northern Lights, BD FACSLytic.

Analysis: SpectroFlo, FlowJo v10, Microsoft Excel.

Compensation was performed using instrument-specific protocols.

2.4 | PBMC Thawing and Preparation

Standard PBMC thawing procedures were used to minimize osmotic shock and maintain sample integrity, with the full detailed protocol provided in the [Supporting Information](#) Section 1.1.1.

2.5 | Cell Mimics Rehydration and Preparation

Lyophilized synthetic controls were rehydrated following standard procedures (see [Supporting Information](#) Section 1.1.2. for full protocol).

After rehydration, samples were centrifuged at $600 \times g$ for 5 min, washed twice with the staining buffer, and resuspended for staining.

2.6 | Staining Protocol

A consistent 100 μ L antibody cocktail was used for all samples; Zombie NIR was used in all PBMC conditions, with full staining details provided in the [Supporting Information](#) Section 1.1.3.

TABLE 1 | Antibody panel used for PBMCs and TBNK Cell Mimic, including fluorochrome, clone, vendor, and catalog number.

Antibody	Fluorochrome	Clone	Vendor	Catalog no.
CD45	eFluor450	2D1	Invitrogen	48-9459-42
CD3	FITC	SK7	Biolegend	344804
CD4	PE-Cy7	SK3	Biolegend	344612
CD19	APC	SJ25C1	Biolegend	363006
CD8	AF700	SK1	Biolegend	344724
CD14	V500	M5E2	BD	561391
CD56	BB700	NCAM 16.2	BD	566573
CD16	PE	B73.1	BD	347617
Zombie NIR	NIR	NA	BioLegend	423106

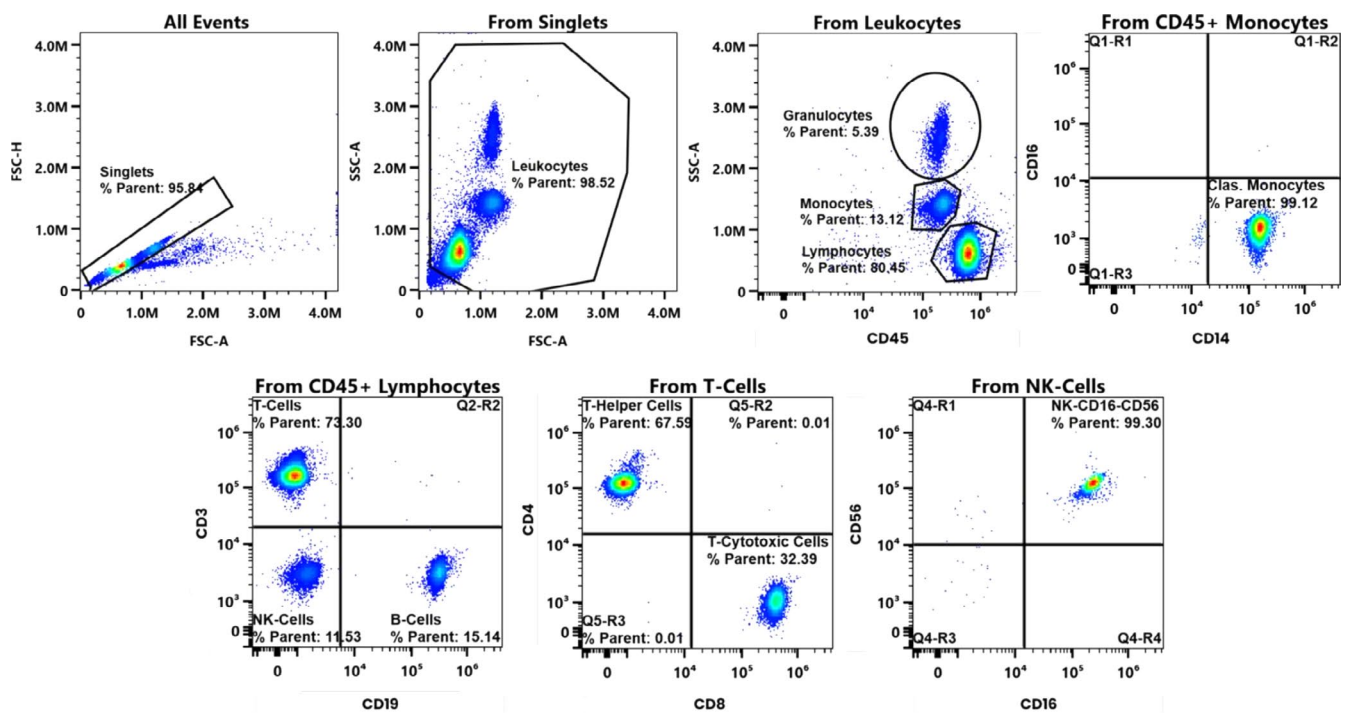


FIGURE 1 | Gating schematic for TBNK immunophenotyping assay using TBNK Cell Mimic.

2.7 | Particle Counting

All samples underwent two standardized wash steps. To evaluate potential particle loss associated with washing, replicate samples were processed identically and total event recovery was quantified following acquisition.

2.8 | Gating Scheme

A standardized gating hierarchy was applied:

- FSC-H versus FSC-A to exclude doublets
- FSC-A versus SSC-A to identify leukocytes.
- CD45 to define leukocyte lineages
- CD3, CD19, CD14, CD16/CD56 to classify subsets.
- The full gating schematic is shown in Figure 1.

2.9 | Product Specification

TBNK Cell Mimic are polymer-based cell mimics with defined scatter properties and attached surface biomarkers. They are designed to replicate the relative characteristics of human lymphocytes, monocytes, and granulocytes, providing a stable, consistent reference material. Table 2 summarizes the key product specifications.

2.10 | Analytical Validation Parameters

The analytical performance of the TBNK immunophenotyping assay was evaluated following established regulatory

TABLE 2 | Product specifications of TBNK Cell Mimic.

Population type	Target value (%)	Key biomarkers
Granulocytes CD45+	~5	CD45+
Monocytes CD45+	~14	CD45+
Lymphocytes CD45+	~81	CD45+
B Cells CD19+	~15	CD45+, CD19+
T Cells CD3+	~74	CD45+, CD3+
T Cytotoxic Cells CD8+	~32	CD45+, CD3+, CD8+
T Helper Cells CD4+	~68	CD45+, CD3+, CD4+
NK Cells CD3- CD19-	~12	CD45+, CD3-, CD19-
NK Cells CD16+ CD56+	~100	CD45+, CD3-, CD19-, CD16+, CD56+
Classical Monocytes CD14+ CD16-	~100	CD45+, CD14+, CD16-

and cytometry-specific guidelines, including the Clinical and Laboratory Standards Institute (CLSI) recommendations [3], the FDA Bioanalytical Method Validation Guidance [4], and validation principles outlined by the International Clinical Cytometry Society (ICCS) [5]. These standards define the core parameters required to demonstrate assay reliability and suitability for quantitative immunophenotyping. The validation experiments described in Table 3 were designed to assess these

TABLE 3 | Summary of analytical validation parameters and acceptance criteria for the TBNK immunophenotyping assay, following CLSI I/LA-33, ICCS, and FDA guidance.

Parameter	Design/purpose	Acceptance criteria (% population)
Precision—repeatability (intra-assay)	Analyze six replicate samples on the same day by the same operator using the same instrument and identical conditions to evaluate within-run variability.	% CV $\leq$ 5%
Precision—intermediate precision (within-laboratory reproducibility)	Assess variability across: Inter-operator: Two operators on the same day using the same instrument. Inter-instrument: One operator using two instruments on the same day. Inter-day: One operator using the same instrument on multiple days.	% CV $\leq$ 5% across conditions.
Linearity	Prepare and analyze a dilution series or samples with known target-cell concentrations spanning the full analytical range.	Coefficient of determination (R^2) $\geq$ 0.98; slope within 0.90–1.10 of expected.
Range	Define the interval between the lowest and highest concentration (or signal intensity) where precision, accuracy, and linearity meet criteria.	Within the validated linear range, showing recovery between 80%–100%.
Limit of detection (LOD)	Determines the lowest signal that can be reliably distinguished from background. (e.g., using blank, low-positive, and replicate analyses).	LOD = mean(blank) + 3 $\times$ SD(blank), or an equivalent approach
Limit of quantitation (LOQ)	Determines the lowest signal that can be reliably quantified with acceptable precision and accuracy (e.g., using blank, low-positive, and replicate analyses).	LOQ = mean(blank) + 10 $\times$ SD(blank) or equivalent.
Accuracy (trueness)	Compare measured results with known or reference values when spike-in or reference materials are available.	Mean bias $\leq$ $\pm$ 5% versus expected.
Specificity/selectivity	Confirm target-specific staining using isotype, FMO, or knockout controls; assess possible interference from other populations or reagents.	Non-target signal $\leq$ 1%–5% of target population frequency; no significant cross-reactivity.
Stability	Evaluate sample stability under variable conditions: Room temperature (25°C) and 37°C	Change in signal $\leq$ 5% relative to time-zero control.
Carryover/cross-contamination	Run a blank buffer or unstained sample immediately after a positive sample.	Carryover < 1% of high-positive signal.
Robustness	Assess assay performance under minor, deliberate deviations (e.g., temperature $\pm$ 2°C, reagent lot variation).	Results remain within % CV $\leq$ 5%

Note: Acceptance criteria are based only on population frequencies (% populations). MFI values are for reference only and are not used for acceptance criteria.

characteristics using standardized approaches consistent with current best practices in flow cytometry method qualification.

within the predefined acceptance criterion of < 5% CV (Table 4). Expanded population and MFI replicates are provided in Tables S1 and S2.

3 | Results

3.1 | Precision—Repeatability (Intra-Assay)

Intra-assay precision determines whether the assay performs consistently under identical conditions^[34]. Six replicates of TBNK Cell Mimics were analyzed to assess population and MFI stability. All populations exhibited $\leq$ 3.0% CV, well

3.2 | Precision—Intermediate Precision (Within-Laboratory Reproducibility)

Intermediate precision assesses reproducibility across normal sources of within-laboratory variation, including operator, instrument, and day-to-day differences. TBNK Cell Mimic demonstrated $\leq$ 4% CV across all evaluated conditions: same-day

same-instrument replicates, cross-day replicates, cross-instrument replicates, and cross-operator replicates, meeting the predefined acceptance criterion of <5% CV (Table 5). These results indicate stable performance independent of operator or platform. Full population and MFI datasets are provided in Tables S3–S11.

Intermediate precision was assessed across key sources of within-laboratory variability, including repeatability (same day, same instrument), day-to-day variation, instrument-to-instrument variation, and operator-to-operator variation, consistent with CLSI I/LA-33 and ICCS guidance.

3.3 | Linearity

Linearity evaluates whether measured values are directly proportional to expected values across the assay's analytical range.

TBNK Cell Mimics were serially diluted with true negative material (the same cell mimics, but with no biomarkers attached to them) to generate a dilution series spanning the full reportable range. Most populations showed slopes within 0.90–1.10 and $R^2 \geq 0.994$ (Table 6). Two populations were slightly outside this range: Granulocytes CD45+ (0.84) showed minor deviations at the lowest dilutions due to sampling variability in low-frequency populations, and B Cells CD19+ (1.15) were slightly overestimated across the series.

Regression plots further confirmed proportional behavior across the validated range (Figure 2). Although population frequencies exhibited strong linearity, MFI % CV values remained below 5%, demonstrating highly stable fluorescence intensity measurements across the dilution series (Table S12). Full datasets for population frequencies and MFIs are provided in Tables S12 and S13.

TABLE 4 | Intra-assay repeatability of TBNK Cell Mimic: Population and MFI across six replicates.

Population type	% populations (avg of 6 reps)	% CV between 6 replicates	MFI (avg of 6 reps)	% CV between 6 replicates
Granulocytes CD45+	5	2.2	148,062	0.5
Monocytes CD45+	14	1.4	290,905	0.8
Lymphocytes CD45+	79	0.3	645,748	0.4
B Cells CD19+	15	0.8	249,264	0.7
T Cells CD3+	74	0.2	144,700	0.9
T Cytotoxic Cells CD8+	33	0.5	290,739	0.8
T Helper Cells CD4+	66	0.3	1,026,908	0.5
NK Cells CD16+ CD56+	99	0.1	285,351 (CD16+)	3.0
			285,719 (CD56+)	0.4
Classical Monocytes CD14+	99	0.1	126,492	1.7

TABLE 5 | Intermediate precision of TBNK Cell Mimic across operators, instruments, and days for population frequencies.

Population type	% CV between three replicates when tested on same day on same instrument	% CV when same operator tested on six different days	% CV when same operator tested on three different instruments on same day	% CV when two different operators tested on same instrument on same day
Granulocytes CD45+	3.8	6.2	3.5	3.3
Monocytes CD45+	2.6	4.7	3.7	1.4
Lymphocytes CD45+	0.5	0.5	1.1	0.7
B Cells CD19+	1.8	0.5	0.8	0.5
T Cells CD3+	0.2	0.2	0.2	0.3
T Cytotoxic Cells CD8+	0.6	0.6	0.7	0.6
T Helper Cells CD4+	0.3	0.3	0.3	0.3
NK Cells CD16+ CD56+	0.3	0.3	1.7	0.2
Classical Monocytes CD14+	0.1	0.05	0.2	0.2

3.4 | Range

The analytical measurement range of a flow cytometry assay defines the interval of cell concentrations over which the assay provides reliable, proportional, and linear results. Linearity of the flow cytometry assay was evaluated using serial dilutions of TBNK Cell Mimic with Slingshot Labs True Negative (Table 7). Recovery at each dilution was calculated as the observed value divided by the expected value and expressed as a percentage. Only consecutive dilutions with 80%–120% recovery were considered within the validated analytical measurement range. The ranges below represent the observed values corresponding to these consecutive dilutions.

TABLE 6 | Linearity assessment of TBNK subset frequencies using serial dilutions of TBNK Cell Mimic: Slope, intercept, and R^2 .

Population type	Slope	Intercept	R^2
Granulocytes CD45+	0.84	0.07	0.997
Monocytes CD45+	0.97	0.08	0.999
Lymphocytes CD45+	0.99	1.23	0.998
B Cells CD19+	1.15	0.09	0.998
T Cells CD3+	1.00	0.97	0.997
T Cytotoxic Cells CD8+	0.97	0.36	0.996
T Helper Cells CD4+	1.02	0.62	0.998
NK Cells CD16+ CD56+	1.05	0.09	0.999
Classical Monocytes CD14+	0.90	0.09	0.998

3.5 | Accuracy

Accuracy assesses how well the assay reflects true population proportions and whether synthetic control derived events behave appropriately when mixed with biological samples. Accuracy was evaluated by spiking TBNK Cell Mimic into cryopreserved PBMCs and comparing measured values with expected target frequencies. Overlay plots demonstrated clear phenotypic separation of Cell Mimic-derived leukocytes and PBMC-derived leukocytes, confirming appropriate marker expression and gating behavior in mixed samples (Figure 3). All evaluated subsets showed minimal bias ($\leq 2.0\%$), indicating that the assay accurately recovers expected population proportions under spike-in conditions (Table 8). Full datasets for population frequencies are provided in Tables S14.

3.6 | Specificity

3.6.1 | Specificity Using FMOs

Specificity confirms that each marker accurately identifies its intended population without interference from non-target signals and is routinely assessed using fluorescence minus one (FMO) controls [2]. FMOs were performed for CD14, CD16, CD19, and CD56, which can show overlapping or variable expression in PBMCs. While these markers are bright and clearly separated in TBNK Cell Mimic, evaluating them with FMOs ensures the assay will perform reliably with real biological samples. Highly expressed markers such as CD3, CD4, CD8, and

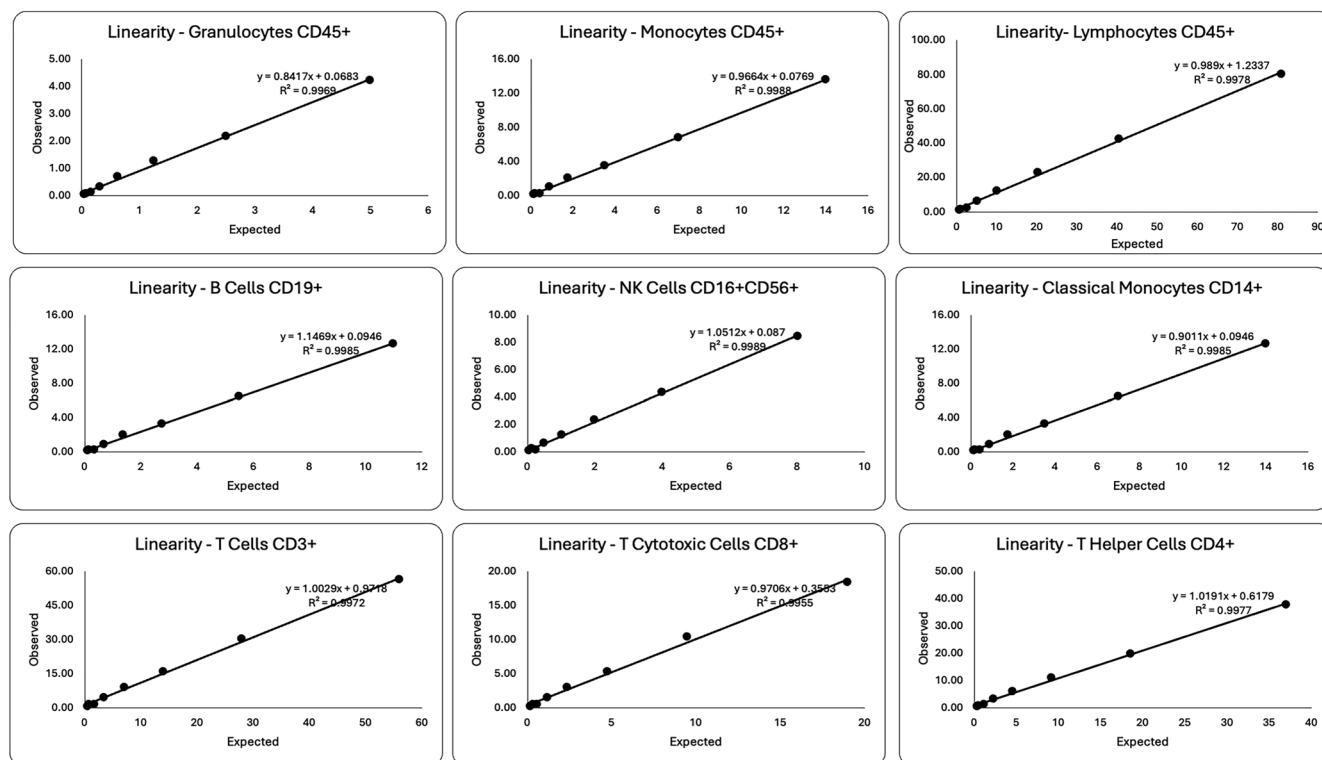


FIGURE 2 | Linearity of TBNK populations across serial dilutions of TBNK Cell Mimic. Scatter plots show observed versus expected frequencies for each population, with regression lines and 95% confidence intervals. All populations exhibited $R^2 > 0.998$ and slopes within acceptable range.

CD45 are well-resolved in both synthetic and biological samples and therefore did not require FMO assessment.

FMO analysis demonstrated minimal non-specific staining (<1%–5% of population frequency) for the evaluated markers

TABLE 7 | Validated analytical measurement ranges of TBNK Cell Mimic by flow cytometry.

Population type	Validated consecutive dilutions	Observed range (% population)	
		Highest value	Lowest value
Granulocytes CD45+	1–16	4.2	0.3
Monocytes CD45+	1–16	14	1.0
Lymphocytes CD45+	1–4	80	23
B Cells CD19+	1–2	11	6.0
T Cells CD3+	1–4	56	16
T Cytotoxic Cells CD8+	1–4	18	5.3
T Helper Cells CD4+	1–4	38	11
NK Cells CD16+ CD56+	1–4	8.4	4.3
Classical Monocytes CD14+	1–16	13	0.9

(Table 9). In the CD19 FMO, only ~0.02% of events fell within the B-cell gate, and in the CD14 FMO, only ~0.01% of events appeared in the monocyte gate, indicating very low background. In the CD16 and CD56 FMOs, the NK gate contained almost no events, reflecting the expected dependence on these markers for NK identification. Overall, the FMOs confirm low background signal and appropriate marker dependence, with all TBNK subsets remaining well resolved when their defining markers were included (Figure 4). Overlay of dotplots are shown in Figure S2.

3.6.2 | Assessing Specificity via Reagent Variation

In addition to fluorescence minus one (FMO) contrasting Cell Mimics were stained with antibodies targeting antigens that are not present on TBNK Cell Mimic to assess potential non-specific binding to the polymer backbone. Antibodies against TCRαβ and CCR7 (ubiquitous T-cell markers) and CD20 (a well-established B-cell marker) were combined in a PE-conjugated dump channel. These markers were selected because they are well-characterized lineage-specific antigens that are not present on TBNK Cell Mimic by design. They serve as negative controls to assess nonspecific antibody interactions. The frequency of events detected in the PE channel was less than 0.05% (Table 10). No detectable staining was observed in the PE channel, indicating minimal nonspecific binding. The complete antibody panel is provided in Table S17, and representative dot plots and histograms are shown in Figure S3.

3.6.3 | TBNK Cell Mimic Evaluation With Commercially Available TBNK Kits

Specificity of TBNK Cell Mimic can also be evaluated by comparing results across different commercially available TBNK

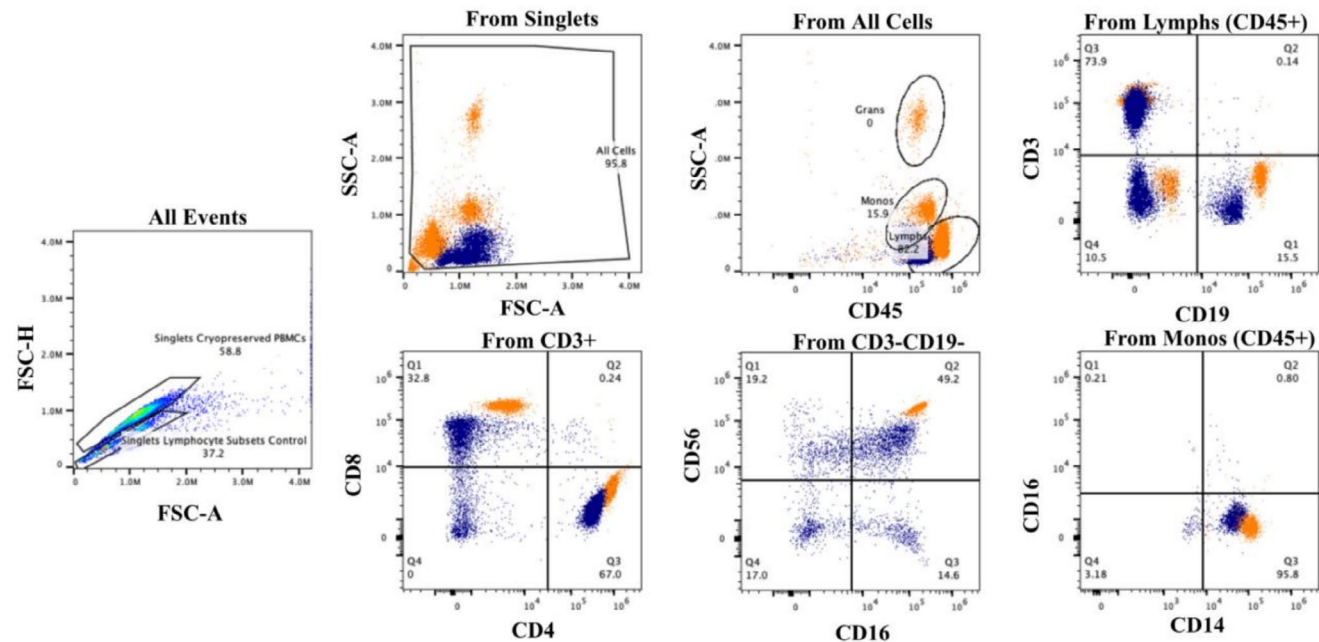


FIGURE 3 | Overlay of PBMC populations and TBNK Cell Mimic from a single spiked sample. Dot plot overlays illustrate distinct clustering of TBNK Cell Mimic T-, B-, and NK-cell mimics alongside PBMC-derived populations, confirming appropriate resolution and marker expression in a mixed-sample analysis. Orange—TBNK Cell Mimic and Navy—Cryopreserved PBMCs.

TABLE 8 | Accuracy evaluation comparing observed population frequency (% population) values from PBMC+TBNK Cell Mimic spike-in experiments with expected TBNK Cell Mimic target values.

Population type	PBMCs spiked in with TBNK cell mimic (spiked % population)	TBNK cell mimic only (expected % population)	Bias (spiked–expected)
Granulocytes CD45+	4	4	0.3
Monocytes CD45+	14	13	0.7
Lymphocytes CD45+	80	81	–2.0
B Cells CD19+	13	13	1.5
T Cells CD3+	74	74	0.3
T Cytotoxic Cells CD8+	33	33	–0.7
T Helper Cells CD4+	67	67	0.7
NK Cells CD16+ CD56+	100	99	0.6
Classical Monocytes CD14+	99	99	–0.1

TABLE 9 | Specificity assessment using FMO controls for major TBNK populations (% population).

Population type	TBNK cell mimic	CD14 FMO	CD16 FMO	CD56 FMO	CD19 FMO
Granulocyte CD45+	4	5	5	5	5
Monocyte CD45+	14	14	14	13	14
Lymphocyte CD45+	79	79	80	80	80
B cells CD19+	15	15	15	15	0.02
T cells CD3+	79	79	80	80	80
T Cytotoxic Cells CD8+	33	33	33	33	33
T Helper Cells CD4+	67	67	67	67	67
NK cells CD16+ CD56+	99	99	0.00	0.00	42 ^a
Classical Monocytes CD14+	98	0.01	99	100	99

^aNK cells (CD16+ CD56+) are 42% in the CD19 FMO because they are gated from the CD3– CD19– population, which is affected in the CD19 FMO.

panels (Figures S4–S6, dot plots). To assess this, population frequencies were measured using Slingshot's in-house panel (Table 1) and three independent reagent kits: Beckman Coulter DURAClone IM, BD Multitest 6-color, and BioLegend Human TBNK 4-color. The % CV across kits was low for most populations (0%–12.8%), with Granulocytes CD45+ showing the

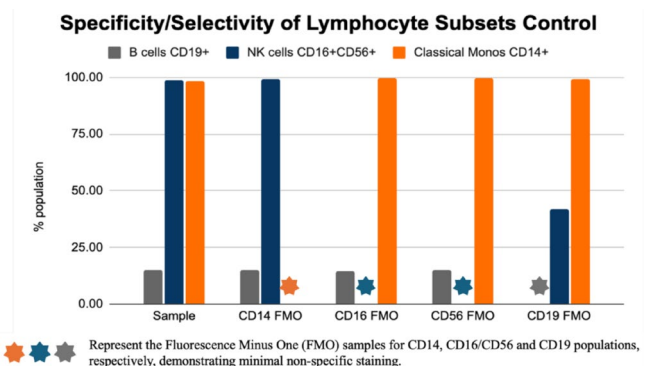


FIGURE 4 | Specificity evaluation using FMOs for CD14, CD16, CD19, and CD56. Representative gating plots illustrate separation between target and non-target populations. Minimal non-specific signal was detected across FMOs, demonstrating high assay specificity.

highest inter-kit variability (12.8% CV) and major lymphocyte subsets (CD3+, CD4+, CD8+, CD19+, and NK cells) demonstrating minimal variation (% CV ≤ 2.2%), indicating that population identification is consistent across different reagent panels (Table 11).

Overall, these data support the specificity and robustness of TBNK Cell Mimic across different reagent formulations, demonstrating that the control material reliably produces expected population distributions regardless of panel choice.

3.6.4 | Antibody Clone Dependence

To evaluate antibody clone dependence across the TBNK panel, TBNK Cell Mimic were stained with three commonly used antibody clones per marker under identical assay conditions (Table S18). For most markers, population frequencies were consistent across commonly used antibody clones, except CD3, which was compatible with two of the four clones evaluated (Table 12).

3.7 | Robustness

Robustness evaluates the sensitivity of an assay to small, routine workflow variations. To demonstrate robustness, TBNK Cell Mimic Controls were subjected to three commonly used sample-processing conditions: no-lyse, lyse/no-wash, and lyse/wash. Population frequencies remained stable ($\leq 2.7\%$ CV) across all conditions (Table 13), and overlay plots confirmed consistent population separation across workflows (Figure 5). These findings indicate that TBNK Cell Mimic maintained assay performance despite procedural differences encountered in typical flow cytometry laboratories.

TABLE 10 | Specificity assessment using reagent variation controls for major TBNK populations (% population).

Population type	% population (average of 3 replicates)
Granulocytes CD45+	4
Monocytes CD45+	12
Lymphocytes CD45+	81
B Cells CD19+	15
T Cells CD3+	74
T Cytotoxic Cells CD8+	33
T Helper Cells CD4+	67
NK Cells CD16+ CD56+	98
Classical Monocytes CD14+	100
TCRab, CD20, CCR7 (Dump Channel)	0.04

TABLE 11 | Specificity assessment using different commercially available immunophenotyping TBNK panel kits. (% population).

Population type	Data from Slingshot's in-house panel	Beckman Coulter DURAClone IM kit	BD multitest 6-color kit	BioLegend human TBNK 4-color kit	% CV between different kits
Granulocytes CD45+	5	4	5	4	12.8
Monocytes CD45+	13	12	13	13	3.9
Lymphocytes CD45+	79	81	81	81	1.2
B Cells CD19+	15	15	15	15	0
T Cells CD3+	75	74	74	75	0.8
T Cytotoxic Cells CD8+	33	34	34	Marker not in panel	1.7
T Helper Cells CD4+	67	66	66	Marker not in panel	0.9
NK Cells CD16+ CD56+	99	100	95	98	2.2
Classical Monocytes CD14+	99	100	Marker not in panel	Marker not in panel	N/A

3.7.1 | Lot-To-Lot Consistency

In addition to procedural robustness, evaluating lot-to-lot consistency is essential for reference materials intended for long-term assay support. Across 11 independent TBNK Cell Mimic lots, population frequencies remained highly consistent, with CVs ranging from 1.3% to 8.8% (Table 14). The small variability observed between lots reflects normal manufacturing tolerances in formulation and antigen loading. Overall, these results confirm that TBNK Cell Mimic maintains stable population frequencies across production batches, supporting their suitability for longitudinal assay validation and routine quality control.

3.8 | Stability

Stability of TBNK Cell Mimic was evaluated under accelerated aging conditions to estimate long-term performance. Studies were conducted at 25°C and 37°C, and equivalent aging intervals were calculated using the Arrhenius model with a Q10 value of 2, a commonly applied assumption for projecting temperature-dependent degradation in non-biological quality control materials [12]. Population frequencies were measured in three replicates per timepoint across accelerated intervals corresponding to up to 36 months of equivalent aging. The accelerated conditions were intended to model thermal degradation on the polymer-based beads and stability of attached surface proteins, which can affect fluorescence intensity and population stability over time.

Stability was assessed under accelerated aging at 25°C (up to 24 months equivalent) and 37°C (up to 36 months equivalent). Most subsets showed % CV below 2% (Table 15), and the absolute change relative to T0 was $\leq 5\%$ for all populations (Table 16). Overlay dot plots comparing T0 and the final timepoints

TABLE 12 | Effect of antibody clone selection on detection of TBNK populations using TBNK Cell Mimic.

Population type	Clone	Positive	Negative
CD3	SK7	74.5	25.5
	OKT3 ^a	0.1	99.7
	UCHT1	74.2	25.8
	HIT3a ^a	0	100
CD4	SK3	49.8	50.2
	OKT4	49.8	50.2
	RPA-T4	49.4	50.6
CD8	SK1	24.8	75.2
	HIT8a	24.7	75.3
	RPA-T4	25.1	74.9
CD14	M5E2	99.3	0.7
	HCD14	99.2	0.8
	61D3	99.3	0.7
CD16	B73.1	10.9	89.1
	CB16	10.8	89.2
	3G8	10.8	89.2
CD19	SJ25C1	14.8	85.2
	HIB19	14.8	85.2
	4G7	15	85
CD45	2D1	99.5	0.5
	HI30	100	0
	MEM-28	100	0
CD56	NCAM 16.2	11.5	88.5
	MEM-188	10.6	89.4
	MY-31	10.9	89.1

Note: Positive and negative population frequencies are shown for representative clones. The original panel fluorochromes (Table 1) were retained for primary clones (italicized), while alternative antibody clones were tested in PE.

^aCD3 OKT3 and HIT3a produced distinguishable positive and negative populations, but overall staining was suboptimal. With fixed gating, the populations appeared poorly resolved, as shown in representative dotplots in Figure S7.

demonstrate consistent gating profiles and minimal shifts in fluorescence patterns (Figure S8).

Accelerated aging studies show that TBNK Cell Mimic maintain strong antigen expression and population stability under thermal stress (Figure 6). Since accelerated conditions may not capture all real-time effects, studies at the recommended storage temperature (−20°C) are ongoing to further validate stability over the full shelf life. Collectively, these data indicate that TBNK Cell Mimic maintain robust antigen expression and population distributions under thermal stress for the assessed accelerated aging period. The observed long-term stability allows a

TABLE 13 | Robustness evaluation under different sample preparation workflows (% population): No lyse, lyse/no-wash, lyse/wash.

Population type	No lyse	Lyse no wash	Lyse wash	% CV
Granulocytes CD45+	5	5	5	2.7
Monocytes CD45+	13	13	13	2.3
Lymphocytes CD45+	80	80	79	0.7
B cells CD19+	15	15	15	0.6
T cells CD3+	74	74	75	0.3
T Cytotoxic Cells CD8+	34	33	34	0.5
T Helper Cells CD4+	66	67	66	0.2
NK cells CD16+ CD56+	100	99	99	0.1
Classical Monocytes CD14+	100	100	100	0.1

single lot of material to be used consistently over extended study periods, without requiring lot-to-lot bridging.

3.9 | Carry-Over

Carryover was assessed using an alternating sequence of high-positive TBNK Cell Mimic samples and blank (stain buffer) samples to determine whether residual events persisted between acquisitions. Across all three alternating runs, blank samples showed negligible event counts, with singlets, leukocytes, and all TBNK subsets approaching zero (Table 17). These findings confirm that the assay and instrument fluidics do not retain measurable residual particles following acquisition of TBNK Cell Mimic, demonstrating minimal carryover and supporting reliable sequential sample analysis.

3.10 | LOD/LOQ

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated for each TBNK population using σ -based statistical estimates derived from the variability observed in the linearity study, together with background signals measured in separately run blank samples. All blanks returned 0 events, confirming the absence of measurable background. Using σ from the linearity residuals, LOD was defined as 3.3σ and LOQ as 10σ , representing the lowest population frequencies that can be distinguished from background and reliably quantified, respectively. The resulting LOD values ranged from 0.33–4.65 and LOQ values ranged from 0.99–14.1 (Table 18), demonstrating that the assay maintains strong sensitivity for all TBNK populations across their validated analytical ranges.

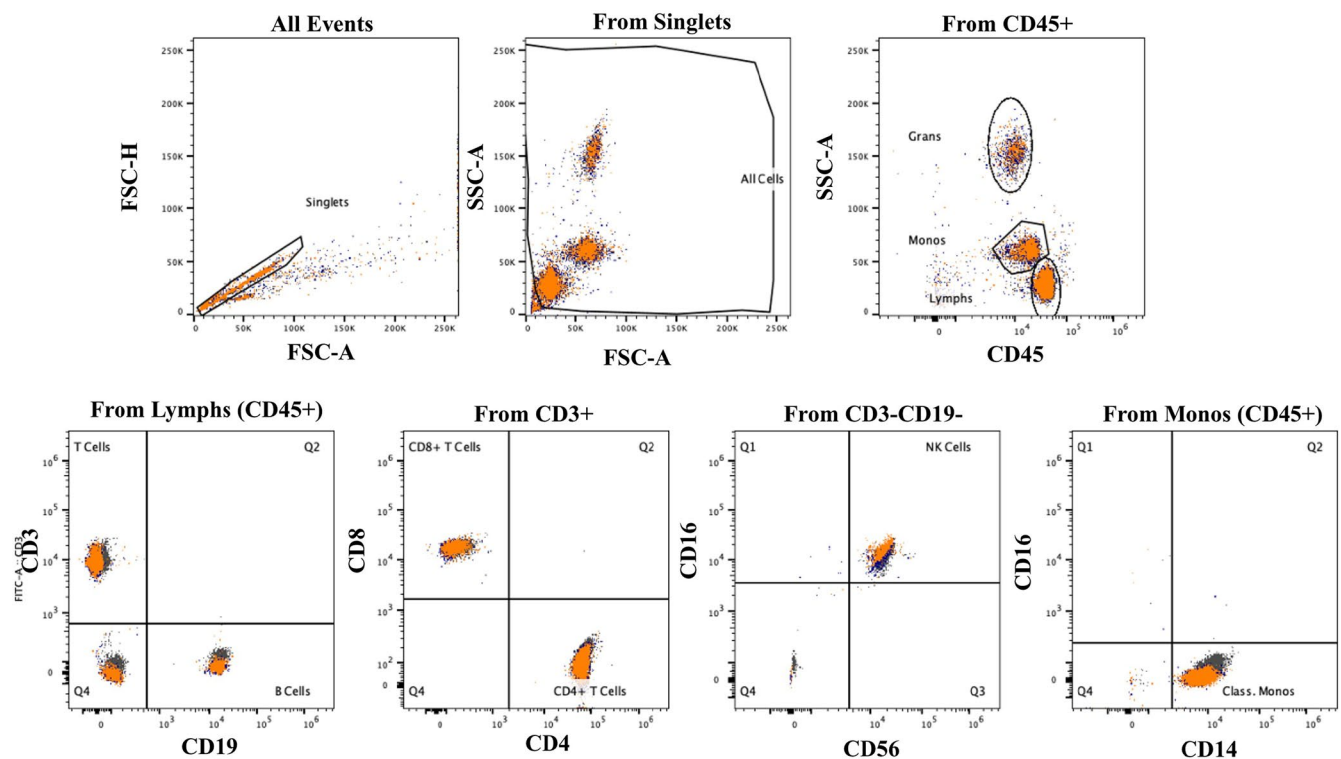


FIGURE 5 | Robustness assessment using no-lyse, lyse/no-wash, and lyse/wash workflows showed stable population frequencies across all sub-sets ($\leq 2.7\%$ CV). Orange—No Lyse, Navy—Lyse wash and Charcoal—Lyse No wash.

TABLE 14 | Lot-to-lot consistency across 11 independent TBNK Cell Mimic lots: Population frequencies.

Population type	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	Lot 10	Lot 11	CV%
Granulocytes CD45+	5	4	6	5	5	5	5	5	5	5	5	8.8%
Monocytes CD45+	16	15	16	14	15	14	15	14	16	14	14	4.6%
Lymphocytes CD45+	79	79	77	80	80	80	79	80	77	80	80	1.3%
B cells CD19+	13	14	17	15	16	15	16	15	15	16	14	7.5%
T cells CD3+	77	76	70	75	74	74	73	75	74	72	73	2.6%
T Cytotoxic CD8+	33	35	35	32	34	34	35	34	34	34	32	2.8%
T Helper CD4+	67	65	65	68	66	67	65	66	66	66	68	1.4%
NK cells CD16+ CD56+	94	100	100	100	100	99	99	100	99	99	99	1.5%
Classical Monos CD14+	94	100	99	99	99	94	98	98	100	99	100	2.1%

3.11 | Particle Counts Post Reconstitution

Each vial of TBNK Cell Mimic is manufactured to provide a minimum working amount of approximately 250,000 cell mimics following reconstitution. This design ensures that, even after routine processing steps such as washing, where minor losses may be expected, the user retains an adequate particle count for analysis. Following two wash steps, replicate recoveries (Table 19) consistently demonstrated particle

counts at or above 250,000, indicating that the washing procedure did not result in meaningful particle loss or introduce frequency bias.

4 | Discussion

This study examined how polymer-based TBNK Cell Mimic performs within a standard TBNK flow cytometry workflow and

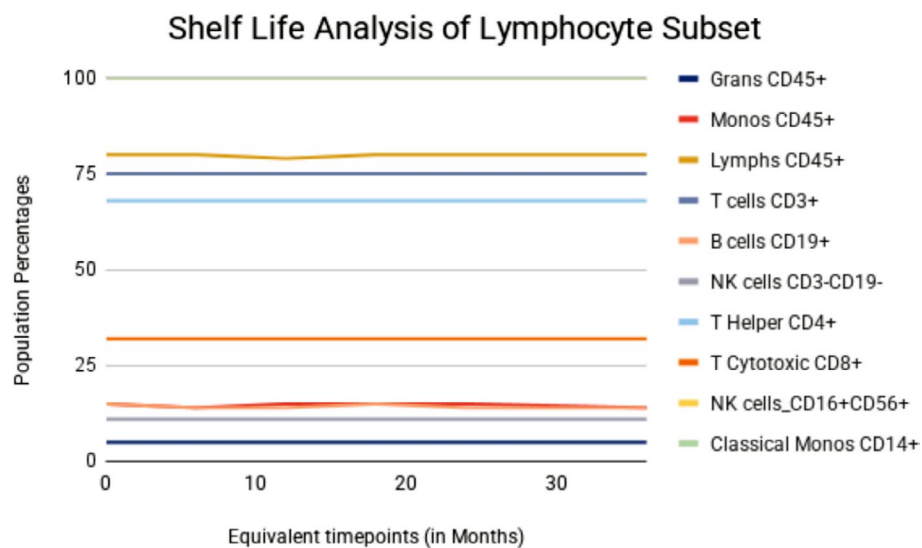
TABLE 15 | Accelerated stability of TBNK Cell Mimic under 25°C and 37°C storage (% population).

Temperature	Population type	Equivalent timepoint (accelerated aging)						All timepoints % CV
		0 months	6 months	12 months	18 months	24 months	36 months	
37°C	Granulocytes CD45+	5	5	5	5	5	5	1.90
	Monocytes CD45+	15	14	15	15	15	14	1.51
	Lymphocytes CD45+	80	80	79	80	80	80	0.36
	B cells CD19+	15	14	14	15	14	14	0.66
	T cells CD3+	75	75	75	75	75	75	0.18
	T Cytotoxic Cells CD8+	32	32	32	32	32	32	0.43
	T Helper Cells CD4+	68	68	68	68	68	68	0.20
	NK cells CD16+ CD56+	100	100	100	100	100	100	0.03
	Classical Monocytes CD14+	100	100	100	100	100	100	0.01
	25°C	Granulocytes CD45+	5	5	5	5	5	N/A
Monocytes CD45+		14	14	14	14	14	N/A	1.06
Lymphocytes CD45+		79	80	80	80	80	N/A	0.27
B cells CD19+		14	15	15	15	14	N/A	0.43
T cells CD3+		75	75	75	75	75	N/A	0.12
T Cytotoxic Cells CD8+		32	32	32	32	32	N/A	0.64
T Helper Cells CD4+		68	68	68	68	68	N/A	0.30
NK cells CD16+ CD56+		100	100	100	100	100	N/A	0.02
Classical Monocytes CD14+		100	100	100	100	100	N/A	0.03

Note: Data shown here is the average of three replicates.

TABLE 16 | Absolute change in % population compared to timepoint zero for accelerated stability of TBNK Cell Mimic under 25°C and 37°C storage.

Temperature	Population type	Equivalent timepoint (accelerated aging)				
		6 months	12 months	18 months	24 months	36 months
37°C	Granulocytes CD45+	1	5	2	3	0.4
	Monocytes CD45+	−1	1	0.8	−0.5	−3
	Lymphocytes CD45+	0.2	−0.6	−0.3	−0.1	0.4
	B cells CD19+	−2	−1	−0.1	−1	−1
	T cells CD3+	0.4	0.2	0.1	0.5	0.2
	T Cytotoxic Cells CD8+	0.6	−0.4	0.5	0.3	−0.4
	T Helper Cells CD4+	−0.3	0.2	−0.2	−0.1	0.2
	NK cells CD16+ CD56+	0.0	0.0	0.0	0.0	0.0
	Classical Monocytes CD14+	0.0	0.0	0.0	0.0	0.0
25°C	Granulocytes CD45+	−0.2	−4	0.0	2	N/A
	Monocytes CD45+	0.1	−2	−1.8	−0.8	N/A
	Lymphocytes CD45+	0.1	0.6	0.5	0.1	N/A
	B cells CD19+	0.6	0.5	0.8	−0.3	N/A
	T cells CD3+	−0.1	−0.3	−0.2	−0.2	N/A
	T Cytotoxic Cells CD8+	−0.9	−0.3	−1.4	0.1	N/A
	T Helper Cells CD4+	0.4	0.1	0.7	−0.1	N/A
	NK cells CD16+ CD56+	0.0	0.0	0.0	0.0	N/A
	Classical Monocytes CD14+	0.0	0.0	−0.1	0.0	N/A

**FIGURE 6** | Percentage population stability is shown over the accelerated aging period at 37°C. All subsets remained within <5% variation from initial values across the assessed time points.

evaluated their performance as stable reference materials for analytical validation and long-term monitoring. These materials provide a consistent and reproducible means of assessing assay performance over time.

Population frequencies remained highly consistent across operators, instruments, and days. This level of stability contrasts with donor PBMCs and whole blood, which show marked inter- and intra-donor variability in subset distributions, antigen

TABLE 17 | Carryover assessment using an alternating sequence of high-positive and blank samples.

Population type	TBNK cell mimic Rep 1	Stain buffer Rep 1	TBNK cell mimic Rep 2	Stain buffer Rep 2	TBNK cell mimic Rep 3	Stain buffer Rep 3
Singlets Count/ μ L	1028.66	1.1	1118.12	2.14	1045.16	1.26
Leukocytes Count (Events/ μ L)	30712	30	33510	57	31478	28
Granulocytes CD45+	5	0	4	0	5	0
Monocytes CD45+	13	0	14	0	13	0
Lymphocytes CD45+	79	0	80	0	79	0
B cells CD19+	15	N/A	15	N/A	15	N/A
T cells CD3+	79	0	80	0	79	0
T Cytotoxic Cells CD8+	33	N/A	34	N/A	33	N/A
T Helper Cells CD4+	67	N/A	66	N/A	67	N/A
NK cells CD16+ CD56+	99	N/A	100	N/A	99	N/A
Classical Monocytes CD14+	98	N/A	99	N/A	99	N/A

TABLE 18 | Limit of detection (LOD) and limit of quantitation (LOQ) for each TBNK population, based on σ -based calculations.

Population type	Sigma (σ)	LOD	LOQ
Granulocyte CD45+	0.088	0.34	1.04
Monocyte CD45+	0.173	0.59	1.79
Lymphocyte CD45+	1.393	4.65	14.1
B Cells CD19+	0.183	0.53	1.59
T Cells CD3+	1.102	3.63	11.0
T Cytotoxic Cells CD8+	0.463	1.57	4.77
T Helper Cells CD4+	0.680	2.20	6.68
NK Cells CD16+ CD56+	0.104	0.33	0.99
Classical Monocytes CD14+	0.183	0.67	2.03

TABLE 19 | Replicate cell counts following reconstitution of TBNK Cell Mimic.

Replicate	Total volume (μ L)	Count/ μ L	Total count
Vial 1	300	874	262,098
Vial 2	300	858	257,463
Vial 3	300	977	293,205
Average	300	903	270,922

expression, and susceptibility to pre-analytical factors such as cryopreservation, processing delays, and isolation methods [10, 13, 14]. Because the synthetic controls do not undergo

biological changes such as activation or apoptosis, their population structure remains stable with repeated handling, providing a reliable baseline for day-to-day assay reproducibility. As shown in Table S15, TBNK Cell Mimic demonstrates less variation in % populations between lots than PBMCs, including off-the-shelf lyophilized or cryopreserved PBMCs, further supporting their robustness as a control material.

Median fluorescence intensities (MFI) were not compared across instruments due to known differences in instrument design, detector sensitivity, and signal amplification settings [8, 11, 15]. These differences persist even when bead-based standards are used, as beads help monitor detector performance but cannot fully harmonize fluorescence signals between cytometers [16]. Though synthetic controls cannot be used to compare MFI across instruments, they are highly effective for monitoring operator-to-operator and day-to-day variability, as shown in Tables S4, S6, and S8. Synthetic controls are ideal for assessing frequency-based metrics and provide a reliable measure of instrument stability over time.

Linearity studies demonstrated proportional responses across a broad concentration range, supporting use of the material in defined intervals where relative quantification is reliable. Slight deviations at the lowest dilutions likely reflect the challenges of measuring very small populations. Because the dilutions were prepared using engineered material rather than biological cells, the resulting measurement range reflects a stable and reproducible control. Biological samples may differ due to variation in antigen density, staining efficiency, or scatter properties [17], emphasizing the value of consistent control. The synthetic controls provided a reliable and reproducible range in which the assay produced values that closely matched expectations.

When mixed with PBMCs, the synthetic populations integrated predictably into standard gating hierarchies. Their forward- and side-scatter characteristics overlapped with the PBMC lymphocyte region (Figure S1), indicating compatibility with conventional lymphocyte gating boundaries. In addition, staining with lineage-specific antibodies to antigens present on T-cells or B-cells but absent on TBNK Cell Mimic by design demonstrated minimal nonspecific binding, supporting clean population resolution without polymer-associated background. This makes the TBNK Cell Mimic material useful for verifying consistency in gate placement without distorting PBMC-derived population boundaries. Minor shifts in NK-related FMOs were consistent with known challenges in CD16 and CD56 gating related to fluorochrome brightness and spectral overlap [18–20]. Overall, the synthetic controls help confirm that major population boundaries are being resolved correctly.

Cryopreserved PBMCs are used in spike-in experiments to provide a controlled approach for evaluating population recovery and gating behavior. However, cryopreservation and freeze-thaw processing can alter surface marker expression and scatter characteristics compared with freshly isolated blood. As a result, the accuracy data presented here are specific to workflows using cryopreserved PBMCs and may not fully reflect assay performance in fresh whole-blood immunophenotyping.

Antibody clone selection is a well-recognized source of variability in flow cytometry-based immunophenotyping, particularly for markers with inconsistent expression. TBNK Cell Mimic exhibited consistent population frequencies for most markers when stained with standard antibody clones, with the exception of CD3, which was compatible with only two of the four clones evaluated. Clone-dependent differences were observed for select markers, demonstrating that the synthetic material responds to antibody variation in a biologically realistic manner.

Performance consistency across no-lyse, lyse/no-wash, and lyse/wash workflows indicates that the material functions reliably despite substantial procedural differences among laboratories. Lot-to-lot comparisons also showed stable population frequencies across independent manufacturing batches, suggesting a well-controlled production process. This type of batch consistency is particularly valuable for longitudinal assay monitoring, where variability in the control material can otherwise hide real assay drift [21, 22].

Stability was supported by minimal changes in population frequencies during accelerated aging at 25°C and 37°C, consistent with expectations for non-biological QC materials. Because accelerated aging relies on thermal acceleration models, such as the Arrhenius assumption, which may not perfectly predict real-time performance [12], an additional real-time stability study is ongoing to confirm the results of the accelerated stability study.

TBNK Cell Mimics are intended for use as gating or assay control for percentage-based immunophenotyping and were evaluated in this study for their ability to reproduce relative population frequencies. They are not designed for use as absolute counting standards unless combined with appropriate counting beads or volumetric cytometry platforms.

In the broader context of flow cytometry reference materials, synthetic controls occupy a distinct role. Donor-derived PBMCs offer biological relevance but are variable and have limited stability; stabilized whole-blood products retain morphology and autofluorescence but vary between lots. Commercially available polystyrene beads are useful for optical standardization such as tracking detector performance, setting compensation, or establishing fluorescence scales but they lack the immunophenotypic complexity required to evaluate cell-based gating strategies [21, 22]. Synthetic controls bridge these gaps by providing reproducible population-level immunophenotypes without donor-dependent variability. Because protein expression and population frequencies (Table 14) can be precisely controlled and reproduced from lot-to-lot, the materials provide biologically relevant structure while maintaining high manufacturing consistency. These attributes enable the products to be used across diverse QC workflows, supporting assessment of assay precision, workflow consistency, and long-term performance trends.

Synthetic controls can be engineered to display a wide range of surface markers, including activation, apoptosis, or exhaustion epitopes, with precise and reproducible expression levels. Unlike living cells, they are not affected by signaling, stress, cytokine exposure, or changes in viability, and they do not exhibit donor-specific variability in antigen density, autofluorescence, or spreading error. This stability provides a reliable reference for gating, compensation, and spectral unmixing, making them particularly well-suited for panels such as TBNK that focus on lymphocyte subsets. The TBNK Cell Mimic platform allows customization of marker expression and population frequencies, providing a consistent and reproducible reference material for supporting assay performance and quality monitoring.

In summary, the polymer-based TBNK Cell Mimic controls bring consistency, precision, and reliability to flow cytometry workflows. With well-defined marker expression and reproducible population frequencies, they provide a robust reference for gating, compensation, and performance monitoring.

5 | Conclusion

This validation demonstrates that TBNK Cell Mimic can provide a consistent benchmark for TBNK immunophenotyping performance across key analytical parameters. The material exhibited stable population profiles (CV < 5%), reproducible measurements across different workflow conditions (CV < 5%), and low lot-to-lot variability (CV < 3%), supporting reliable evaluation of assay precision, linearity, specificity, stability, and robustness. These results indicate that TBNK Cell Mimic can serve as a dependable reference material for monitoring assay performance in both research and regulated cytometry environments. Their stability and controllability make them a complementary reference material to biological samples, supporting consistent and reliable assay performance.

Author Contributions

Swetha Pratyusha Gunturu: writing – original draft, validation, data curation, methodology, formal analysis. **Armando Martinez:** data

curation. **Kanwal Palla:** writing – review and editing, supervision. **Louisa D'Lima:** supervision, resources. **Brian Kerfs:** data curation. **Subhanip Biswas:** conceptualization.

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The authors have nothing to report.

Conflicts of Interest

Authors are employees of Slingshot Biosciences, the manufacturer of the synthetic controls evaluated in this study.

Data Availability Statement

The data that supports the findings of this study are available in the [Supporting Information](#) of this article.

References

1. R. Y. Ward, M. Stevens, and S. Bashir, "Metrological Traceability in Flow Cytometry? Evaluation of a New Volumetric Method for Lymphocyte Subsets," *International Journal of Laboratory Hematology* 46, no. 3 (2023): 488–494, <https://doi.org/10.1111/ijlh.14219>.
2. J. J. M. Van Dongen, M. Van Der Burg, T. Kalina, et al., "EuroFlow-Based Flowcytometric Diagnostic Screening and Classification of Primary Immunodeficiencies of the Lymphoid System," *Frontiers in Immunology* 10 (2019): 1271, <https://doi.org/10.3389/fimmu.2019.01271>.
3. Clinical and Laboratory Standards Institute (CLSI), Validation of Assays Performed by Flow Cytometry (CLSI Guideline I/LA-33), 2023.
4. U.S. Food and Drug Administration, Bioanalytical Method Validation: Guidance for Industry, 2018.
5. International Clinical Cytometry Society (ICCS), "ICCS/ICSH Guidelines for Validation of Flow Cytometry Methods," *Cytometry Part B: Clinical Cytometry* 84, no. 5 (2013): 315–323.
6. L. Wang and R. A. Hoffman, "Standardization, Calibration, and Control in Flow Cytometry," *Current Protocols in Cytometry* 79, no. 1 (2017): 1.3.1–1.3.27, <https://doi.org/10.1002/cpcy.14>.
7. J. G. Burel, Y. Qian, C. L. Arlehamn, et al., "An Integrated Workflow to Assess Technical and Biological Variability of Cell Population Frequencies in Human Peripheral Blood by Flow Cytometry," *Journal of Immunology* 198, no. 4 (2017): 1748–1758, <https://doi.org/10.4049/jimmunol.1601750>.
8. J. C. Wood, "Evaluating Fluorescence Sensitivity on Flow Cytometers: An Overview," *Cytometry* 33, no. 2 (1998): 256–259, [https://doi.org/10.1002/\(SICI\)1097-0320\(19981001\)33:2<256::AID-CYTO22>3.0.CO;2-S](https://doi.org/10.1002/(SICI)1097-0320(19981001)33:2<256::AID-CYTO22>3.0.CO;2-S).
9. R. A. Hoffman, "Standardization, Calibration, and Control in Flow Cytometry," *Current Protocols in Cytometry* 32, no. 1 (2005): 1.3.1–1.3.27, <https://doi.org/10.1002/0471142956.cy0103s32>.
10. H. T. Maecker, J. P. McCoy, and R. Nussenblatt, "Standardizing Immunophenotyping for the Human Immunology Project," *Nature Reviews. Immunology* 12, no. 3 (2012): 191–200, <https://doi.org/10.1038/nri3158>.
11. T. Kalina, J. Flores-Montero, V. H. J. Van Der Velden, et al., "EuroFlow Standardization of Flow Cytometer Instrument Settings and Immunophenotyping Protocols," *Leukemia* 26, no. 9 (2012): 1986–2010, <https://doi.org/10.1038/leu.2012.122>.
12. A. Ebrahim, K. DeVore, and T. Fischer, "Limitations of Accelerated Stability Model Based on the Arrhenius Equation for Shelf-Life

Estimation of in Vitro Diagnostic Products," *Clinical Chemistry* 67, no. 4 (2020): 684–688, <https://doi.org/10.1093/clinchem/hvaa282>.

13. B. Li, C. Yang, G. Jia, et al., "Comprehensive Evaluation of the Effects of Long-Term Cryopreservation on Peripheral Blood Mononuclear Cells Using Flow Cytometry," *BMC Immunology* 23, no. 1 (2022): 30, <https://doi.org/10.1186/s12865-022-00505-4>.

14. S. P. Perfetto, D. Ambrozak, R. Nguyen, P. Chattopadhyay, and M. Roederer, "Quality Assurance for Polychromatic Flow Cytometry," *Nature Protocols* 1, no. 3 (2006): 1522–1530, <https://doi.org/10.1038/nprot.2006.250>.

15. H. T. Maecker and J. Trotter, "Flow Cytometry Controls, Instrument Setup, and the Determination of Positivity," *Cytometry, Part A* 69A, no. 9 (2006): 1037–1042, <https://doi.org/10.1002/cyto.a.20333>.

16. S. P. Perfetto, P. K. Chattopadhyay, and M. Roederer, "Seventeen-Colour Flow Cytometry: Unravelling the Immune System," *Nature Reviews. Immunology* 4, no. 8 (2004): 648–655, <https://doi.org/10.1038/nri1416>.

17. T. Kalina, K. Lundsten, and P. Engel, "Relevance of Antibody Validation for Flow Cytometry," *Cytometry, Part A* 97, no. 2 (2019): 126–136, <https://doi.org/10.1002/cyto.a.23895>.

18. I. Filipovic, I. Sönnnerborg, B. Strunz, et al., "29-Color Flow Cytometry: Unraveling Human Liver NK Cell Repertoire Diversity," *Frontiers in Immunology* 10 (2019): 2692, <https://doi.org/10.3389/fimmu.2019.02692>.

19. A. Poli, T. Michel, M. Thérésine, E. Andrès, F. Hentges, and J. Zimmer, "CD56bright Natural Killer (NK) Cells: An Important NK Cell Subset," *Immunology* 126, no. 4 (2008): 458–465, <https://doi.org/10.1111/j.1365-2567.2008.03027.x>.

20. L. Rebuffet, J. E. Melsen, B. Escalière, et al., "High-Dimensional Single-Cell Analysis of Human Natural Killer Cell Heterogeneity," *Nature Immunology* 25, no. 8 (2024): 1474–1488, <https://doi.org/10.1038/s41590-024-01883-0>.

21. J. K. Nicholson, "Evaluation of Stabilized Whole Blood Control Materials for Lymphocyte Immunophenotyping," *Cytometry* 33, no. 2 (1999): 256–259, [https://doi.org/10.1002/\(sici\)1097-0320\(19991215\)38:6<268::aid-cyto2>3.0.co;2-k](https://doi.org/10.1002/(sici)1097-0320(19991215)38:6<268::aid-cyto2>3.0.co;2-k).

22. M. Bergeron, A. Shafaie, T. Ding, et al., "Evaluation of Stabilized Blood Cell Products as Candidate Preparations for Quality Assessment Programs for CD4 T-Cell Counting," *Cytometry* 50, no. 2 (2002): 86–91, <https://doi.org/10.1002/cyto.10090>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Overlay of TBNK Cell Mimic (Orange) with Cryopreserved PBMCs from All Cells (Navy) **Table S1:** Replicate data used to assess intra-assay repeatability of TBNK Cell Mimic. Population frequency measurements are presented to evaluate within-run precision. **Table S2:** Replicate data used to assess intra-assay repeatability of TBNK Cell Mimic. Median fluorescence intensity (MFI) values are shown to evaluate within-run precision. **Table S3:** Intermediate precision assessment of TBNK Cell Mimic across operators. Population frequency measurements were compared to evaluate inter-operator variability. **Table S4:** Intermediate precision assessment of TBNK Cell Mimic across operators. Mean fluorescence intensity (MFI) values were compared to evaluate inter-operator variability. **Table S5:** Intermediate precision evaluation of TBNK Cell Mimic Population frequencies obtained from same-day, same-operator replicate analyses are presented to characterize assay repeatability. **Table S6:** Intermediate precision evaluation of TBNK Cell Mimic. Mean fluorescence intensity (MFI) values obtained from same-day, same-operator replicate analyses are presented to characterize assay repeatability. **Table S7:** Intermediate precision assessment of TBNK Cell Mimic across different days. Population frequency measurements were compared to evaluate inter-day variability. **Table S8:**

Intermediate precision assessment of TBNK Cell Mimic across different days. Mean fluorescence intensity (MFI) values were compared to evaluate inter-day variability. **Table S9:** Intermediate precision assessment of TBNK Cell Mimic using Rainbow bead-normalized MFIs across multiple days. **Table S10:** Intermediate precision assessment of TBNK Cell Mimic across different instruments on the same day. Population frequency measurements were compared to evaluate inter-instrument variability. **Table S11:** Intermediate precision assessment of TBNK Cell Mimic across different instruments on the same day. Mean fluorescence intensity (MFI) values were compared to evaluate inter-instrument variability. **Table S12:** Linearity assessment of TBNK subset frequencies using serial dilutions of TBNK Cell Mimic: population frequencies **Table S13:** Linearity assessment of TBNK subset frequencies using serial dilutions of TBNK Cell Mimic: MFI **Table S14:** Replicate data for Accuracy evaluation comparing observed values from PBMC + TBNK Cell Mimic spike-in experiments with expected TBNK Cell Mimic target values. **Table S15:** TBNK Cell Mimic demonstrates less variation in % populations between lots than PBMCs. **Figure S2:** Overlay of TBNK Cell Mimic Immune Cell Populations with Fluorescence minus one sample (FMOs). This figure shows overlays of FMOs (Charcoal—CD19 FMO, Blue CD56 FMO, Navy—CD16 FMO, Yellow—CD14 FMO) with the TBNK Cell Mimic (in Orange). **Figure S3:** Specificity assessment of TBNK Cell Mimic using non-specific markers (TCRab, CD20 and CCR7 in PE). Right plot—demonstrates CD3 versus CD19, middle plot—demonstrates CD3 versus PE channel with all the non-specific markers, Left plot—histogram of PE channel. **Figure S4:** Schematic representation of TBNK Cell Mimic Immune Cell Analysis using DURAClone IM Phenotyping kit **Figure S5:** Schematic representation of TBNK Cell Mimic Immune Cell Analysis using BD Multitest 6-color TBNK kit **Figure S6:** Schematic representation of TBNK Cell Mimic Immune Cell Analysis using Human 4-color TBNK kit **Figure S7:** Representative dot plots showing positive and negative populations for CD3 alternate clones (UCHT1, OKT3 and HIT3a). **Figure S8:** Overlay of TBNK Cell Mimic Immune Cell Populations Across Two Accelerated Stability Study Timepoints **Table S16:** TBNK Antibody Panel Information **Table S17:** TBNK Antibody Panel Information for assessing Specificity **Table S18:** TBNK Cell Mimic clone compatibility.