

Highly Sensitive B-Cell Monitoring Following B-Cell-Depleting Therapies: An Optimized Flow Cytometry Assay for Residual B-Cell Quantification

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PURPOSE

Accurate absolute quantification of peripheral B cells at very low concentrations remains challenging using conventional TBNK flow cytometry assays. Increasing use of B-cell depleting therapies has created a need for highly sensitive and robust methods capable of measuring residual B-cell populations and supporting pharmacodynamic assessments in clinical studies. The objective of this work was to develop and characterize a high-sensitivity whole-blood flow cytometry assay for absolute enumeration of B cells and major lymphocyte subsets. The assay was designed to achieve an initial target lower limit of quantification (LLOQ) of 0.1 cells/ μ L, representing a ten-fold improvement in sensitivity compared with standard TBNK methodologies, while maintaining analytical robustness suitable for clinical sample testing.

METHOD

A single-tube flow cytometry assay was developed to provide absolute counts (cells/ μ L) of B cells, T cells, CD4+ T cells, CD8+ T cells, and natural killer (NK) cells directly from whole blood. Antibodies are listed in **Table 1** The method utilizes a lyse/no-wash workflow to maximize recovery of rare cell populations and minimize sample manipulation. Enhanced sensitivity was achieved through optimization of sample acquisition volume, staining conditions, and analytical workflow. Performance characteristics were evaluated using whole blood samples from 3 healthy donors at both normal levels and at B cell-depleted levels. B cell depleted blood samples were obtained following incubation with CD19 Pan B Dynabeads (**Figure 1**).

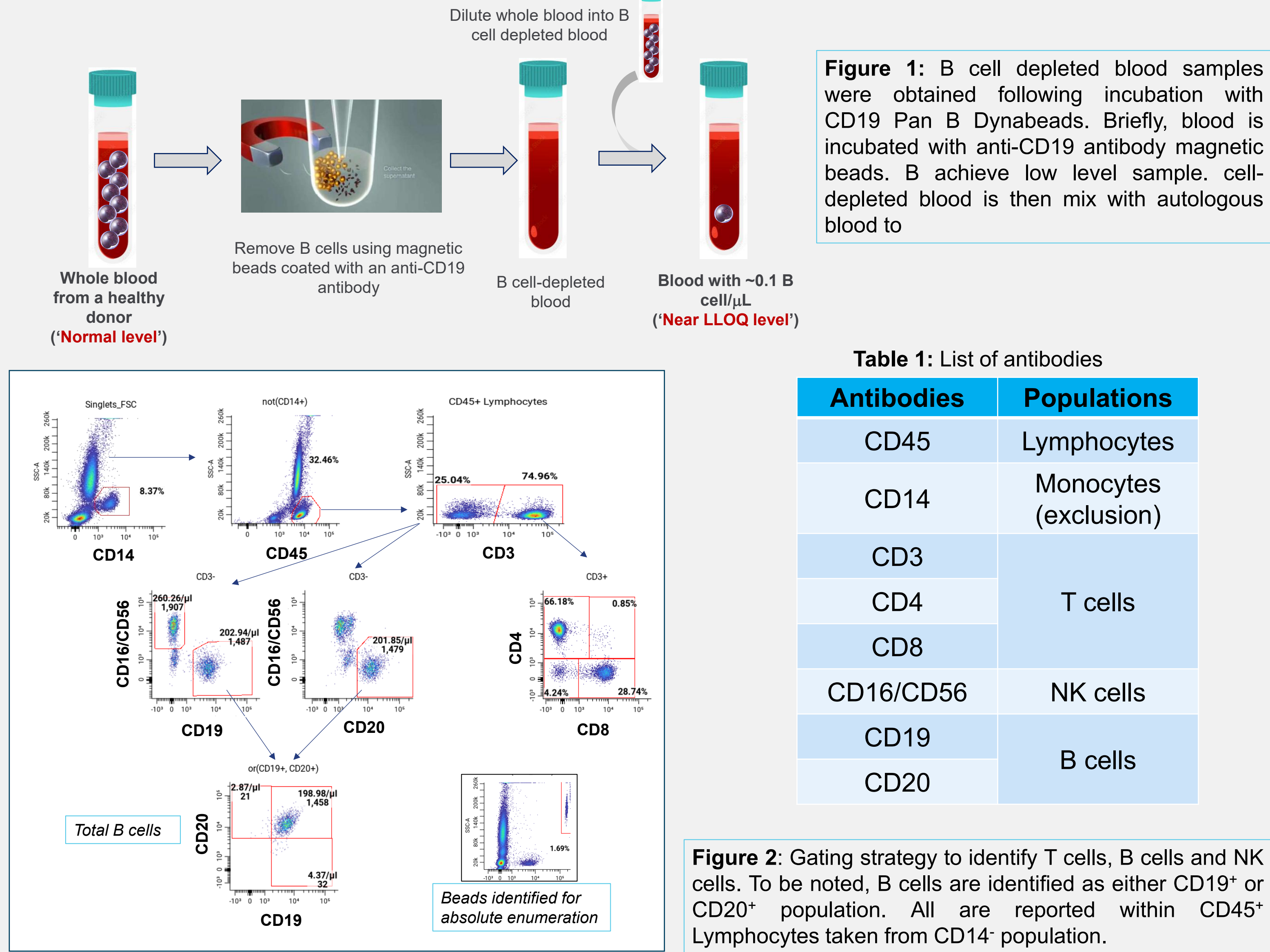


Table 1: List of antibodies

Antibodies	Populations
CD45	Lymphocytes
CD14	Monocytes (exclusion)
CD3	
CD4	T cells
CD8	
CD16/CD56	NK cells
CD19	
CD20	B cells

Figure 2: Gating strategy to identify T cells, B cells and NK cells. To be noted, B cells are identified as either CD19+ or CD20+ population. All are reported within CD45+ Lymphocytes taken from CD14- population.

RESULTS

Precision

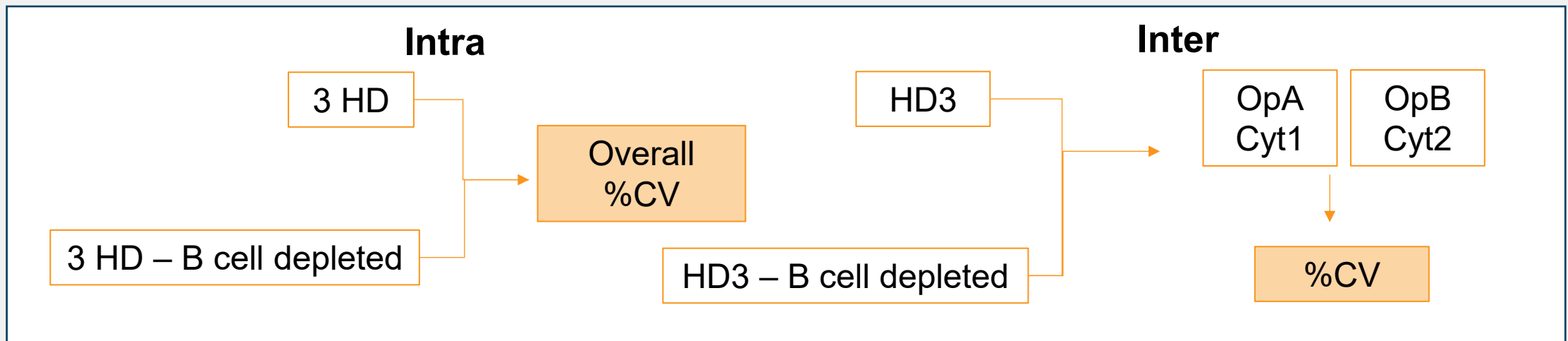


Table 2: Precision data for absolute count and frequency readouts

Population	Normal Samples				B cell-depleted Samples			
	Average	HD1	HD2	HD3	Average	HD1	HD2	HD3
Absolute Count (cells / μ L)								
NK cells	98.2	653.7	92.7	4.7	4.9	91.1	536.0	94.8
Total B cells	157.8	156.4	225.5	6.1	3.8	0.10	0.09	0.16
CD19+ B cells	157.6	156.1	225.4	6.0	3.8	0.09	0.08	0.15
CD20+ B cells	154.4	151.7	220.2	6.1	3.8	N/R	N/R	N/R
Total T cells	1086.7	691.1	843.8	5.3	3.0	986.8	567.0	778.7
CD4+ T cells	578.7	387.7	460.3	5.5	3.3	524.2	315.9	426.5
CD8+ T cells	451.3	266.4	333.2	5.0	3.0	410.4	219.8	306.1
Percent of CD45+ Lymphocytes (%)								
NK cells	7.1	42.5	7.6	2.9	5.4	8.4	47.9	10.4
Total B cells	11.3	10.2	18.5	1.5	1.6	0.009	0.008	0.017
CD19+ B cells	11.3	10.1	18.5	1.5	1.6	0.008	0.007	0.016
CD20+ B cells	11.1	9.9	18.1	1.4	1.5	N/R	N/R	N/R
Total T cells	78.1	44.9	69.2	0.5	0.4	90.9	50.7	85.7
CD4+ T cells	41.6	25.2	37.8	0.6	0.6	48.3	28.2	46.9
CD8+ T cells	32.4	17.3	27.3	0.9	0.8	37.8	19.6	33.7

Precision: For normal samples, %CV of all reportable readouts was below 10% for both intra and inter-assay precision. For B-cell depleted samples, B cell readouts displayed %CV <15% for intra and <20% for inter-assay precision.

Post-staining Stability

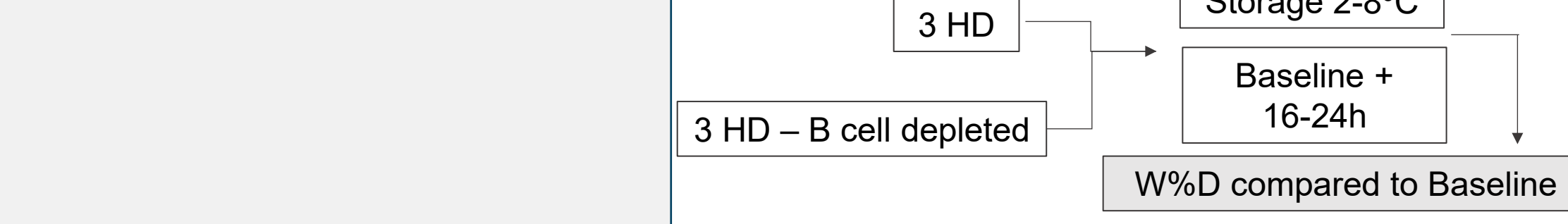


Table 4: Post-staining stability summary

Population	Normal Samples			B cell-depleted Samples		
	HD1	HD2	HD3	HD1	HD2	HD3
Absolute Count (cells / μ L)						
Total B cells	5.8	3.1	10.2	0.3	0.5	N/R
CD19+ B cells	5.8	3.1	10.2	3.3	5.8	43.2
CD20+ B cells	6.1	3.0	10.4	N/R	N/R	N/R
Percent of CD45+ Lymphocytes (%)						
Total B cells	0.2	0.4	1.0	0.09	2.0	N/R
CD19+ B cells	0.2	0.4	1.0	3.1	2.9	N/R
CD20+ B cells	0.03	0.2	0.8	N/R	N/R	N/R

Post-staining Stability: Post-staining stability was assessed with three healthy donors with or without B cell depletion for up to 23h following staining. At least 2/3 donors displayed <25 W%D compared to baseline measurement (within 2h post-staining).

Legend: %CV – Coefficient of Variation, Cyt – Cytometer, HD – Healthy Donor, LLOQ – Lower limit of quantification, N/R – Non-reportable (below threshold, or failed %CV), TP – Timepoint, W%D – Weighted % of Difference

Linearity and LLOQ

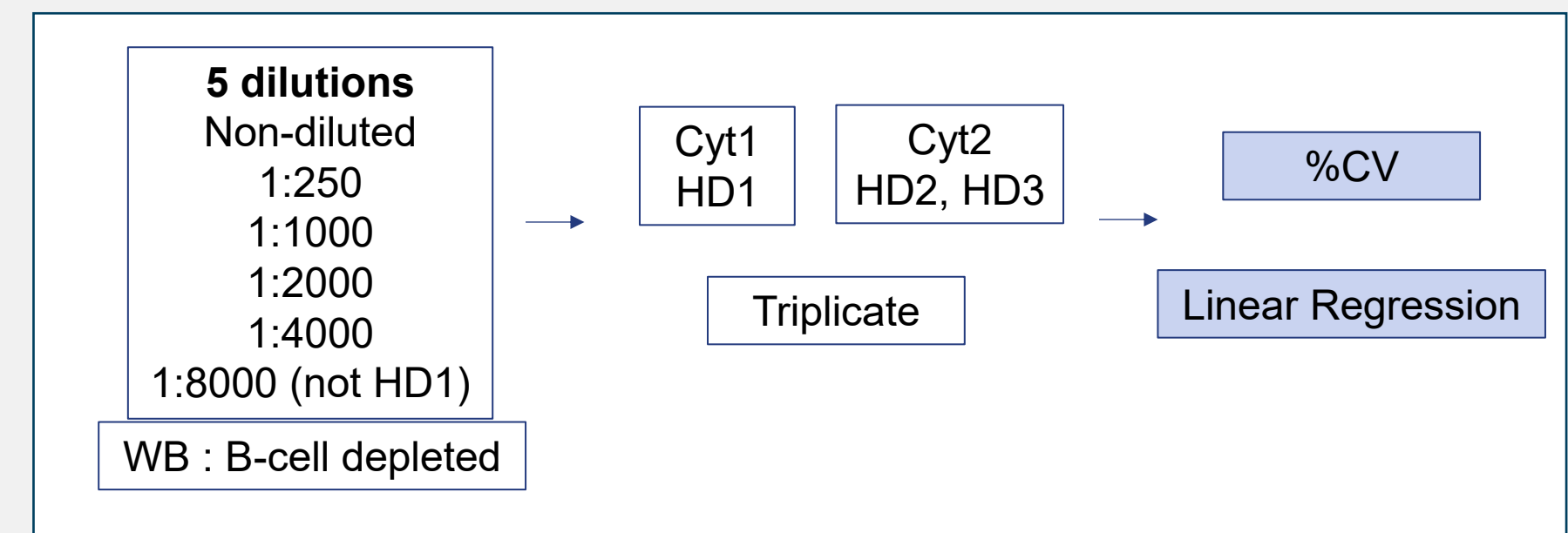


Table 3: LLOQ values with associated event counts

Readout	Population	LLOQ	%CV	Event Count Mean
Absolute	Total B cells	0.04	18.7	11
Count (cells / μ L)	CD19+ B cells	0.02	18.3	6
	CD20+ B cells	0.15	8.3	44
Percent of	Total B cells	0.003	23.5	8
	CD19+ B cells	0.002	16.7	6
	CD20+ B cells	0.01	18.9	77

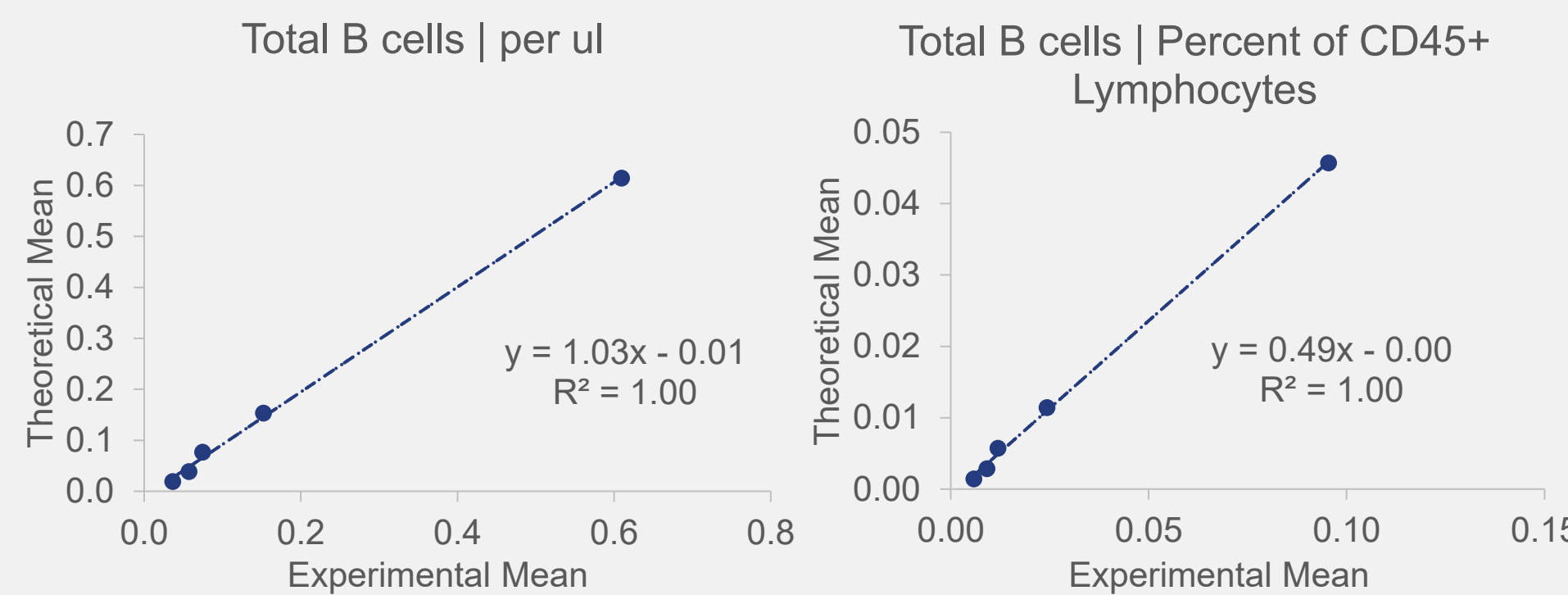


Figure 3: Representative linear regression for total B cell population (absolute count on the left and percent of CD45+ Lymphocytes on the right) for HD3.

Linearity and LLOQ: LLOQ level were identified at the lowest value with <25.0%CV from all 3 donors tested without any event count cut-off. The identified LLOQ were between 0.02 and 0.15 cells/ μ L and 0.002 and 0.01% of CD45+ Lymphocytes with 6-77 gated events.

This supports reliable quantification of rare residual B-cell populations. The CD19/CD20 gating strategy enabled consistent identification of B cells at low frequencies and contributed to robust detection.

Whole Blood Stability

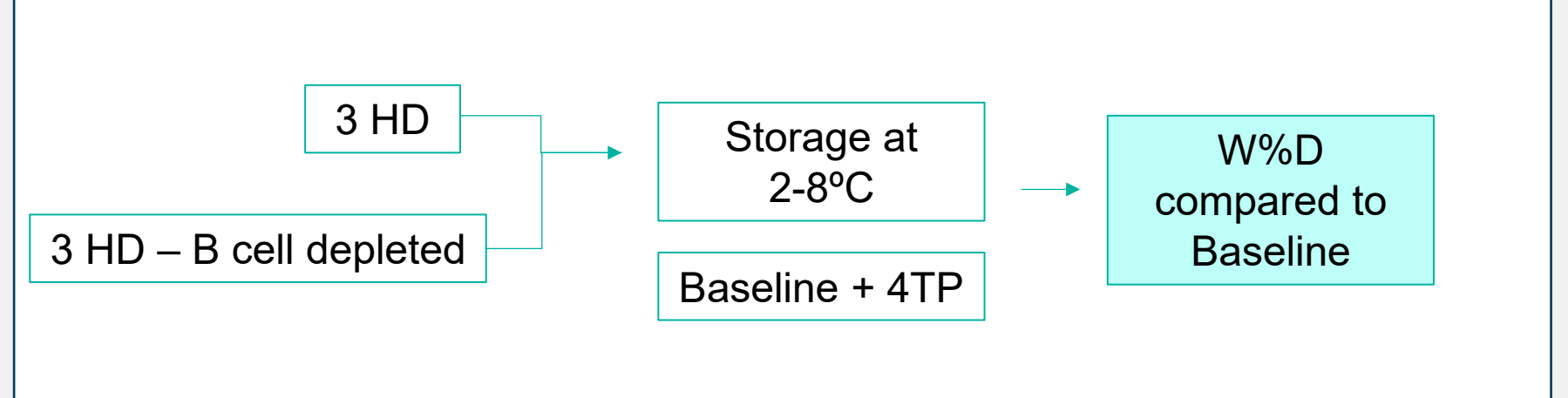


Table 5: Whole blood stability summary data

Population	Normal Samples			B cell-depleted Samples		
	Baseline Average	HD1	HD2	Baseline Average	HD1	HD2
Absolute Count (cells / μ L)						
NK cells	130.6	313.8	219.6	6.0	0.5	32.1
Total B cells	142.8	326.5	243.9	0.7	8.0	3.7
CD19+ B cells	142.7	326.4	243.7	2.9	5.2	32.2
CD20+ B cells	141.8	323.5	241.6	7.0	5.3	0.1
Total T cells	848.4	1702.1	1557.4	14.3	0.2	35.0
CD4+ T cells	579.0	1009.6	957.6	14.7	0.4	36.1
CD8+ T cells	212.1	629.9	446.6	17.6	4.3	36.2
Percent of CD45+ Lymphocytes (%)						
NK cells	11.2	13.0	10.7	9.4	1.1	1.4
Total B cells	12.3	13.5	11.9	1.9	3.9	1.1
CD19+ B cells	12.3	13.5	11.9	1.1	2.4	3.3
CD20+ B cells	12.2	13.4	11.8	0.6	0.9	8.0
Total T cells	73.0	70.4	76.1	1.0	0.9	0.1
CD4+ T cells	49.8	41.8	46.8	1.8	0.7	1.3
CD8+ T cells	18.3	26.1	21.8	4.0	5.6	1.3

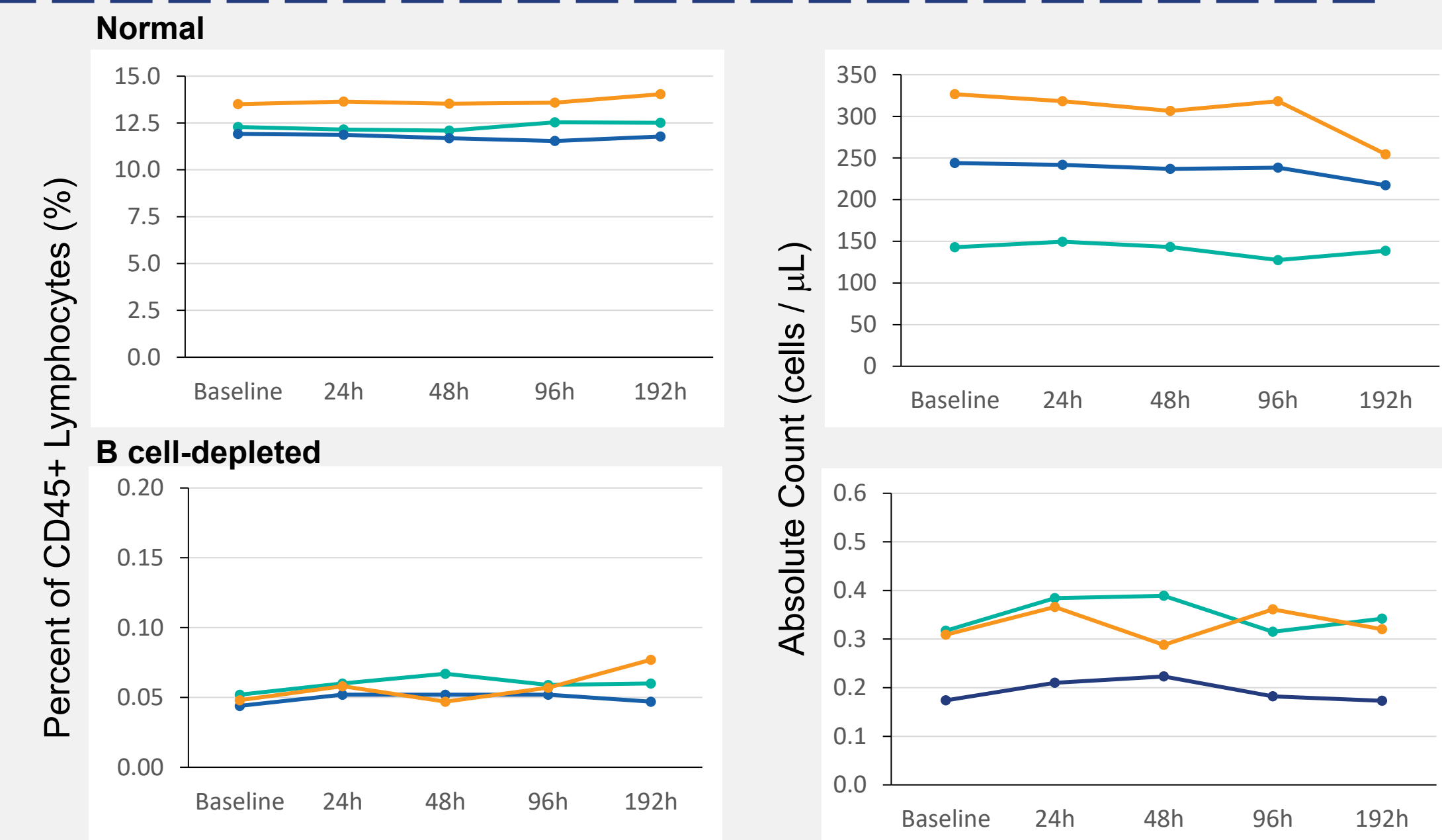


Figure 4: Whole blood stability of Total B cells population in Normal Samples (top row) and in B cell-depleted Samples (bottom row). Each line represents a different donor (total 3 donors).

Whole Blood Stability: Whole blood stability was assessed with three healthy donors with or without B cell depletion for up to 8 days post-collection. At least 2/3 donors displayed <25 W%D compared to baseline measurement (day of collection in Cyto-Chex®).

The extended stability experiment demonstrated adequate sample stability to ensure global clinical trial deployment.

CONCLUSION AND NEXT STEPS

A highly sensitive whole-blood flow cytometry assay was successfully developed and characterized for enumeration of B cells and major lymphocyte subsets. Through optimization of sample processing, acquisition strategy, and B-cell identification using CD19 and CD20 markers, the assay achieved an **LLOQ of ≤ 0.15 cells/ μ L** while maintaining acceptable precision and stability characteristics. The method provides a robust platform for quantification of low-level B-cell populations and supports pharmacodynamic assessments in studies involving B-cell depleting therapies. Future work will focus on expanded validation activities and generation of data packages supporting broader clinical and regulatory applications. In addition to achieving high analytical sensitivity, development of a low-level QC material near the assay LLOQ provides a practical strategy for long-term monitoring of assay performance in studies involving profoundly B-cell-depleted subjects. Additional characterization and standardization of this low-level QC material are ongoing.

