Analytical Constraints of the Standard 6-Color TBNK Panel in Allogeneic CAR-T cell Trials and Strategies for Resolution

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Introduction

Chimeric Antigen Receptor (CAR) T-cell therapy has improved the treatment of hematological malignancies and is showing promise in solid tumors. This immunotherapy uses genetically engineered T-cells that express a receptor targeting specific tumor antigens, enhancing immune-mediated tumor killing. Initially, therapies relied on autologous CAR T cells derived from the patient, but allogeneic CAR T cells from healthy donors are advancing in clinical trials. Allogeneic CAR T cells provide an "off-the-shelf" option with faster availability and scalable production, compared to autologous CAR T therapy. However, they introduce risks such as graft-versus-host disease and rejection by the patient's immune system.

It is common to use the standard 6-color (CD45, CD3, CD4, CD8, CD19, CD16/56) TBNK assay to enumerate T-cells, B-cells, and NK-cells in clinical trials involving CAR-T therapy. However, allogeneic CAR T-cells are knocked out for TCRαβ to reduce the risk of a graft-versus-host reaction and as such lack surface expression of CD3. As such, in patients treated with allogeneic CAR T-cells, the $CD3^-$ compartment not only includes $CD19^+$ B cells, $CD16^+/56^+$ NK cells, but also CAR T-cells. An extra layer of complication is the possible expression of CD56 on the surface of the allogeneic CAR T cells because of activation. In this case, using the standard TBNK assay will lead to erroneous inclusion of $CD16/56^+CD3^-CAR^+$ T cells as NK-cells. To overcome this, NK cell marker NKp46 was added to the standard TBNK assay and CD16 and CD56 were split over two separate fluorochromes. This required redesign of the panel to get an optimized configuration, and a modified gating strategy to identify all populations, including a proper distinction of host NK cells from $CD56^+CD3^-$ CAR T-cells. In this gating strategy NK cells are identified as $CD45^+CD3^-CD4^-CD8^{+/-}CD56^+CD16^{+/-}NKp46^+$.

Here we present an optimized assay, which is similar to the standard TBNK lyse/no wash assay in a BD Trucount™ tube, to determine absolute count of each population. The newly developed assay was validated based on CLSI H62* guideline to monitor precision (repeatability and reproducibility), between instrument variability and between-operator variability. Sample stability of the optimized TBNK assay was established at 120 hours when samples were maintained at ambient temperature. Moreover, we present a successful experimental design that allowed us to determine sensitivity of the lymphocyte detection with an LLoD of 2 cells per µl and an LLoQ of 6 cells per µl. As such we validated the optimized TBNK assay for secondary endpoint use in clinical trials for allogeneic CAR T therapy.

* CLSI (Ed.). Validation of assays performed by flow cytometry, 1st ed. CLSI document H62. Wayne, PA: Clinical Laboratory Standards Institute, (2021).

Panel configuration (BD FACSLyric™)

Detector (laser)	FL1 (405)	FL2 (405)	FL3 (405)	FL4 (405)	FL5 (405)	FL6 (488)	FL7 (488)		FL8 (488)	FL9 (488)	FL10 (640)	FL11 (640)	FL12 (640)
Fluorochrome (Filter)	BV421 (448/45)	BV510 (528/45)	BV605 (606/36)	BV711 (715/50)	BV785 (755LP)	FITC (527/32)	PE (586/42)		PerCP-Cy5.5 (700/54)	RB780 (783/56)	APC (660/10)	APC-R700 (720/30)	APC-Cy7 (783/56)
Tube 1*			CD45		CD8	CD4	CD19	CD20	CD16	NKp46		CD56	CD3

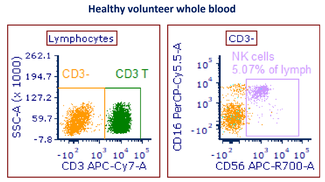
**Trucount tube; Lyse/No Wash*

Optimization of gating strategy

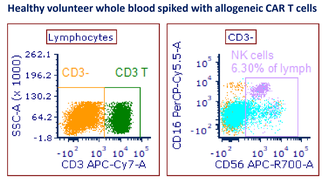
1. Traditional gating leads to inclusion of $CD56^+CD3^-$ allogeneic CAR T cells in the NK-cell population

Absence of CD3 expression in allogeneic CAR T cells raises a unique challenge to identify NK cells when using the standard gating strategy from the 6-color (CD45, CD3, CD4, CD8, CD19, CD16/56) TBNK assay. In patients treated with allogeneic CAR T-cells, the $CD3^-$ compartment not only includes $CD19^+$ B cells, $CD16^+/56^+$ NK cells, but also CAR T-cells. An extra layer of complication is the expression of CD56 on the surface of the allogeneic CAR T cells because of activation. This was demonstrated by comparing a healthy volunteer whole blood (WB) sample with a WB sample from the same healthy volunteer spiked with allogeneic CAR T cells. As shown in the figure below, the CAR T cells (depicted in turquoise and identified as $CD3^-CD4^+$orCD8$^+$) that show expression of CD56 are contaminating the NK cell gate. Hence, using the traditional gating from the 6-color TBNK assay leads to erroneous inclusion of $CD56^+CD3^-$ CAR T cells in the CD16/$CD56^+CD3^-$ NK-cell compartment.

Healthy volunteer whole blood

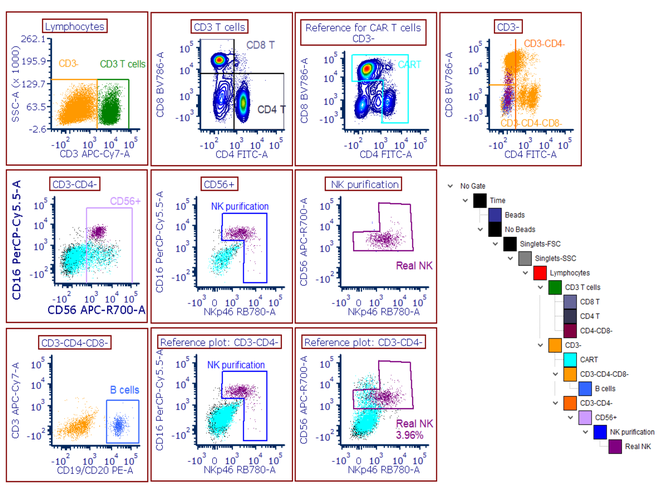


Healthy volunteer whole blood spiked with allogeneic CAR T cells



2. Addition of NK cell marker NKp46 allows for proper distinction of host NK cells from $CD56^+CD3^-$ CAR T cells

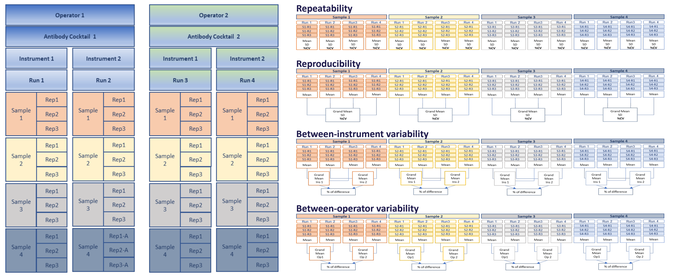
To allow proper distinction of host NK cells from $CD56^+CD3^-$ allogeneic CAR T cells, the panel design was adjusted with addition of NK cell marker NKp46 and splitting of CD16 and CD56 over two separate fluorochromes. Next, an optimized gating strategy was developed to purify the NK cell gate from possible $CD56^+CD3^-$ contaminating CAR T cells. In this new gating strategy, B cells are now defined as $CD19/CD20^+$ cells within the $CD3^-CD4^-CD8^-$ lymphocytes. Furthermore, extra purification gates were added in the hierarchy to separate real NK cells from possible $CD56^+$ allogeneic CAR T cells. First, $CD56^+$ cells are selected within the $CD3^-CD4^-$ lymphocytes. This step already excludes $CD4^+$ CAR T cells. At this stage in the gating hierarchy, it is not possible to exclude $CD8^+$ CAR T cells yet, because NK cells can express $CD8^+$ in a subset. In the next step, called 'NK purification', NK cells are further purified from $CD56^+$ CAR T cells, using CD16 and the additional NK cell marker NKp46.



Panel Validation

1. Precision

Precision was determined on four WB samples from apparently healthy donors, of which two donors were spiked with allogeneic CAR T cells. Samples were processed in triplicates by two operators and acquired on two instruments for a total of four different runs. For repeatability (or intra-assay precision) and reproducibility (or inter-assay precision), acceptance criteria of ≤25%CV between replicates/runs were applied. Higher imprecision (35%CV) was accepted for rare populations (≤5% of parent or ≤100 events) or populations with dimly expressed antigens. For between-instrument and between-operator variability, acceptance criteria of ≤20% difference between instruments/operators were applied.



Reportables	Repeatability %CV (average of 4 samples)	Reproducibility %CV (range over 4 samples)	Inter-instrument % difference (range over 4 samples)	Inter-operator % difference (range over 4 samples)
Lymphocytes	2.85	0.90 - 6.50	0.11 - 2.66	1.06 - 11.07
T cells (% of Lymph)	0.70	1.21 - 2.15	0.05 - 3.06	0.30 - 2.06
T cells (abs.)	2.86	1.93 - 5.90	1.05 - 3.03	1.35 - 9.30
CD4 T cells (% of Lymph)	1.10	1.20 - 2.00	0.03 - 3.03	0.06 - 1.48
CD4 T cells (% of T cells)	0.70	0.13 - 0.62	0.03- 0.58	0.02 - 0.38
CD4 T cells (abs.)	2.95	1.55 - 6.12	1.15 - 3.04	1.13 - 9.73
CD8 T cells (% of Lymph)	1.26	1.31 - 3.25	0.60 - 3.32	0.24 - 4.56
CD8 T cells (% of T cells)	0.74	0.57 - 2.57	0.11 - 0.63	0.86 - 4.25
CD8 T cells (abs.)	3.22	1.51 - 4.89	0.45 - 3.25	1.58 - 7.19
B cells (% of Lymph)	1.44	0.99 - 2.48	0.05 - 4.00	0.64 - 1.54
B cells (abs.)	3.17	1.45 - 6.22	1.16 - 4.01	0.73 - 9.70
NK cells (% of Lymph)	2.45	2.57 - 10.88	0.00 - 4.88	4.13 - 13.30
NK cells (abs.)	4.34	4.17 - 11.42	0.92 - 3.91	6.67 - 14.53

All reportables met the acceptance criteria for repeatability, reproducibility, between-instrument variability, and between-operator variability. Noticeably, precision of all reportables is below 12% CV and between-instrument and between-operator is less than 15% difference for all reportables, indicating a highly robust assay.

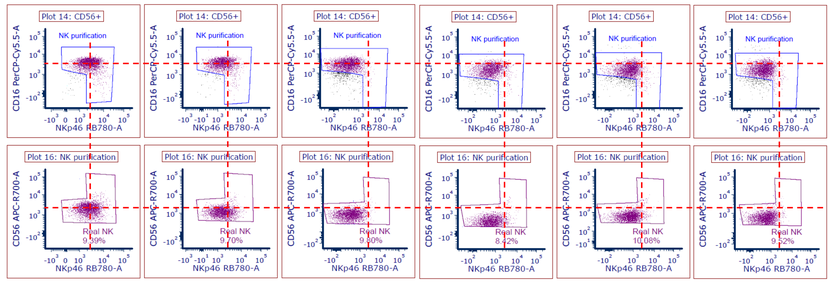
2. Sample Stability and Post-Stain Stability

Sample stability was determined on six WB samples from apparently healthy donors, processed at baseline (within 2 hours post-collection), 24 hours, 48 hours, 72 hours, 96 hours and 120 hours from collection time. In between timepoints, samples were stored at ambient temperature (18-26°C). Post-stain stability was determined on six WB samples from apparently healthy donors, acquired at baseline (within 30 minutes after staining), 1 hour, 3 hours and 24 hours from baseline. Stained samples were stored at ambient temperature up to 3 hours after baseline and further stored refrigerated (2-6°C) until 24 hours.

Sample stability and post-stain stability were both established at the latest time point where ≤20% change from the baseline was achieved for at least 80% (5 out of 6) of the samples.

Results showed that samples are stable up to 120 hours post-collection for all reportables. However, for the NK cells, the profile showed a decrease in signal for NKp46, and to a lesser extent for CD16 and CD56, over time. However, the gating strategy using all three markers and the design with CD56 and CD16 spread over two fluorochromes, still allows for proper identification of the NK cell population.

Post-stain stability was established up to 3 hours after staining when stored at room temperature, and up to 24 hours after processing when stored refrigerated.



3. Sensitivity – Lower Limit of Detection (LLoD) and Lower Limit of Quantitation (LLoQ) Assessment for Lymphocytes Absolute Count

Severe lymphodepletion is required before treatment with allogeneic CAR T cells to limit the risk of host-versus-graft disease . Therefore, it is essential to determine LLoD and LLoQ for the absolute lymphocyte count of the optimized TBNK assay.

LLoD for lymphocytes absolute count was determined using two whole blood samples from apparently healthy donors in a total of 10 CD45-negative runs, as depicted in below experimental setup. Next, LLoD was calculated as: $\mathrm{LLoD} = \mathrm{Mean} + 3\,\mathrm{SD}$. The results showed an **LLoD of 2 cells/µl** for lymphocytes absolute count.

Experimental setup	Sample 1	Sample 2
Processing	CD45 FMO*	CD45 FMO*
Acquisition	5 x per tube = 5 runs	5 x per tube = 5 runs

Results	Mean (cells/µl)	SD (cells/µl)	LLoD (cells/µl)
Lymphocytes (abs.)	0.60	0.52	2.15

**Stained with all antibodies except CD45 (FMO)*

LLoQ for lymphocytes absolute count was determined using three whole blood samples from apparently healthy donors. Each sample was processed twice: once with the normal full stain (FS) antibody cocktail and once with an unlabeled CD45 antibody (no other antibodies). Next, 5 different levels of serial dilution were created in triplicate, as shown in example below.

For each sample and each dilution, the mean, SD and %CV were calculated for lymphocytes absolute count and LLoQ was established at the level where ≤ 35% CV was achieved in at least 2 out of 3 samples when a minimum of 50 events were present in the population gate and all triplicates were above LLoD. Based on all three samples, the **LLoQ** for lymphocytes absolute count was established at **6 cells/µl.**

Experimental setup for 1 sample	Processed with CD45 unlabeled antibody	Serial dilution	Dilution factor
Tube 1	458 µl	42 µl stained with FS cocktail	12
Tube 2	375 µl	125 µl from tube 1	48
Tube 3	375 µl	125 µl from tube 2	192
Tube 4	375 µl	125 µl from tube 3	768
Tube 5	375 µl	125 µl from tube 4	3072

Results	Sample 1			Sample 2			Sample 3		
Dilution	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
1:12	100.67	3.51	3.49	95.00	8.54	8.99	106.00	3.00	2.83
1:48	35.00	1.00	2.86	22.00	3.00	13.64	23.33	1.53	6.55
1:192	8.33	0.58	6.93	5.67	0.58	10.19	6.00	0.00	0.00
1:768	2.67	0.58	21.65	1.33	0.58	43.30	1.33	0.58	43.30
1:3072	0.67	0.58	86.60	0.00	0.00	UC	0.33	0.58	173.21

Table Legend
Pass (<35%CV)
Fail (>35%CV)
Unable to calculate (UC)
Used for LLoQ
Below LLoD (< 2 cells/µl)

Conclusion

The data presented in this poster highlight how our optimized TBNK assay is able to **accurately identify NK-cells from $CD56^+$ allogeneic CAR T cells**. Moreover, we showed that the assay was **validated according to the CLSI H62 guidelines for use as secondary endpoint in clinical trials**. We determined a sample stability of 120 hours and post-stain stability of 24 hours, which allows for testing at our centrals laboratories during an extended time window. Finally, we showed high sensitivity of the optimized assay with an LLoD of 2 cells/µl and an LLoQ of 6 cells/µl for $CD45^+$ lymphocytes.

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