Method validation of a flow cytometry assay for sensitive detection of CD20 CAR$^+$ T cells in peripheral blood

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Introduction

Autologous T cells, genetically modified to express a second-generation chimeric antigen receptor (CAR), are under investigation for treatment of several human hematopoietic and other malignancies. Since CAR T cell persistence after autologous T cell transfer correlates with effective disease clearance and protection from recurrence, the quantification of such cells in the blood of patients is a valuable monitoring tool. Multicolor flow cytometry offers the opportunity to analyze the presence as well as the phenotype of CAR T cells during follow-up. Importantly, such an assay should deliver reproducible, highly precise and sensitive results for reliable evaluation of CAR T cell persistence. Here we present the results obtained during validation of a flow cytometry assay developed for the detection and monitoring of CD20 CAR$^+$ T cells using a specific CD20 CAR detection reagent in combination with additional T cell and lineage markers in 8-color panels.

Methods

1 Detection and enumeration of CD20 CAR$^+$ T cells

As a test system, peripheral blood (obtained from healthy donors) with or without spiking with CD20 CAR–transduced T cells (generated by the CliniMACS Prodigy®) were used. Per sample, 600 µL of peripheral EDTA-anticoagulated blood, stored for 24 h, were lysed and resuspended in a final volume of 100 µL. As **"Negative control"**, lysed blood was used without further additions. For a **"Sample"**, lysed blood was spiked with CD20 CAR–transduced T cells. For the **"Positive control"**, lysed blood was mixed with CAR control beads (CD20 antibody–loaded MACSiBead™ Particles), which served as a reagent and gating control. All samples were stained according to a standard operating procedure with CD45RA-VioBlue®, CD4-VioGreen™, CD3-FITC, CD20 CAR detection reagent (PE), 7-AAD, dump markers (CD14-PerCP-Vio® 700, CD15-PerCP-Vio 700), CD62L-PE-Vio 770, CD45RO-APC, and CD8-APC-Vio 770. Staining was followed by a washing and centrifugation step, aspiration of the supernatant, and resuspension of cell pellets in 500 µL of PBS supplemented with 0.5% BSA and 2 mM EDTA. The flow cytometric analysis of 450 µL cell suspension was performed on a MACSQuant® Analyzer 10 by a software tool for automated acquisition and analysis (Express Mode CAR_T_Cell_Persistence_h_01). In total, 1.7 to 3.5×10^6 events were acquired per sample. Within the automated analysis, samples were gated for CD3$^+$ cells after exclusion of debris, SSC^{high} cells, 7-AAD$^+$, and dump marker–positive cells. Then CAR$^+$CD3$^+$ cells were identified in all samples (fig. 1B; "Negative control", "Sample", and "Positive control", all acquired events are shown).

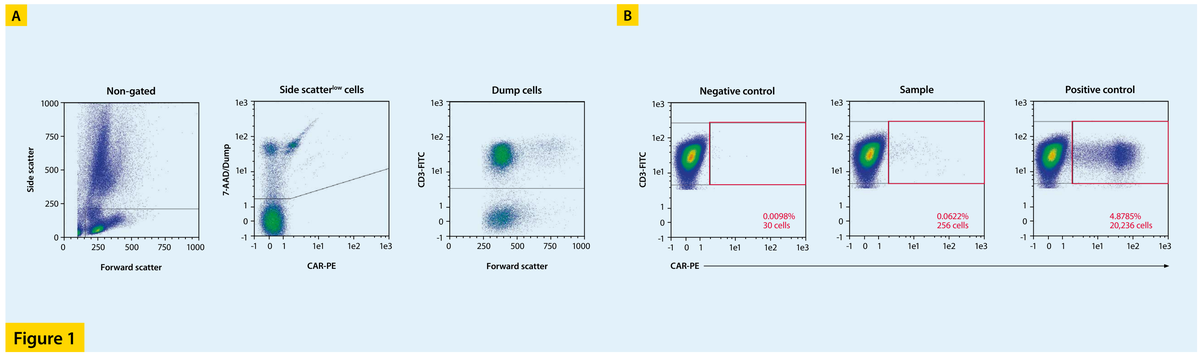
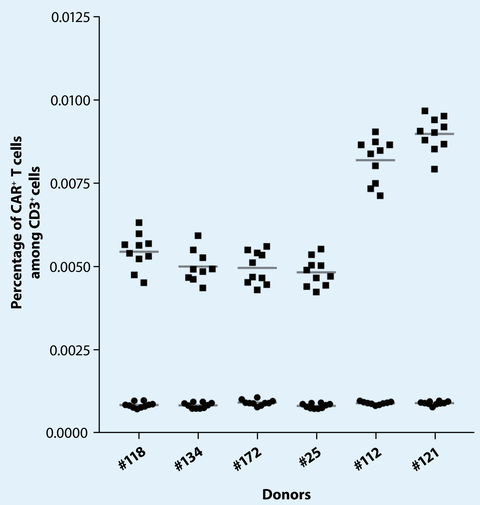


Figure 1

Results

1 Repeatability of CD20 CAR$^+$ T cell detection

Repeatability of CD20 CAR$^+$ T cell detection was determined by using EDTA-anticoagulated whole blood from six different donors for processing and analyzing ten replicates each of "Negative controls" and CD20 CAR$^+$ T cell–spiked "Samples" under the same operating conditions over a short interval of time. The mean and coefficient of variation (CV) of CAR$^+$ cells among CD3$^+$ cells were calculated for each donor. The CV of "Blank" samples ranged from 5.11% to 9.99%, whereas the CV of CAR$^+$ T cell–spiked "Samples" ranged from 5.81% to 9.88%.



	Blank (Negative control)			Sample		
	Mean	%CV	N	Mean	%CV	N
#118	0.0084	9.99	10	0.0545	9.88	10
#134	0.0083	9.97	10	0.0501	9.70	9
#172	0.0092	9.06	10	0.0496	9.80	10
#25	0.0082	8.70	10	0.0483	8.70	10
#112	0.0090	5.11	9	0.0820	8.10	10
#121	0.0090	5.98	10	0.0899	5.81	10

Figure 2

2 Analytical sensitivity of the assay

Analytical sensitivity was determined according to the CLSI guideline EP17-A2, which defines the limit of blank (LoB) as the highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte are tested.[1,2] Therefore, ten replicate "Blanks" from six different donors were analyzed with regard to overall mean (0.0087% CAR$^+$ cells among CD3$^+$ cells) and standard deviation (0.0007% CAR$^+$ cells among CD3$^+$ cells). Then, the LoB was calculated according to the formula below (A) with an α-failure of 5%, B = number of blank results (= 59) and K = number of different samples (= 6).

$$\text{(A)}\quad \text{LoB} = \text{Mean}_{blank} + c_{LoB} \times 1.645\ \text{SD}_{blank} \quad \text{with } c_{LoB} = \frac{1.645}{1-\left(\frac{1}{4\times(B-K)}\right)}$$

$$\text{(B)}\quad \text{LoD} = \text{LoB} + c_{LoD} \times 1.645\ \text{SD}_{low} \quad \text{with } c_{LoD} = \frac{1.645}{1-\left(\frac{1}{4\times(B-K)}\right)}$$

LoB was determined to be 0.0098% CAR$^+$ cells among CD3$^+$ cells (fig. 3) with a standard error (SE) of 0.0001% and a confidence interval (CI) of 0.0002%. Furthermore, the LoB of the CAR$^+$ cell count was determined to be 33.93 CAR$^+$ cells (SE: 0.41 cells; CI: 0.83 cells; data not shown).
Limit of detection (LoD) was determined by utilizing both the measured LoB and the values measured for "Samples" spiked with low concentration of analyte (i.e. CAR$^+$ T cells). Ten low-positive, spiked "Sample" replicates (six donors) were analyzed with regard to overall mean (0.0387% CAR$^+$ cells among CD3$^+$ cells) and standard deviation (0.0025% CAR$^+$ cells among CD3$^+$ cells). The LoD was calculated according to the formula above (B) with a β-failure of 5%, B = number of blank results (= 59) and K = number of different samples (= 6).
The LoD was determined to be 0.0139% CAR$^+$ cells among CD3$^+$ cells (fig. 3) with SE of 0.0010% and CI of 0.0026%. Furthermore, the LoD of the CAR$^+$ cell count was determined to be 60.38 CAR$^+$ cells (SE: 7.15 cells; CI: 19.84 cells; data not shown).

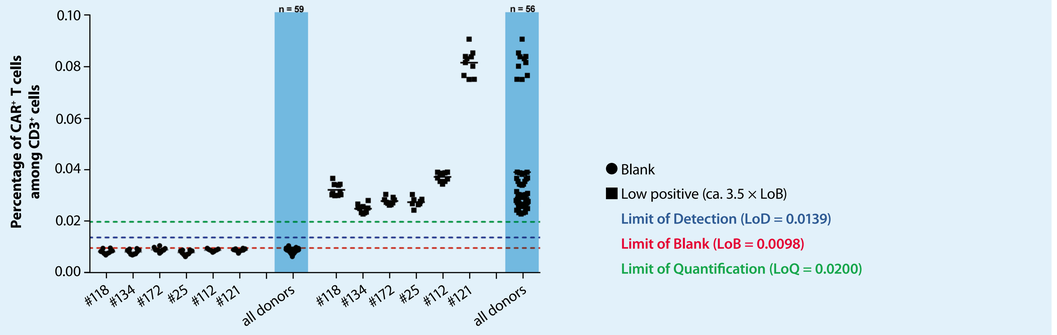


Figure 3

3 Linearity of the assay

For evaluation of assay linearity, CD20 CAR–transduced T cells were spiked into bulk-lysed, EDTA-anticoagulated whole blood from three different donors (triplicates per spike-in) to generate samples with different frequencies of CD3$^+$CAR$^+$ cells over a broad range, starting from 18.32% CAR$^+$ cells among CD3$^+$ cells down to 0.09% CAR$^+$ cells among CD3$^+$ cells. The obtained coefficient of determination for CD3$^+$CAR$^+$ cell frequency averaged at R^2 = 0.996 (range: 0.992–0.999; fig. 4A), and the coefficient of determination for CD3$^+$CAR$^+$ cell counts averaged at R^2 = 0.994 (range: 0.992–0.998; fig. 4B). The "functional sensitivity" (limit of quantification; LoQ) was estimated to be approximately equal to the LoD with its SE and CI (stated LoQs with safety range: percentage of CAR$^+$ cells among CD3$^+$ cells = 0.0200: CAR$^+$ cell count = 90).

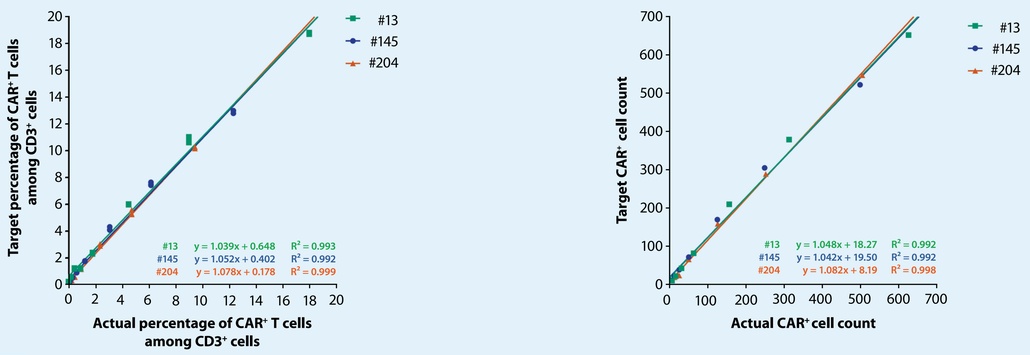


Figure 4

Conclusion

The results obtained for reproducibility and linearity of the assay, as well as determination of LoB, LoD, and LoQ demonstrate that our analytical flow cytometry method is well suited for determination of the persistence of CD20 CAR–transduced T cells in EDTA-anticoagulated whole blood.

References
- Wood, B. *et al.* (2013) Cytometry B Clin. Cytom. 84: 315–323.
- Clinical and Laboratory Standards Institute (2012) Evaluation of detection capability for clinical laboratory measurement procedures; approved guideline (second edition). CLSI document EP17-A2. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.