# Flow cytometric assays with automated data acquisition and analysis for CAR T cell manufacturing and patient immunomonitoring

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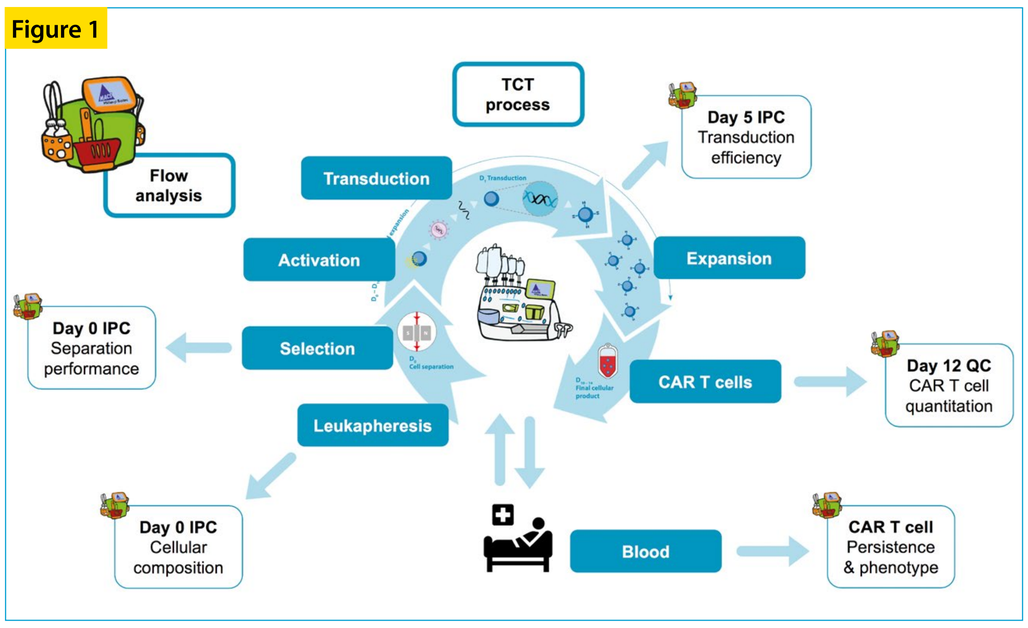
## Introduction

Unprecedented potency in B cell malignancies has been demonstrated using adoptive cell therapy with genetically engineered chimeric antigen receptor (CAR) T cells, offering new hope for curative responses in patients suffering from cancer. However, the CAR T cell manufacturing process is highly complex and has extensive demands on personnel and infrastructure, which is still a major obstacle for their routine clinical use. Using a device for automated cell processing, the CliniMACS Prodigy®, helps to overcome these hurdles, allowing generation of CAR T cells in a single automated and closed system.
For assessment of CAR T cells during the manufacturing process and for later patient immunomonitoring we provide a set of flow cytometric assays along with CAR detection reagents. These CAR detection reagents bind specifically to the antigen-recognition domain of the receptor, which helps to discriminate between various CAR constructs, allows to assess the potency of the CAR to bind to its target, and can be used to enumerate CAR T cells during manufacturing and immunomonitoring. Those detection reagents are used in our flow cytometric assays which were developed for 1) in-process control, QC release testing, and concomitant research during cell manufacturing, and 2) for determination of CAR T cell persistance and cell phenotyping during patient immunomonitoring.
For all flow assays mentioned above so-called Express Modes have been programmed, that allow an automated acquisition and analysis of stained samples on MACSQuant® Analyzers. These Express Modes feature predefined experiment settings, automate the sample measurement, and apply a fully automated gating strategy with computational gate adjustment for each individual data file. This allows for a high standardization by reducing operator variability, full reproducibility of data analysis, and integration into automated workflows.

**Figure 1**




## Methods

### 1 Flow cytometry assays for analysis of CAR T cells during cell manufacturing and immunomonitoring

**Table 1**

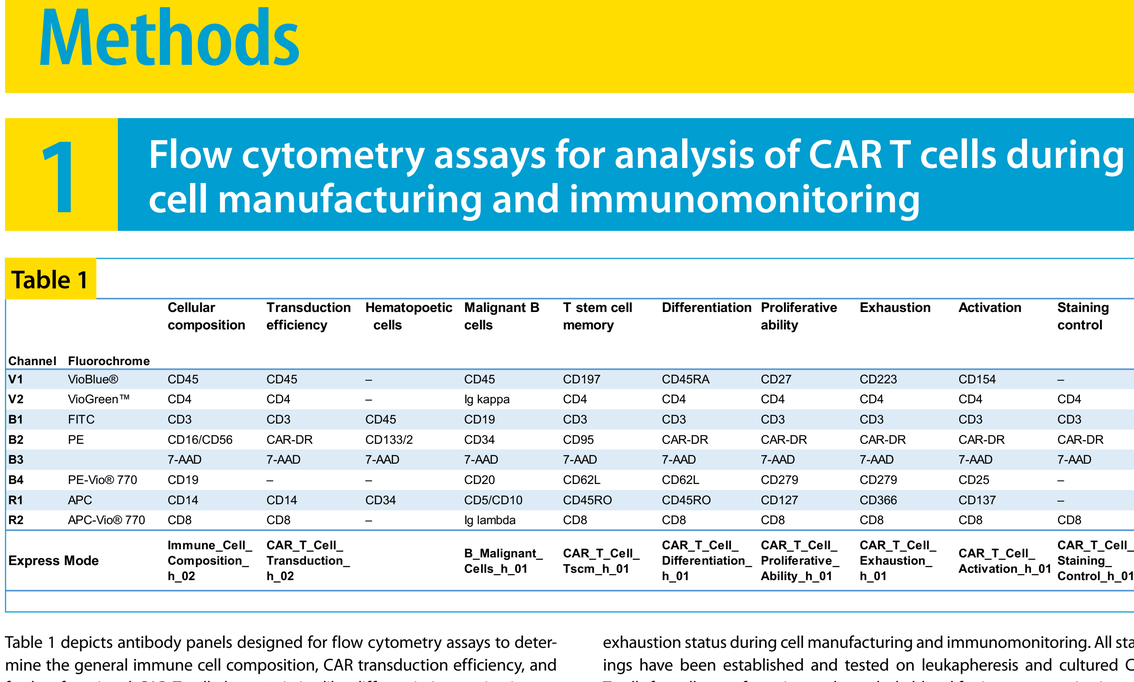
| | | Cellular composition | Transduction efficiency | Hematopoetic cells | Malignant B cells | T stem cell memory | Differentiation | Proliferative ability | Exhaustion | Activation | Staining control |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Channel** | **Fluorochrome** | | | | | | | | | | |
| **V1** | VioBlue® | CD45 | CD45 | – | CD45 | CD197 | CD45RA | CD27 | CD223 | CD154 | – |
| **V2** | VioGreen™ | CD4 | CD4 | – | Ig kappa | CD4 | CD4 | CD4 | CD4 | CD4 | CD4 |
| **B1** | FITC | CD3 | CD3 | CD45 | CD19 | CD3 | CD3 | CD3 | CD3 | CD3 | CD3 |
| **B2** | PE | CD16/CD56 | CAR-DR | CD133/2 | CD34 | CD95 | CAR-DR | CAR-DR | CAR-DR | CAR-DR | CAR-DR |
| **B3** | | 7-AAD | 7-AAD | 7-AAD | 7-AAD | 7-AAD | 7-AAD | 7-AAD | 7-AAD | 7-AAD | 7-AAD |
| **B4** | PE-Vio® 770 | CD19 | – | – | CD20 | CD62L | CD279 | CD279 | CD279 | CD25 | – |
| **R1** | APC | CD14 | CD14 | CD34 | CD5/CD10 | CD45RO | CD45RO | CD127 | CD366 | CD137 | – |
| **R2** | APC-Vio® 770 | CD8 | CD8 | – | Ig lambda | CD8 | CD8 | CD8 | CD8 | CD8 | CD8 |
| **Express Mode** | | **Immune_Cell_ Composition_ h_02** | **CAR_T_Cell_ Transduction_ h_02** | | **B_Malignant_ Cells_h_01** | **CAR_T_Cell_ Tscm_h_01** | **CAR_T_Cell_ Differentiation_ h_01** | **CAR_T_Cell_ Proliferative_ Ability_h_01** | **CAR_T_Cell_ Exhaustion_ h_01** | **CAR_T_Cell_ Activation_h_01** | **CAR_T_Cell_ Staining_ Control_h_01** |

Table 1 depicts antibody panels designed for flow cytometry assays to determine the general immune cell composition, CAR transduction efficiency, and further functional CAR T cell characteristics like differentiation, activation, or exhaustion status during cell manufacturing and immunomonitoring. All stainings have been established and tested on leukapheresis and cultured CAR T cells for cell manufacturing and on whole blood for immunomonitoring.

## Results

### 1 Enumeration of immune cell subsets in leukapheresis products

**Figure 2**



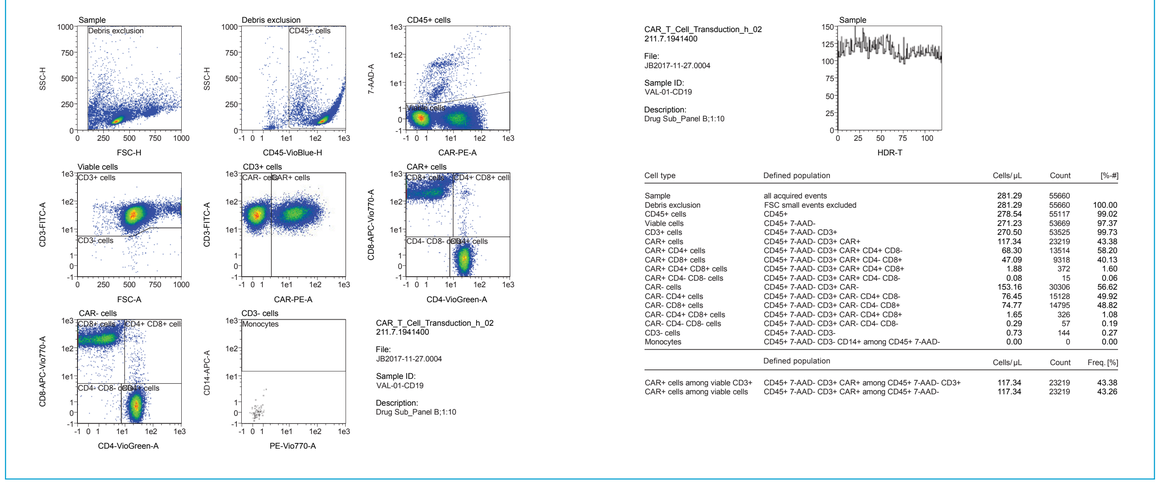

| Cell type | Defined population | Cells/µL | Count | [%] among viable CD45+ |
|---|---|---|---|---|
| Sample | all acquired events | 1887.18 | 376494 | |
| Debris exclusion | FSC small events excluded | 1887.18 | 376494 | |
| CD45+ cells | CD45+ | 1787.33 | 356575 | |
| Viable cells | CD45+ 7-AAD- | 1739.30 | 346992 | |
| CD3+ cells | CD45+ 7-AAD- CD3+ | 950.63 | 189652 | 54.66 |
| CD3- cells | CD45+ 7-AAD- CD3- | 788.67 | 157340 | 45.34 |
| T cells | CD45+ 7-AAD- CD3+ CD56- CD16- | 899.85 | 179521 | 51.74 |
| NKT cells | CD45+ 7-AAD- CD3+ CD56+ CD16+ | 50.78 | 10131 | 2.92 |
| CD4+ cells | CD45+ 7-AAD- CD3+ CD56- CD16- CD4+ CD8- | 578.33 | 115377 | 33.25 |
| CD8+ cells | CD45+ 7-AAD- CD3+ CD56- CD16- CD4- CD8+ | 284.25 | 56708 | 16.34 |
| CD4+ CD8+ cells | CD45+ 7-AAD- CD3+ CD56- CD16- CD4+ CD8+ | 5.73 | 1144 | 0.33 |
| CD4- CD8- cells | CD45+ 7-AAD- CD3+ CD56- CD16- CD4- CD8- | 31.54 | 6292 | 1.81 |
| Monocytes | CD45+ 7-AAD- CD3- CD14+ | 357.99 | 71419 | 20.58 |
| B cells | CD45+ 7-AAD- CD3- CD19+ | 110.02 | 21949 | 6.33 |
| Neutrophils | CD45+ 7-AAD- CD3- CD14- CD19- CD56+ CD16+ SSC hi | 118.49 | 23638 | 6.81 |
| Eosinophils | CD45+ 7-AAD- CD3- CD14- CD19- CD56- CD16- SSC hi | 3.47 | 693 | 0.20 |
| CD56+ CD16+ cells | CD45+ 7-AAD- CD3- CD14- CD19- CD56+ CD16+ SSC lo | 125.08 | 24954 | 7.19 |

| | Defined population | Cells/µl | Count | [%-#] |
|---|---|---|---|---|
| Viability of CD3+ cells | CD45+ CD3+ 7-AAD- among CD45+ CD3+ | 950.63 | 189652 | 97.99 |
| % CD3+ in viable CD45+ | CD45+ 7-AAD- CD3+ among CD45+ 7-AAD- | 950.63 | 189652 | 54.66 |

Staining of cells from a leukapheresis product with flow panel "Cellular composition" and automated acquisition and analysis using Express Mode "Immune_Cell_Composition_h_02" allowed quantification of various cellular subsets in the starting material (fig. 2). Using the same panel on the final cellular product allowed assessment of cellular impurities and quantification of T cells.

### 2 Assessment of transduction efficiency and viability of CAR T cells

**Figure 3**



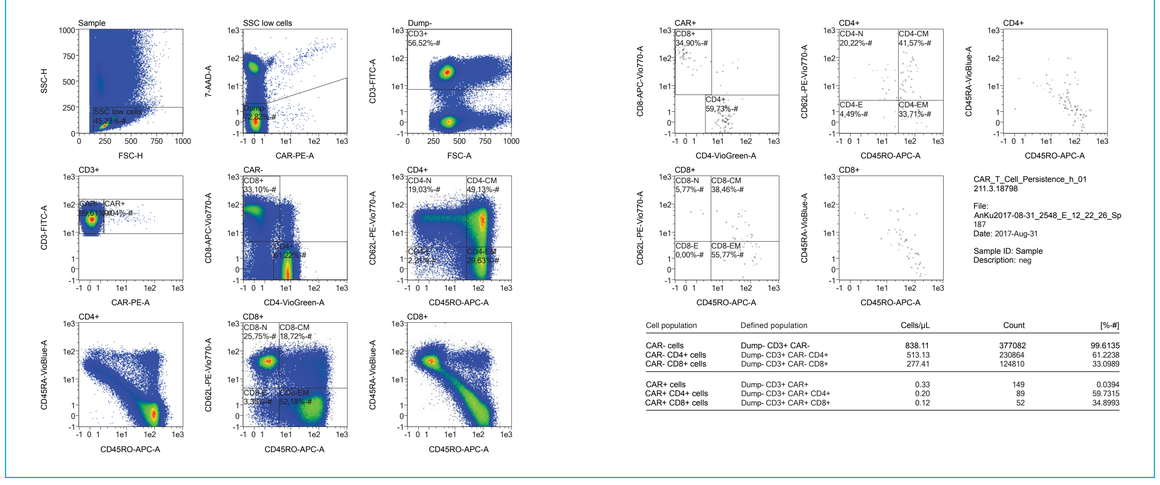

| Cell type | Defined population | Cells/µL | Count | [%-#] |
|---|---|---|---|---|
| Sample | all acquired events | 281.29 | 55660 | |
| Debris exclusion | FSC small events excluded | 281.29 | 55660 | 100.00 |
| CD45+ cells | CD45+ | 278.54 | 55117 | 99.02 |
| Viable cells | CD45+ 7-AAD- | 271.23 | 53669 | 97.37 |
| CD3+ cells | CD45+ 7-AAD- CD3+ | 270.50 | 53525 | 99.73 |
| CAR+ cells | CD45+ 7-AAD- CD3+ CAR+ | 117.34 | 23219 | 43.38 |
| CAR+ CD4+ cells | CD45+ 7-AAD- CD3+ CAR+ CD4+ CD8- | 68.30 | 13514 | 58.20 |
| CAR+ CD8+ cells | CD45+ 7-AAD- CD3+ CAR+ CD4- CD8+ | 47.09 | 9318 | 40.13 |
| CAR+ CD4+ CD8+ cells | CD45+ 7-AAD- CD3+ CAR+ CD4+ CD8+ | 1.88 | 372 | 1.60 |
| CAR+ CD4- CD8- cells | CD45+ 7-AAD- CD3+ CAR+ CD4- CD8- | 0.08 | 15 | 0.06 |
| CAR- cells | CD45+ 7-AAD- CD3+ CAR- | 153.16 | 30306 | 56.62 |
| CAR- CD4+ cells | CD45+ 7-AAD- CD3+ CAR- CD4+ CD8- | 76.45 | 15128 | 49.92 |
| CAR- CD8+ cells | CD45+ 7-AAD- CD3+ CAR- CD4- CD8+ | 74.77 | 14795 | 48.82 |
| CAR- CD4+ CD8+ cells | CD45+ 7-AAD- CD3+ CAR- CD4+ CD8+ | 1.65 | 326 | 1.08 |
| CAR- CD4- CD8- cells | CD45+ 7-AAD- CD3+ CAR- CD4- CD8- | 0.29 | 57 | 0.19 |
| CD3- cells | CD45+ 7-AAD- CD3- | 0.73 | 144 | 0.27 |
| Monocytes | CD45+ 7-AAD- CD3- CD14+ among CD45+ 7-AAD- | 0.00 | 0 | 0.00 |

| | Defined population | Cells/µL | Count | Freq.[%] |
|---|---|---|---|---|
| CAR+ cells among viable CD3+ | CD45+ 7-AAD- CD3+ CAR+ among CD45+ 7-AAD- CD3+ | 117.34 | 23219 | 43.38 |
| CAR+ cells among viable cells | CD45+ 7-AAD- CD3+ CAR+ among CD45+ 7-AAD- | 117.34 | 23219 | 43.26 |

Staining of CAR T cells from day-12 cultures with flow panel "Transduction efficiency" and automated acquisition and analysis using Express Mode "CAR_T_Cell_Transduction_h_02" enabled the determination of CAR T cell transduction efficiency and viability (fig. 3).

### 3 Analysis of CAR T cell persistence and differentiation during immunomonitoring

**Figure 4**

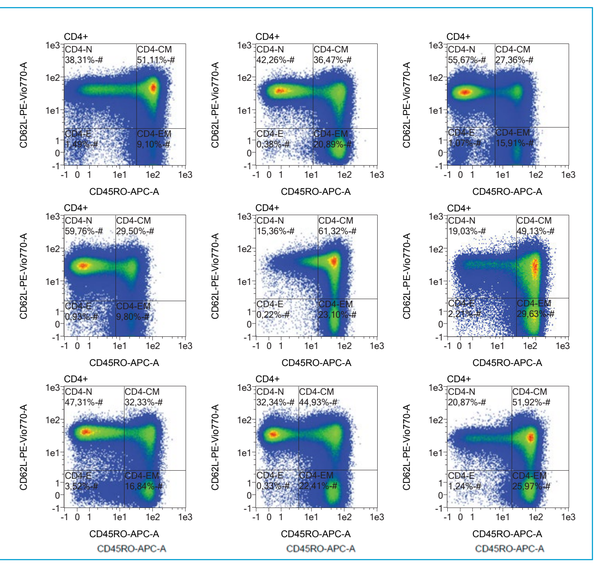



| Cell population | Defined population | Cells/µL | Count | [%-#] |
|---|---|---|---|---|
| CAR- cells | Dump- CD3+ CAR- | 838.11 | 377082 | 99.6135 |
| CAR- CD4+ cells | Dump- CD3+ CAR- CD4+ | 513.13 | 230864 | 61.2238 |
| CAR- CD8+ cells | Dump- CD3+ CAR- CD8+ | 277.41 | 124810 | 33.0989 |
| CAR+ cells | Dump- CD3+ CAR+ | 0.33 | 149 | 0.0394 |
| CAR+ CD4+ cells | Dump- CD3+ CAR+ CD4+ | 0.20 | 89 | 59.7315 |
| CAR+ CD8+ cells | Dump- CD3+ CAR+ CD8+ | 0.12 | 52 | 34.8993 |

Staining of CAR T cells, spiked into whole blood, with flow panel "Persistence" and automated acquisition and analysis using Express Mode "CAR_T_Cell_Persistence_h_01" was used as a model for the analysis of CAR T cell persistence and differentiation after adoptive transfer during immunomonitoring (fig. 4).

### 4 Evaluation of donor-dependent variations and automated Express Mode gating

**Figure 5**




**Full automation of flow data acquisition and analysis on the MACSQuant Analyzer 10**

- Predefined experiment settings
- Automated sample measurement
- Computational gate adjustment
- Full statistics and data export

Express Modes

**Safety**: Human error is reduced as much as possible

**Convenience**: Automation of machine set-up, acquisition, and data analysis

**Standardization**: No operator variability due to lack of manual intervention

**Reproducibility**: Flow analysis is identical independent of the user

**Integration**: Can be incorporated in automated workflows

**Data handling**: Automated export of flow statistics after analysis

**Documentation friendly**: Quick to integrate into SOPs for robust analysis

Figure 5 shows example plots depicting T cell differentiation in leukapheresis products from various donors, processed on different days and run on different MACSQuant Instruments to evaluate sample-to-sample variations. The fully automated gating procedure of the Express Mode adapts to each data file via pre-defined algorithms, which yields a high level of standardization across samples, lacks operator variability, and ensures full reproducibility.

## Conclusion

Elaborate flow assays specifically designed for CAR T cells, run with specific CAR detection reagents, high-quality recombinant antibodies, and fully automated flow analysis, provide a robust assessment of cell manufacturing and patient immunomonitoring. This will help with establishing complex individualized therapies and will enable us to understand in greater detail the phenotypic changes occuring throughout the life time of a CAR T cell.