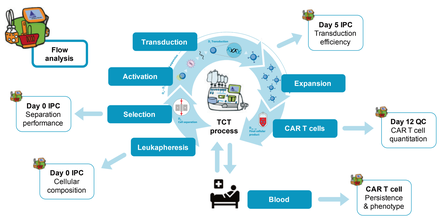
Flow cytometric assays for CAR T cell manufacturing and patient monitoring, involving specific CAR detection reagents, stabilized pre-formulated cocktails, and automated data acquisition and analysis

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Introduction

Unprecedented efficacy in targeting cancer has been demonstrated with CAR T cell therapy. However, the CAR T cell manufacturing process is still highly complex and has extensive demands on personnel and infrastructure. 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. However, also the necessary QC procedures and any accompanying CAR T cell phenotyping efforts require robust and reliable assays. Therefore, to streamline the assessment of CAR T cells during manufacturing and patient monitoring, we aimed to establish a set of flow cytometric assays prepared from recombinant antibodies along with CAR detection reagents. These assays were complemented by stabilized pre-formulated cocktails and additional tools for automated data acquisition and analysis, to achieve the highest level of standardization, reproducibility, and convenience. Our flow cytometric assays were developed for 1) in-process control, QC release testing, and CAR T cell phenotyping during cell manufacturing, and 2) for determination of CAR T cell persistance and phenotyping during patient immunomonitoring (Fig. 1). Currently already used in clinical trials, these assays allow to determine the general immune cell composition, the CAR transduction efficiency, and functional CAR T cell phenotyping like differentiation, activation, or exhaustion status. To further improve assay standardization, to simplify and speed up the IPC/QC process, and to reduce risks from manual handling, we developed stabilized pre-formulated antibody cocktails for these flow assays, featured in a single-tube format. In addition, so-called Express Modes were programmed that allow an automated sample acquisition and analysis 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.

Figure 1



Results

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

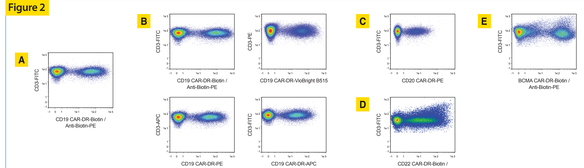
Table 1

Channel	Fluorochrome	Cellular composition	Transduction efficiency	Hematopoietic cells	Malignant B cells	T stem cell memory	Differentiation / Persistence	Proliferative ability	Exhaustion	Activation	Staining control
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	CD56/CD66	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	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		**CART_Immune_Composition_h_03**	**CART_Transduction_h_03**		**B_Malignant_Cells_h_01**	**CART_Tscm_h_03**	**CART_Differentiation_/ Persistence_h_03**	**CART_Proliferative_Ability_h_03**	**CART_Exhaustion_h_03**	**CART_Activation_h_03**	**CART_Staining_Control_h_03**

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.

2 CAR Detection Reagents

Figure 2



Example plots of CAR T cells stained with various CAR detection reagents, based on CD19 protein (Fig. 2a), a CD19 CAR FMC63 idiotype antibody (REA1297) conjugated to Biotin and various dyes (Fig. 2b), on CD20 protein (Fig. 2c), CD22 protein (Fig. 2d), or on BCMA protein (Fig. 2e).

3 Evaluation of donor-dependent variations and automated Express Mode gating

Figure 3



Full automation of flow data acquisition and analysis on the MACS Quant Analyzer

Predefined instrument settings

Automated sample measurement

Computational gate adjustment

Full statistics and data export

Safety: Human error 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 into automatic workflows

Data handling: Automated export of flow statistics after analysis

Documentation friendly: Quick to integrate into SOPs for robust analysis

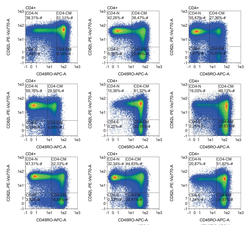
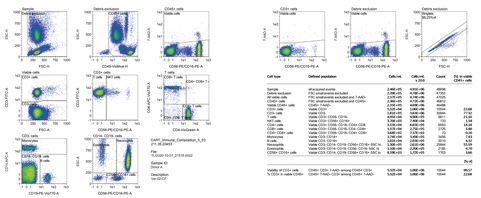


Figure 3 shows example plots depicting T cell differentiation in leukapheresis products from various donors, processed on different days and run on different MACSQuant Instruments to evaluate sample-to-sample variations. The fully automated gating procedure of the Express Mode adapts to each data file via pre-defined algorithms, which yields a high level of standardization across samples, lacks operator variability, and ensures full reproducibility.

4 Enumeration of immune cell subsets in leukapheresis products

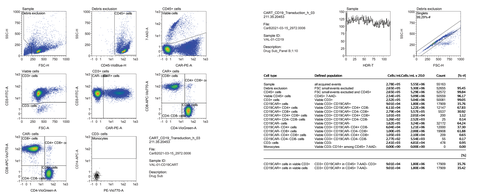
Figure 4



Staining of cells from whole blood with flow panel "Cellular composition" and automated acquisition and analysis using Express Mode "CART_Immune_Composition_h_03" allowed quantification of various cellular subsets in the starting material (Fig. 4). Using the same panel on the final cellular product allowed assessment of cellular impurities and quantification of T cells.

5 Assessment of transduction efficiency and viability of CAR T cells

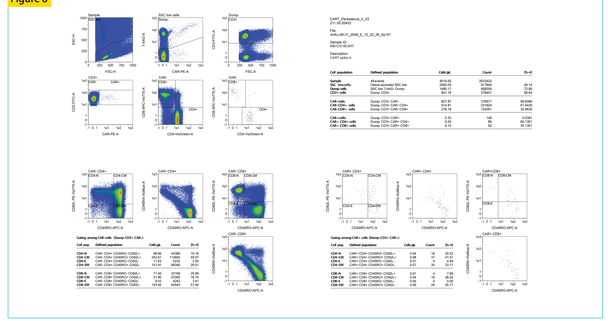
Figure 5



Staining of CAR T cells from day-12 cultures with flow panel "Transduction efficiency" and automated acquisition and analysis using Express Mode "CART_Transduction_h_03" enabled the determination of CAR T cell transduction efficiency and viability (Fig. 5).

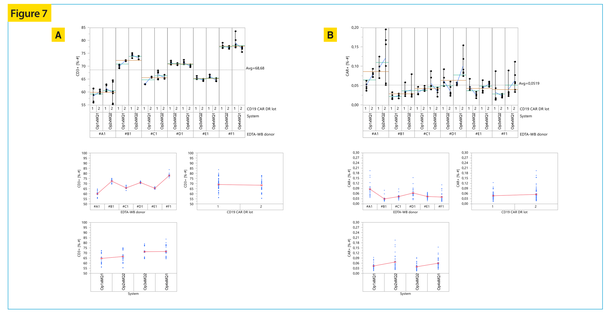
6 Analysis of CAR T cell persistence and differentiation during immunomonitoring

Figure 6



Staining of CAR T cells spiked into whole blood with panel "Persistence" and acquisition and analysis using Express Mode "CART_Persistence_h_03" was used as a model for the analysis of CAR T cell persistence and differentiation after adoptive transfer during immunomonitoring (Fig. 6).

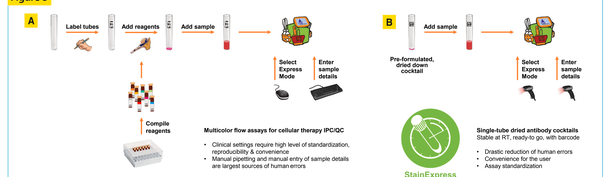
Figure 7



Partition of variation analysis for CD3 cell percentage (Fig. 7a) and CAR frequency (Fig. 7b) on whole blood from six healthy donors (without any CAR T cells spiked in) stained with panel "Persistence", measured by several operators with two different lots of CD19 CAR detection reagent.

7 Dried antibody cocktails for assay standardization and workflow simplification

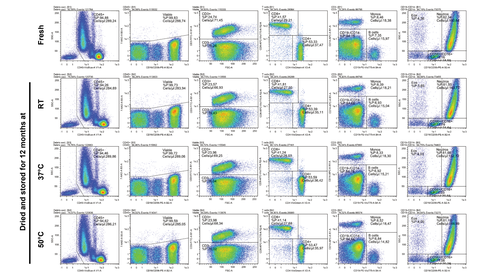
Figure 8



Workflow for manual setting up of flow assay staining cocktails, selection of corresponding Express Modes, and entering sample details (Fig. 8a), and simplification of this workflow by using pre-formulated and dried down StainExpress antibody cocktails along with barcoded sample tubes to allow automation of Express Mode selection and specification of sample details (Fig. 8b).

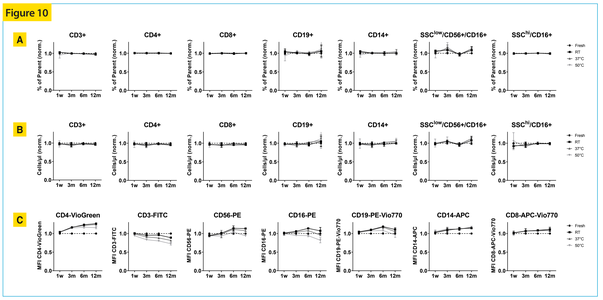
8 Performance evaluation and stability testing of dried antibody cocktails

Figure 9



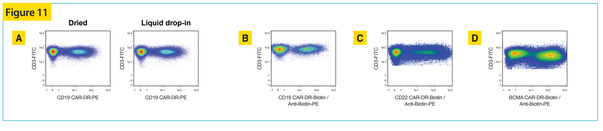
Dried antibody cocktails of flow panel "Cellular composition" were stored for 12 months at room temperature (RT), 37°C, or 50°C, and analyzed in parallel to liquid reference reagents ("fresh") in order to compare population distributions, cell percentages and counts, and fluorescent intensities of the dyes used (Fig. 9).

Figure 10



Dried antibody cocktails were stored for up to 12 months at room temperature (RT), 37°C, or 50°C, and repeatedly compared to liquid reference reagents ("fresh"). Values for percent of parent (Fig. 10a), cell count (Fig. 10b), and MFI (Fig. 10c) are displayed normalized to the liquid reference reagents. An increase in MFI probably indicates a decrease in performance of the liquid reagent, stored for the same time frame in the fridge, rather than an increase in performance of the dried reagent.

Figure 11



Example plots of CAR T cells stained with various CAR detection reagents within dried antibody cocktails of flow panel "Transduction efficiency", based on a CD19 CAR FMC63 idiotype antibody (REA1297) conjugated to PE dried down along with the cocktail or as a liquid drop-in (Fig. 11a), or based on CD19 protein (Fig. 11b), CD22 protein (Fig. 11c), or on BCMA protein (Fig. 11d), all of which were used as a liquid drop-in.

Conclusions

Elaborate flow assays specifically designed for CAR T cells, run with specific CAR detection reagents, high-quality recombinant antibodies, provided in a highly standardized dried-down state, along with 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.

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