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Important note

The present protocols are research protocols, based on laboratory experience. Miltenyi Biotec cannot and will not accept any liability as to the outcome of procedures. The procedures are for research use only, not for clinical use.

1. Description

1.1 Background

The CliniMACS Prodigy enables the automated production of chimeric antigen receptor (CAR) T cells. During the process, cell samples are collected and monitored by flow cytometry for quantity and composition of immune cell populations and presence of CAR T cells. The MACSQuant® Analyzer 10, MACSQuant X, and MACSQuant Analyzer 16 in conjunction with the CAR T Cell Express Mode Package can be used for automated analysis of immune cell composition and for detection of CAR T cells at all stages required during the CAR T cell manufacturing process. The number and frequencies of cell populations can be determined from the flow cytometric statistics generated at the end of the CAR T Cell Express Mode analysis. This protocol describes the preparation of samples, setup of the instrument, and analysis of data using the CAR T Cell Express Mode Package.

1.2 Reagent and instrument requirements

- MACSQuant Analyzer 10 (# 130-096-343), MACSQuant X (# 130-105-100), or MACSQuant Analyzer 16 (# 130-109-803) installed with a compatible MACSQuantify™ Software version:
 - For MACSQuant Analyzer 10: The CAR T Cell Express Mode Package (# 160-002-376) compatible with MACSQuantify Software version 2.11.5 and higher.
 - For MACSQuant X: The CAR T cell Express Mode Package (# 160-002-376) is only compatible with MACSQuantify Software versions 2.11.5, 2.13.0, and 2.13.2. MACSQuant X is not compatible with software versions 2.13.1 and 2.13.3.
 - For MACSQuant Analyzer 16: The CAR T Cell Express Mode Package (# 160-002-376) is compatible with MACSQuantify Software version 2.13.3.
- ▲ **Note:** It is recommended to verify the compatibility of the MACSQuantify Software and the respective CAR T Cell Express Mode Package.
- MACS® MiniSampler Plus (# 130-105-745) and Chill 5 Rack (# 130-092-951).
 - ▲ **Note:** The MACS MiniSampler Plus in combination with the Chill 5 Rack will be necessary if not using the Single Tube Rack.
- Refrigerator.
- Centrifuge suitable for 12×75 mm tubes with cap.
- Vortex mixer.
- Micropipettes with disposable tips.
- 50 mL conical tubes.
- PBS/EDTA buffer, e.g., autoMACS® Rinsing Solution (# 130-091-222).
- BSA, e.g., MACS BSA Stock Solution (# 130-091-376).
- Red Blood Cell Lysis Solution (10×) (# 130-094-183).
 - ▲ **Note:** Red blood cell lysis should only be necessary for the analysis of starting material.
- Double-distilled water (ddH₂O).
- Inside Fix, a component of the Inside Stain Kit (# 130-090-477).
 - ▲ **Note:** Fixation is mandatory for samples to be handled under specific laboratory safety requirements. Please follow local regulations.
- Antibodies and reagents as listed below in tables 1 and 2.

Product	Order no.
StainExpress Immune Cell Composition Cocktail, human	130-127-637
StainExpress CAR T Transduction Cocktail, human	130-127-638

Table 1: StainExpress Cocktails required for flow cytometric analysis of immune cell composition and CAR T transduction (CAR T T) efficiency. For each of these reagents use the volume, incubation time, and dilution indicated in the respective data sheet.

Product	Order no.
CD19 CAR FMC63 Idiotype Antibody, PE, REAfinity™	130-127-342
CD19 CAR FMC63 Idiotype Antibody, Biotin, REAfinity*	130-127-345
CD22 CAR Detection Reagent, human, Biotin*	130-126-727
BCMA CAR Detection Reagent, human, Biotin*	130-126-090
CD19 CAR Detection Reagent, human, Biotin*	130-129-550
CD33 CAR Detection Reagent, human, Biotin*	130-127-642

Table 2: Recommendation of antibodies and CAR Detection Reagents to be added to the StainExpress CAR T Transduction Cocktail. Choose one of the above-mentioned CAR Detection Reagents or refer to www.miltenyibiotec.com for additional CAR Detection Reagent options. For each of these reagents use the volume, incubation time, and dilution indicated in the respective data sheet.

*Use Biotin Antibody, PE, REAfinity (# 130-110-951) in combination with these reagents.

1.3 Preparation of solutions

▲ Solutions should be prepared freshly. Discard unused solutions at the end of the day.

- 1× Red Blood Cell Lysis Solution
Prepare 2 mL of 1× Red Blood Cell Lysis Solution by diluting Red Blood Cell Lysis Solution (10×) 1:10 with ddH₂O, e.g., dilute 200 µL Red Blood Cell Lysis Solution (10×) with 1.8 mL of ddH₂O and mix well. Adjust for multiple samples accordingly. Store and use at room temperature (19–25 °C).
- Buffer: Prepare PBS/EDTA buffer supplemented with 0.5% BSA by diluting MACS® BSA Stock Solution 1:20 with, e.g., autoMACS Rinsing Solution. Store at 2–8 °C.

1.4 Compensation

▲ For optimal compensation refer to the data sheets of the StainExpress Immune Cell Composition Cocktail, human (# 130-127-637) and the StainExpress CAR T Transduction Cocktail, human (# 130-127-638).

▲ **Note:** For optimal compensation always use the provided StainExpress Compensation Set matching the StainExpress Cocktails used. To ensure they match, check for identical order number-related lot numbers indicated on the pouches next to the expiration date.

2. Staining protocol

2.1 Sample preparation

1. Collect the respective samples for the analysis of immune cell composition (ICC) and CAR T transduction (CAR T T) efficiency as described in table 3. If desired, an additional in-process control (IPC) can be performed between day 9 and day 11. CliniMACS Prodigy Process Samples are obtained using the sampling adapter. A minimum of 100 µL sample volume with a maximal concentration of 1×10⁷ cells/mL is required for each analysis.

▲ **Note:** If the concentration is higher, dilute the cell suspension with buffer and keep the respective dilution factor in mind. It will be needed for cell count calculations at the end of the analysis.

Sampling date	Sample type	Analysis
Day 0	Starting material and enriched T cells	ICC
Day 5	CliniMACS Prodigy sample	ICC, CAR T T
Day 9-11 (optional)	CliniMACS Prodigy sample	ICC, CAR T T
Day 12	CliniMACS Prodigy sample and final cell product	ICC, CAR T T

Table 3: Exemplary sampling date, sample type, and recommended analysis.

2. Label the required number of tubes with the panel (ICC or CAR T T) and a suitable sample ID.

2.2 Labeling of cells with StainExpress Cocktails

2.2.1 Staining protocol for immune cell composition

▲ To avoid loss of cells and changes in cell composition, the ICC panel should be applied as a 'no-wash protocol'.

1. Add 100 µL of cell suspension to one tube of StainExpress Immune Cell Composition Cocktail, human.
2. Incubate for 10–20 seconds, then vortex at high speed for 5 seconds and incubate for 10 minutes in the dark at 19–25 °C.
▲ **Note:** Working at lower temperatures requires increased incubation times.
3. If the sample does not require red blood cell lysis or fixation, add 1900 µL of buffer for analysis by flow cytometry.

or

If the sample does require red blood cell lysis but no fixation, add 1900 µL of 1× Red Blood Cell Lysis Solution and immediately vortex thoroughly for 3 seconds. Incubate for 15 minutes in the dark at 19–25 °C.

or

If the sample requires fixation, follow the user instruction of an appropriate fixative reagent, e.g. Inside Fix, a component of the Inside Stain Kit. In case Inside Fix is used, it is recommended to adjust the sample volume to 250 µL by adding 150 µL of buffer prior to adding 250 µL of Inside Fix. Mix well and incubate for 20 min at room temperature in the dark. Add 1500 µL of buffer for analysis.

▲ **Note:** Fixation is mandatory for samples to be handled under specific laboratory safety regulations. Please follow local regulations.

▲ **Note:** Do not wash the sample after fixation. Adjust the volume to 2 mL using buffer.

4. Store the samples at 2–8 °C protected from light until acquisition on the MACSQuant Analyzer. Acquire samples within one hour after staining.

2.2.2 Staining protocol for CAR T transduction efficiency

1. Add 100 µL of cell suspension to one tube of StainExpress CAR T Transduction Cocktail, human.
2. Add suitable amount of CAR Detection Reagent. Refer to the respective data sheet for more information.
3. Incubate for 10–20 seconds, then vortex at high speed for 5 seconds and incubate for 10 minutes in the dark at 19–25 °C.
▲ **Note:** Working at lower temperatures requires increased incubation times.
4. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
5. (Optional) If using a biotin-conjugated CAR Detection Reagent, follow steps 5 a–c:
 - a. Resuspend cells in 98 µL of buffer and add 2 µL of Biotin Antibody, PE, REAfinity.
 - b. Vortex shortly at high speed and incubate for 10 minutes in the dark at 19–25 °C.
 - c. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
6. If the sample does not require fixation, resuspend cell pellet in 2 mL of buffer for analysis by flow cytometry.

▲ **Note:** It is recommended to resuspend the cells in a smaller volume first (e.g. 200 µL) and then to fill up to 2 mL.

or

If the sample requires fixation, follow the user instruction of an appropriate fixative reagent, e.g., Inside Fix, a component of the Inside Stain Kit. In case Inside Fix is used, it is suggested to adjust the sample volume to 250 µL with buffer prior to adding 250 µL of Inside Fix. Mix well and incubate for 20 min at room temperature in the dark. Add 1500 µL of buffer for analysis.

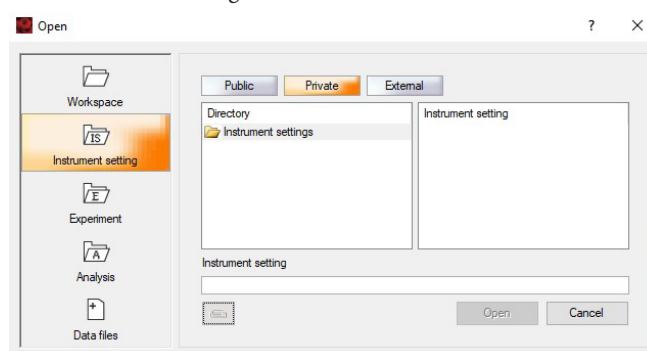
7. Store samples at 2–8 °C protected from light until analysis. Acquire samples within one hour after staining.

3. Example of a flow cytometric data acquisition and analysis using Express Modes

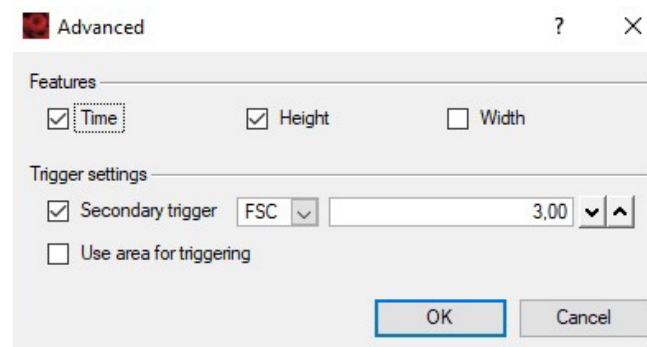
3.1 Data acquisition using Express Modes

The MACSQuant Analyzers in conjunction with the respective CAR T Cell Express Mode Package provide predefined experiment settings for automated acquisition and analysis. For accurate data acquisition, ensure that the instrument has been set up appropriately by performing PMT calibration and compensation by measurement of the respective compensation controls. After setup, do not change any instrument settings during acquisition of one experiment series. A detailed protocol for the usage of the Express Modes **Immune_Cell_Composition_h_02** or **CART_Immune_Composition_h_03** and **CAR_T_Cell_Transduction_h_02** or **CART_Transduction_h_03** is described in the following section. The protocol can be adapted for manual experiment setup and data analysis accordingly.

1. Click on the **Open icon** to select and open the current valid instrument settings.



2. Go to the **Channels** tab and click on the **Advanced** button to verify that **Height** is switched on.



3. Go to the Experiment tab.

▲ **Note:** For manual setup of the experiment, refer to the figure below, select the respective settings, and annotate the channels by hand.

a. Depending on the sample number, select the **Single Tube Rack** or the **Chill 5 Rack** from the **Rack drop-down menu**.

b. If the Chill 5 Rack is used, select the appropriate number of sample positions (indicated by closed green circles) and verify that all samples are selected for editing (indicated by the orange rim). For colorblind users, the settings can be adjusted to closed grey circles and orange rims.

c. Select the respective Express Mode:

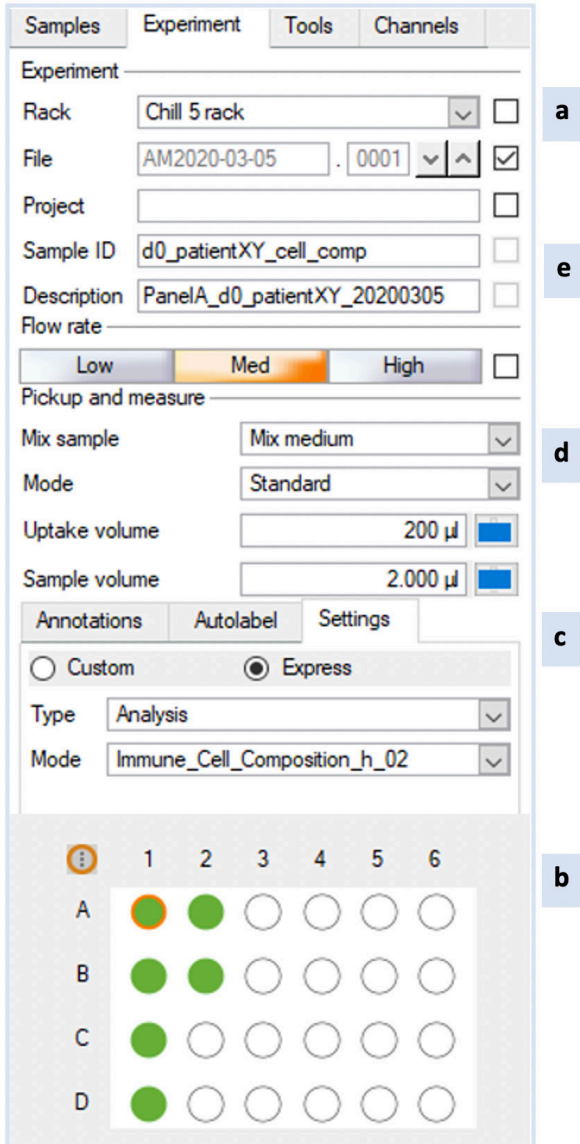
(**Immune_Cell_Composition_h_02** or **CART_Immune_Composition_h_03** or **CAR_T_Cell_Transduction_h_02**

or **CART_Transduction_h_03**, respectively) from the dropdown list within the **Settings tab**. All pre-defined experiment settings (flow rate, annotations, sample, and uptake volume) are loaded automatically, except for the sample mixing which has to be selected manually.

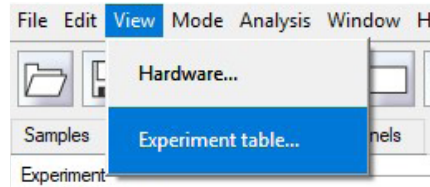
d. Select an appropriate sample mixing (e.g. Mix medium).

▲ **Note:** If no sample mixing is chosen, the cells might sediment before sample uptake. Sedimentation will influence the results.

e. Click on a single well and enter relevant sample information in the **Sample ID** and **Description** field. Proceed with the next well and continue for all wells to be measured.



4. Go to **View** → **Experiment table** to verify that annotations and settings for all samples have been entered correctly.



5. Mix your samples by vortexing and place them in the respective position in the Chill 5 Rack. Further check for sufficient amount of buffers and ensure that the waste bottle is empty.

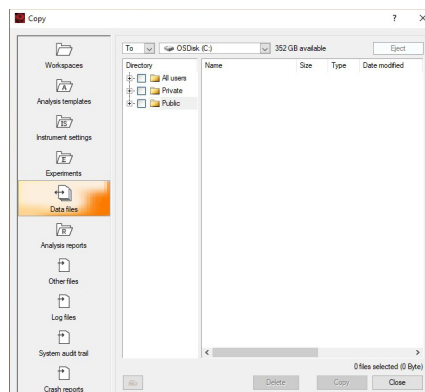
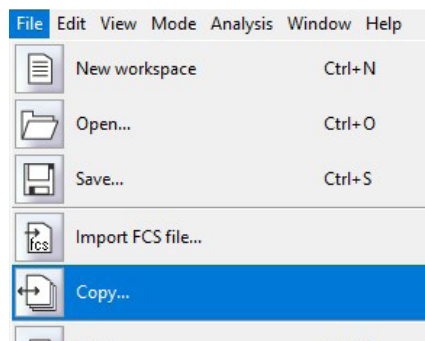
▲ **Note:** Although sample mixing has been selected on the MACSQuant Analyzer 10, MACSQuant X or MACSQuant Analyzer 16, it is recommended to vortex samples if multiple samples will be measured.

6. Start the acquisition.

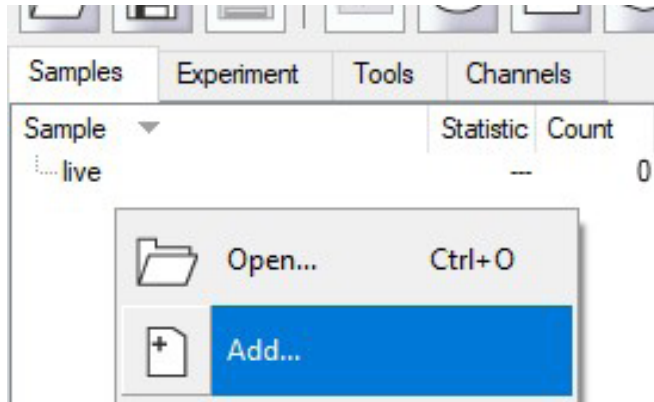
3.2 Data analysis using Express Modes

▲ If the Single Tube Rack is used, the analysis is automatically performed directly after acquisition. If the Chill 5 Rack is used, the analysis is immediately replaced by the subsequent sample for acquisition and can be done afterwards. However, it is recommended to use a separate PC (equipped with the same MACSQuantify Software version and CAR T Cell Express Mode Package) for data analysis and backing up the data files, since export of data to Microsoft Excel is only possible when analysis is performed on a separate PC. If data analysis has to be performed on the MACSQuant Analyzer itself, analysis should be started after finishing acquisition.

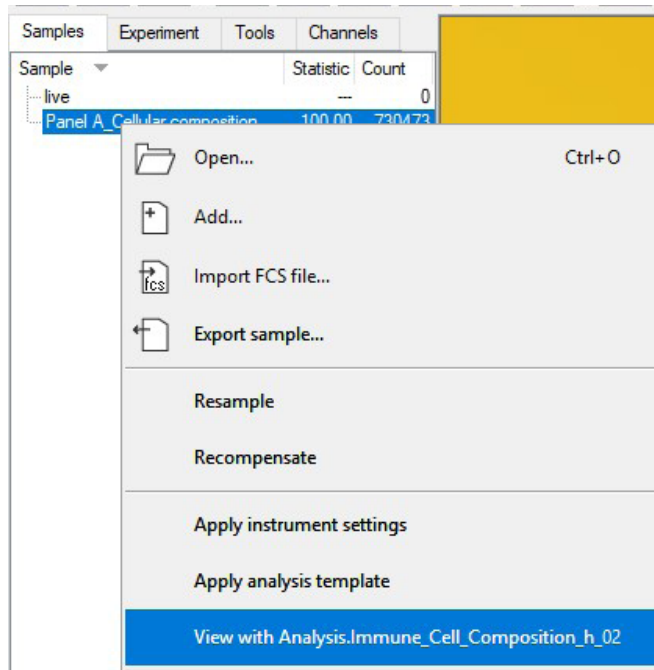
1. After acquisition, data can be copied to the analysis PC. Go to **File** → **Copy** and select the respective data files and directory for copying.



- On the analysis PC, right click within the **Samples** tab and select **Add...** to upload data files to the MACSQuantify Software.



- Right click on the first data file and select **View with Analysis.Immune_Cell_Composition_h_02** or **View with Analysis.CART_Immune_Composition_h_03** or **View with Analysis.CAR_T_Cell_Transduction_h_02** or **View with Analysis.CART_Transduction_h_03**, respectively, for accessing the Express Mode analysis.

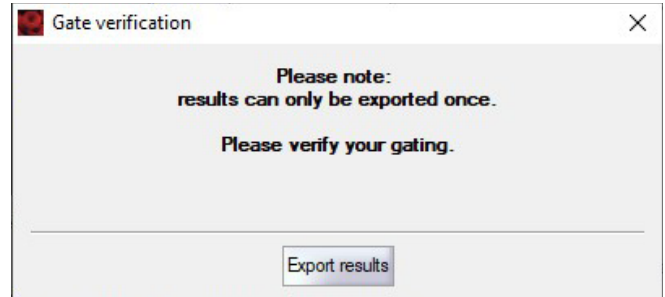


- The appropriate analysis pages will be displayed after analysis has been finished (see figure 3 and figure 4). During this step, the gates are created and individually adjusted to the selected sample.

▲ **Note:** Please be aware that depending on the Express Mode version it might be necessary to enter the total dilution factor (refer to section 4) of the sample and to choose the display unit of the cell concentration (cells/mL vs. cells/μL).

- If necessary, adjust the gates manually by moving the **Gate verification window** to the side before selecting **Export results**. Do not close this window or select Export results before adjusting the gates.

▲ **Note:** Export of data can only be done once within the automated Express Mode analysis. Make sure all regions are set properly before exporting the data. If adjustments are necessary after the results have been exported, start the analysis again and perform manual adjustments to the gates before selecting Export results. The Gate verification window must be closed before commencing a new Express Mode analysis. When the analysis is performed on the MACSQuant Analyzer itself, the Gate verification window is not shown and therefore data export cannot be performed.



- By selecting **Export results**, the Express Mode software version, the file name, sample ID, description, and statistics are exported to an Excel file ('data file name_results') and saved in the same directory as the data file.
- To print the analysis, go to **File → Print**.
- Accomplish the analysis, gate adjustment, and export of results for the remaining data files.

▲ **Note:** For manual analysis, refer to the gating hierarchies and plots in figures 1–4 and perform the gating and export of results by hand.

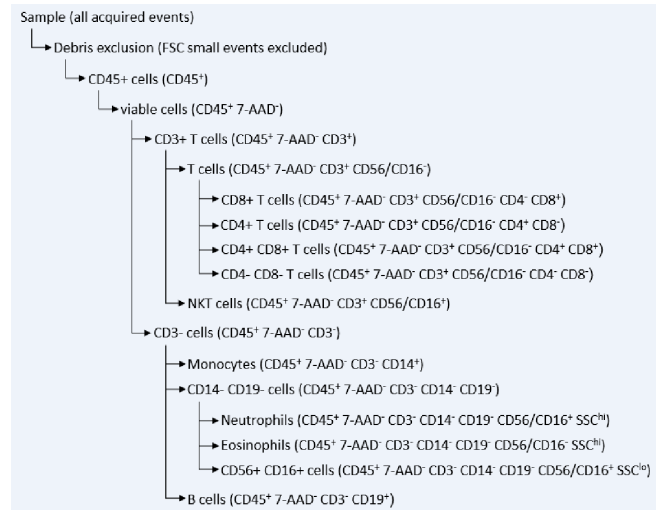


Figure 1: Hierarchical gating strategy for the ICC analysis. Subtypes of T cells (CD4 and/or CD8), as well as NKT cells, monocytes, neutrophils, eosinophils, and B cells can be determined based on the marker expression indicated in brackets.

9. As shown in figure 1 and figure 3, subtypes of T cells (CD4 and/or CD8), NKT cells, monocytes, neutrophils, eosinophils, NK cells, and B cells can be determined with the antibodies included in the ICC panel.

▲ **Note:** Keep in mind that populations other than T cells might be absent when analyzing the enriched T cell sample of the CliniMACS Prodigy Process Samples.

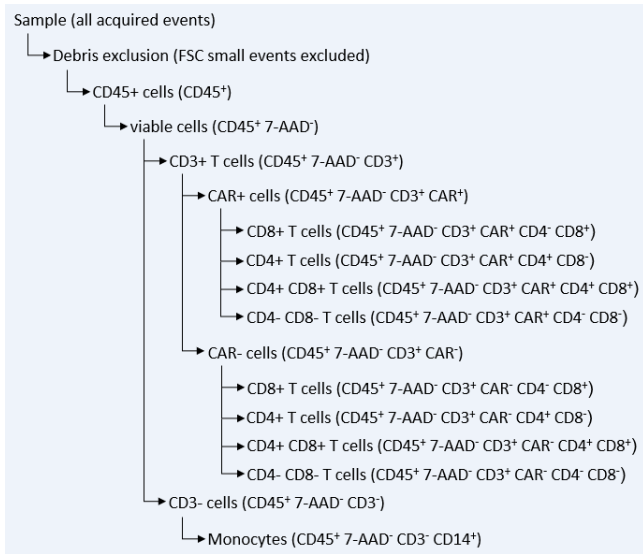


Figure 2: Hierarchical gating strategy for the CAR T transduction efficiency analysis. CAR⁺ and CAR⁻ T cells can be determined and further subdivided for their expression of CD4 and/or CD8. Monocytes that might be partially enriched during T cell enrichment should be excluded via CD14 staining.

10. CliniMACS Prodigy Process Samples are stained with the respective antibodies for the CAR T T panel to detect CAR⁺ and CAR⁻ T cells, and to analyze these for CD4/CD8 subtypes (see figure 2 and figure 4).

▲ **Note:** Due to a possible CD4^{dim} expression, it cannot be excluded that monocytes are partially enriched during CD4/CD8 T cell enrichment. To exclude presence of monocytes during the CliniMACS Prodigy Process, staining for CD14 in the CAR T panel is mandatory (see figure 4).

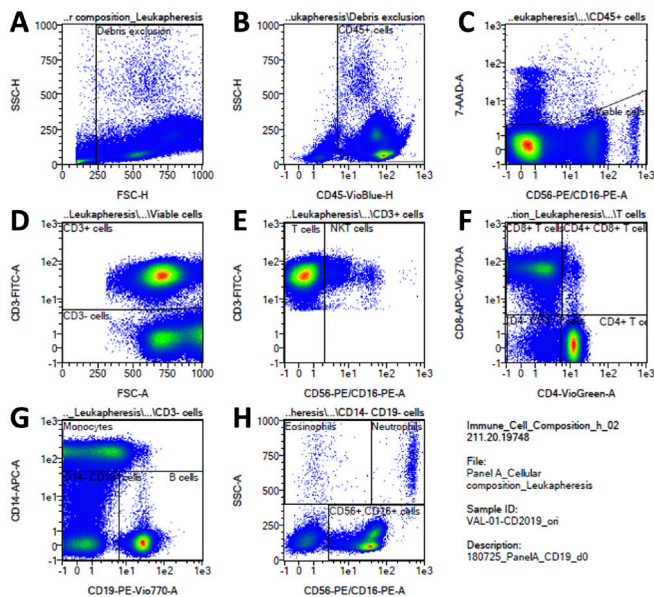


Figure 3: Gating strategy and populations that can be determined by using the Immune_Cell_Composition_h_02 or CART_Immune_Composition_h_03 Express Mode. Data from a healthy donor leukapheresis sample at day 0 of the CliniMACS Prodigy T cell

manufacturing process is depicted. After debris exclusion (A) and gating for CD45⁺ cells (leukocytes, B), the viable cells are detected by exclusion of 7-AAD (C). Subsequently, cells can be divided in CD3⁺ cells and CD3⁻ cells (D). Based on CD56/CD16 expression, CD3⁺ T cells can further be divided into NKT cells (CD56/CD16⁺) and conventional T cells (E); these conventional T cells can be distinguished by their expression of CD4 and/or CD8 (F). CD3⁻ cells comprise B cells (CD19⁺ CD14⁻), monocytes (CD14⁺ CD19⁻), NK cells (CD14⁻ CD19⁻ CD56/CD16⁻ SSC^{lo}), eosinophils (CD14⁻ CD19⁻ CD56/CD16⁻ SSC^{hi}), and neutrophils (CD14⁻ CD19⁻ CD56/CD16⁻ SSC^{hi}) respectively (G, H). Keep in mind that data will look different in terms of cell composition when analyzing CliniMACS Prodigy Process Samples.

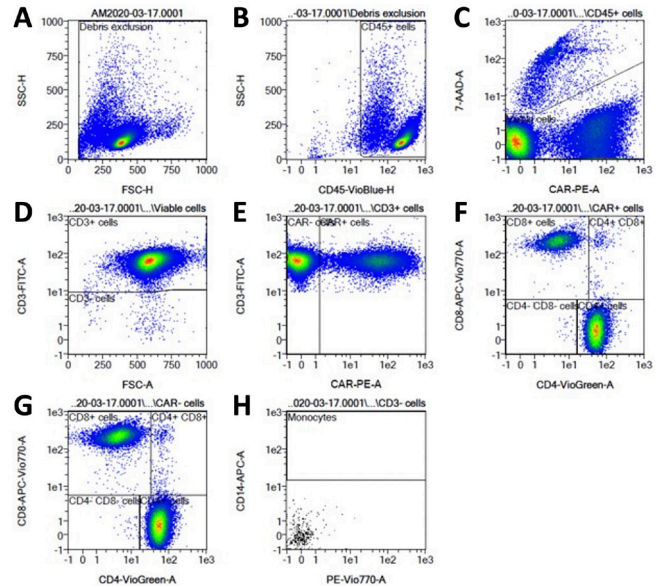


Figure 4: Gating strategy and populations that can be determined by using the CAR_T_Cell_Transduction_h_02 or CART_Transduction_h_03 Express Mode. Data from an IPC sample at day 12 of the CliniMACS Prodigy T cell manufacturing process is depicted. After debris exclusion (A) and gating for CD45⁺ cells (leukocytes, B), the viable cells were detected by exclusion of 7-AAD (C). Subsequently, cells were divided into CD3⁺ and CD3⁻ cells (D). With CD19 CAR Detection Reagent, CD3⁺ cells were further divided into CAR⁺ and CAR⁻ cells (E). Both subpopulations (CAR⁺ cells, F; CAR⁻ cells, G) can be distinguished by their expression of CD4 and/or CD8. Additionally, CD14 expression should be determined to exclude the presence of monocytes (CD3⁻ CD14⁺, H).

4. Analysis of results

Calculation of dilution factor

As the sample is diluted during sample preparation, the total dilution factor needs to be calculated. For this purpose, the pre-dilution factor, the total staining volume (e.g. 2 mL), and the sample volume (e.g. 100 µL) have to be considered. This is only applicable if the sample had to be adjusted for an appropriate concentration before staining. If no pre-dilution has been performed, the pre-dilution factor is “1”.

Total dilution factor =

$$\text{Pre-dilution factor} \times \frac{\text{Total staining volume}}{\text{Sample volume}}$$

Calculation of cell concentration

The final cell concentration (exemplary for CD3⁺ cells) is calculated using the MACSQuant Analysis Report (ICC analysis) and the dilution factor.

Final cell concentration CD3⁺ cells (cells/µL) =

$$\text{Cell concentration CD3}^+ \text{ cells (cells/}\mu\text{L)} \times \text{Total dilution factor}$$

To determine the final cell concentration of CD3⁺ cells per mL (cells/mL), multiply the final cell concentration of CD3⁺ cells (cells/µL) by 1,000.

Final cell concentration CD3⁺ cells (cells/mL) =

$$\text{Cell concentration CD3}^+ \text{ cells (cells/}\mu\text{L)} \times 1,000$$

▲ **Note:** If a dilution factor was entered during the Express Mode analysis and the unit of cell concentration cells/mL was selected, the section “Calculation of cell concentration” can be neglected and it can directly be proceeded with “Calculation of CD3⁺ cell count”.

Calculation of CD3⁺ cell count

To determine the total amount of CD3⁺ cells (e.g. in the entire leukapheresis product), use the calculated final CD3⁺ cell concentration and multiply by the total volume of cell suspension.

Total CD3⁺ cell count =

$$\text{Final cell concentration CD3}^+ \text{ cells (cells/mL)} \times \text{Total volume of cell suspension (mL)}$$

Calculation of CAR⁺ cell concentration

To determine the CAR⁺ cell concentration, use the final CD3⁺ cell concentration (cells/mL, ICC analysis) and multiply by the observed frequency of CAR⁺ cells among viable CD3⁺ cells (CAR T T analysis). For the total CAR⁺ cell count, multiply this concentration by the total volume of the suspension (inside the CliniMACS Prodigy system).

CAR⁺ CD3⁺ cell concentration (cells/mL) =

$$\text{Final cell concentration CD3}^+ \text{ cells (cells/mL) (ICC)} \times \frac{\% \text{ CAR}^+ \text{ among viable CD3}^+}{100}$$

Total CAR⁺ cell count =

$$\text{CAR}^+ \text{ CD3}^+ \text{ cell concentration (cells/mL)} \times \text{Total volume of cell suspension (mL)}$$

Refer to www.miltenyibiotec.com for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

The CliniMACS System components, including Reagents, Tubing Sets, Instruments, and PBS/EDTA Buffer, are designed, manufactured and tested under a quality system certified to ISO 13485.

In the EU, the CliniMACS System components are available as CE-marked medical devices for their respective intended use, unless otherwise stated. The CliniMACS Reagents and Biotin Conjugates are intended for in vitro use only and are not designated for therapeutic use or direct infusion into patients. The CliniMACS Reagents in combination with the CliniMACS System are intended to separate human cells. Miltenyi Biotec as the manufacturer of the CliniMACS System does not give any recommendations regarding the use of separated cells for therapeutic purposes and does not make any claims regarding a clinical benefit. For the manufacturing and use of target cells in humans, the national legislation and regulations - e.g. for the EU the Directive 2004/23/EC (“human tissues and cells”), or the Directive 2002/98/EC (“human blood and blood components”) - must be followed. Thus, any clinical application of the target cells is exclusively within the responsibility of the user of a CliniMACS System.

In the US, the CliniMACS CD34 Reagent System, including the CliniMACS Plus Instrument, CliniMACS CD34 Reagent, CliniMACS Tubing Sets TS and LS, and the CliniMACS PBS/EDTA Buffer, is FDA approved as a Humanitarian Use Device (HUD), authorized by U.S. Federal law for use in the treatment of patients with acute myeloid leukemia (AML) in first complete remission. The effectiveness of the device for this indication has not been demonstrated. Other products of the CliniMACS Product Line are available for use only under an approved Investigational New Drug (IND) application, Investigational Device Exemption (IDE) or FDA approval.

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