Miltenyi Biotec

Determination of immune cell composition and CAR T cells from the CliniMACS Prodigy® T Cell Transduction Process

Contents

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 T Cell Transduction (TCT) process 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 and MACSQuant Analyzer 16 in conjunction with the CAR T Cell Express Mode Package can be used for automated analysis of immune cell composition (ICC) and for detection of CAR T cells at all stages required during the TCT 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) or MACSQuant Analyzer 16 (# 130-109-803) installed with MACSQuantify™ Software version 2.11.1746.19438, 2.11.1812.19581, 2.11.1817.19623, 2.11.1907.19925, or 2.13.1+64.g1143ad24
- For MACSQuant Analyzer 10: CAR T Cell Express Mode Package (# 160-002-376)

  For MACSQuant Analyzer 16: CAR T Cell Essential Express Mode Package (# 160-002-759)

  ▲ **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
- 12×75 mm tubes with cap
- 50 mL conical tubes
- PBS/EDTA buffer, e.g., MACSQuant Running Buffer (# 130-092-747)
- 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 (ddH_2O)
- 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 table 1.

Product	Clone	Order no.
CD45 Antibody, anti-human, VioBlue®, REAfinity™*	REA747	130-110-637
CD4 Antibody, anti-human, VioGreen™, REAfinity*	REA623	130-113-230
CD3 Antibody, anti-human, FITC, REAfinity*	REA613	130-113-138
CD16 Antibody, anti-human, PE, REAfinity*	REA423	130-113-393
CD56 Antibody, anti-human, PE, REAfinity*	REA196	130-113-312
CD19 CAR Detection Reagent, human, Biotin		130-115-965
Biotin Antibody, PE, REAfinity	REA746	130-110-951
7-AAD Staining Solution*		130-111-568

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Product	Clone	Order no.
CD19 Antibody, anti-human, PE-Vio® 770, REAfinity*	REA675	130-113-647
CD14 Antibody, anti-human, APC, REAfinity*	REA599	130-110-520
CD8 Antibody, anti-human, APC-Vio 770, REAfinity*	REA734	130-110-681

*Reagents are also available in CE-IVD and ASR quality. For each of these reagents use the volume, incubation time and dilution indicated in the respective data sheet.

Table 1: Antibodies and reagents required for flow cytometric analysis of ICC and CAR TCT efficiency.

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 ddH_2O, e.g., dilute 200 µL Red Blood Cell Lysis Solution (10×) with 1.8 mL of ddH_2O and mix well. Adjust for multiple samples accordingly. Store at room temperature (+20 °C to +25 °C).
- Buffer: Prepare PBS/EDTA buffer supplemented with 0.5% BSA by diluting MACS® BSA Stock Solution 1:20 with MACSQuant® Running Buffer. Store at +2 °C to +8 °C.

1.4 Preparation of staining cocktails

▲ Staining cocktails for ICC and TCT efficiency analysis should be prepared freshly as indicated in table 2 and 3, respectively.

▲ The final volume of the cocktails including antibodies, buffer, and 7-AAD Staining Solution should be 50 µL. The indicated volumes are for staining of one sample and need to be adjusted for multiple samples accordingly.

▲ **Note:** CD19 CAR Detection Reagent in the TCT panel had to be stained first and cannot be included in the staining cocktail.

▲ **Note:** The titer for 7-AAD Staining Solution is optimized for the current protocol and thus differs from the respective data sheet.

Antibody	Titer	Volume	
CD45-VioBlue®	1:50	3 µL	
CD4-VioGreen™	1:50	3 µL	
CD3-FITC	1:50	3 µL	
CD16-PE	1:50	3 µL	Fill up with buffer to 50 µL
CD56-PE	1:50	3 µL	
7-AAD Staining Solution	1:10	15 µL	
CD19-PE-Vio® 770	1:50	3 µL	
CD14-APC	1:50	3 µL	
CD8-APC-Vio 770	1:50	3 µL	

Table 2: Required antibody and reagent volumes for ICC analysis. The indicated volumes are for staining of one sample. The dilution factor (titer) refers to the final antibody dilution.

Antibody	Titer	Volume	
(CD19 CAR Detection Reagent, human, Biotin)	(1:11)	(10 µL)	Pre-stained
CD45-VioBlue	1:50	3 µL	Fill up with buffer to 50 µL
CD4-VioGreen	1:50	3 µL	
CD3-FITC	1:50	3 µL	
Biotin-PE	1:50	3 µL	
7-AAD Staining Solution	1:10	15 µL	
CD14-APC	1:50	3 µL	
CD8-APC-Vio 770	1:50	3 µL	

Table 3: Required antibody and reagent volumes for TCT efficiency analysis. The indicated volumes are for staining of one sample. The dilution factor (titer) refers to the final antibody dilution.

2. Staining protocol

2.1 Sample preparation

1. Collect the respective samples for the analysis of ICC and CAR TCT efficiency as described in table 4. If desired, an additional in-process control (IPC) can be performed between day 9 and day 11. CliniMACS Prodigy® TCT Process samples are obtained using the sampling adaptor. A minimum of 100 µL sample volume with a maximal concentration of 1×10^7 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 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 TCT sample	ICC, TCT
(Optional) Day 9-11	CliniMACS Prodigy TCT sample	ICC, TCT
Day 12	CliniMACS Prodigy TCT sample and final cell product	ICC, TCT

Table 4: Sampling date, sample type, and recommended analysis.

2. Label the required number of tubes with the panel (ICC or TCT) and a suitable sample ID.
3. Transfer 100 µL of cell sample containing a maximum number of 1×10^6 cells into each tube.

2.2 Labeling of cells with staining cocktails

2.2.1 Staining protocol for ICC

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

1. Add 50 µL of ICC staining cocktail to 100 µL cell sample. Cap the tube and mix the cells by vortexing. Incubate the cells for 10 minutes in the dark in the refrigerator (+2 °C to +8 °C).
2. If the sample does not require red blood cell lysis or fixation, add 1.85 mL buffer and return the cap to the tube.

   or

   If the sample does not require fixation but red blood cell lysis, add 1.85 mL of Red Blood Cell Lysis Solution (1×) to each tube. Return the cap, vortex for 5 seconds, and incubate for 10 minutes at room temperature (+20 °C to +25 °C) in the dark.

▲ **Note:** Red blood cell lysis should only be necessary for the analysis of starting material.

or

If the sample needs to be fixed, add 100 µL of buffer and 250 µL of Inside Fix (a component of the Inside Stain Kit) to each sample. Mix well by pipetting up and down and incubate for 20 minutes at room temperature (+20 °C to +25 °C) in the dark. After incubation, add 1.5 mL of buffer to the sample and mix by vortexing.

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

4. Store the samples at +2 °C to +8 °C in the dark until acquisition on the MACSQuant® Analyzer 10. Acquire samples within one hour after staining.

2.2.2 Staining protocol for CAR TCT efficiency

1. Add 10 µL of CD19 CAR Detection Reagent, Biotin to 100 µL cell sample. Cap the tube and mix the cells by vortexing. Incubate for 10 minutes at room temperature (+20 °C to +25 °C) in the dark.
2. Wash cells 2× with 1 mL of buffer. Centrifuge at 300×g for 5 minutes at room temperature. After the second washing step, carefully remove the supernatant and resuspend cells in 100 µL of buffer.
3. Add 50 µL of TCT staining cocktail to 100 µL cell sample. Cap the tube and mix the cells by vortexing. Incubate the cells for 10 minutes in the dark in the refrigerator (+2 °C to +8 °C).
4. Add 1 mL of buffer and centrifuge at 300×g for 5 minutes at room temperature. Carefully remove the supernatant.
5. If the sample does not require fixation, resuspend the cells in 2 mL of buffer and return the cap to the tube.

   ▲ 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 needs to be fixed, resuspend the cells in 250 µL of buffer before adding 250 µL of Inside Fix (a component of the Inside Stain Kit) to each sample. Mix well by pipetting up and down and incubate for 20 minutes at room temperature (+20 °C to +25 °C) in the dark. After incubation, add 1.5 mL of buffer to the sample and mix by vortexing.

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

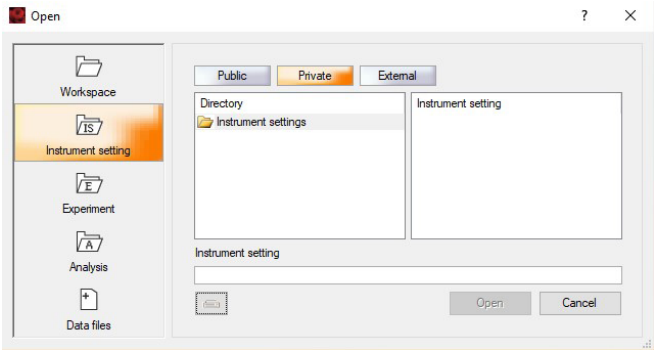
4. Store the samples at +2 °C to +8 °C in the dark until acquisition on the MACSQuant® Analyzer 10. Acquire samples within one hour after staining.

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

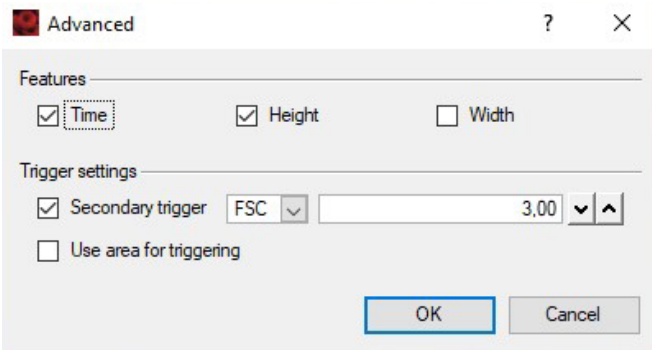
3.1 Data acquisition using Express Modes

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 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 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.

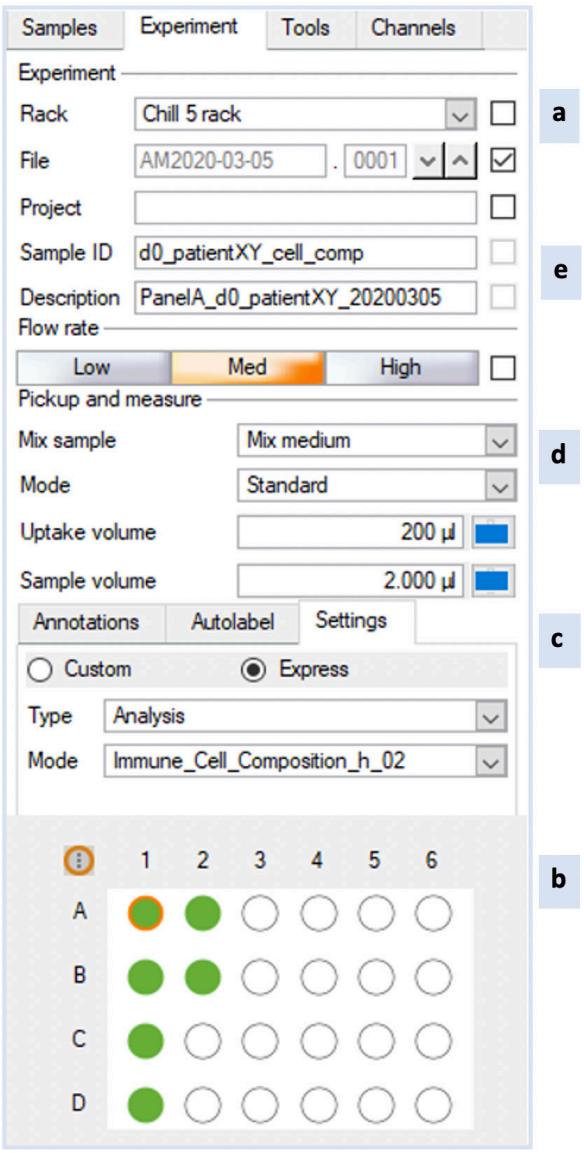
   c. Select the respective Express Mode: (**Immune_Cell_Composition_h_02** or **CART_Immune_Composition_h_03** or **CAR_T_Cell_Transduction_h_02**

or **CART_Transduction_h_03**, respectively) from the drop-down 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.

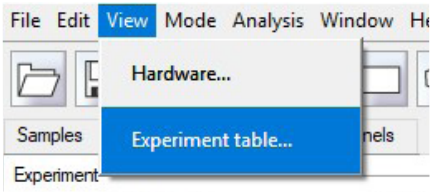
d. Select an appropriate sample mixing (e.g. Mix medium).

▲ **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.

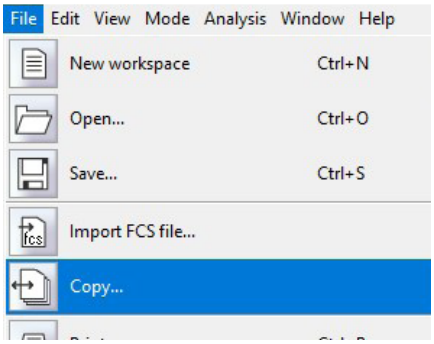
   ▲ **Note:** Although sample mixing has been selected on the MACSQuant® Analyzer 10 or MACSQuant Analyzer 16, it is recommended to vortex samples if multiple samples will be measured.

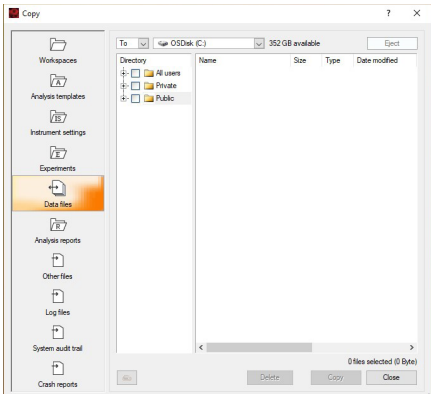
6. Start the acquisition.

3.2 Data analysis using Express Modes

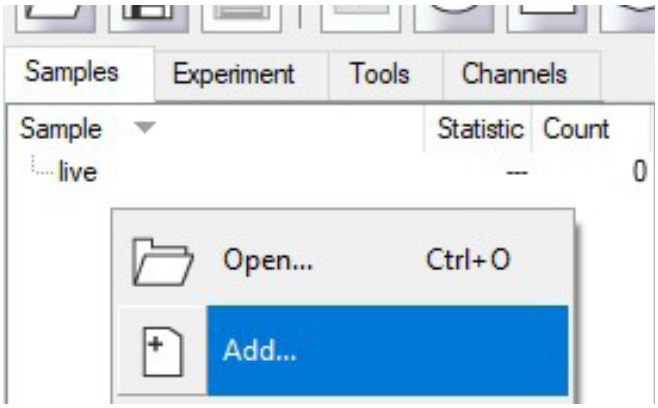
▲ If the Single Tube Rack is used, the analysis is automatically performed directly after acquisition. If the Chill 5 Rack is used, the analysis is immediately replaced by the subsequent sample for acquisition and can be done afterwards. However, it is recommended to use a separate PC (equipped with 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.

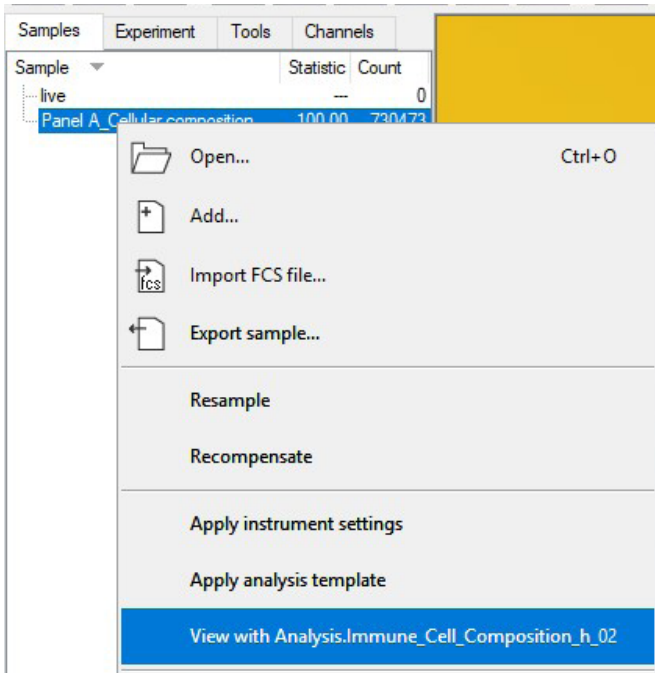




2. On the analysis PC, right click within the **Samples tab** and select **Add...** to upload data files to the MACSQuantify Software.



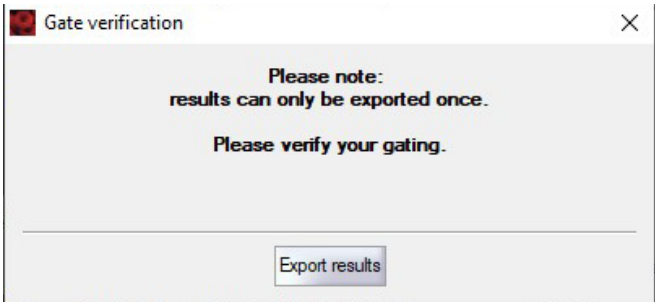
3. Right click on the first data file and select **View with Analysis. Immune_Cell_Composition_h_ 02** or **View with Analysis. CART_Immune_Composition_h_03** or **View with Analysis. CAR_T_Cell_Transduction_h_02 or View with Analysis. CART_Transduction_h_03**, respectively, for accessing the Express Mode analysis.



4. 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.

5. 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.



6. 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.
7. To print the analysis, go to **File → Print**.
8. 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.

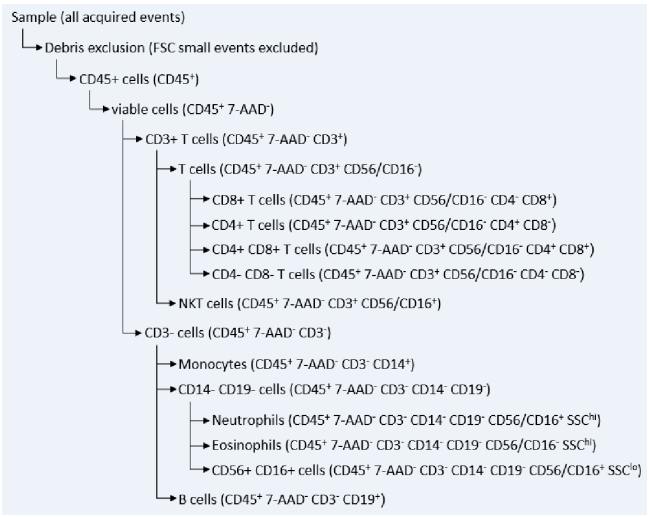


Figure 1: Hierarchical gating strategy for the ICC analysis. Subtypes of T cells (CD4 and/or CD8), as well as NKT cells, monocytes, neutrophils, eosinophils, NK cells, and B cells can be determined based on the receptor expression indicated in brackets.

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 at day 0 or the CliniMACS Prodigy® TCT process samples.

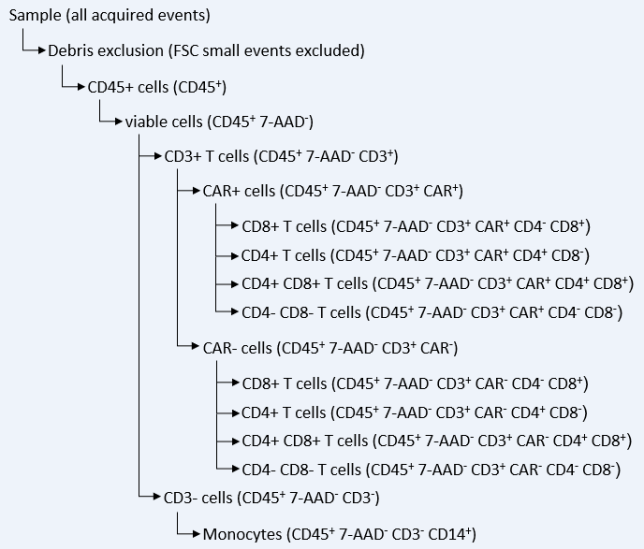


Figure 2: Hierarchical gating strategy for the CAR TCT efficiency analysis. CAR^+ and CAR^- T cells can be determined and further subdivided for their expression of CD4 and/or CD8. Monocytes that might be partially enriched during T cell enrichment should be excluded via CD14 staining.

10. CliniMACS Prodigy® TCT process samples are stained with the respective antibodies for the TCT 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 at day 0. To exclude presence of monocytes during the CliniMACS Prodigy TCT Process, staining for CD14 in the TCT panel is mandatory (see figure 4).

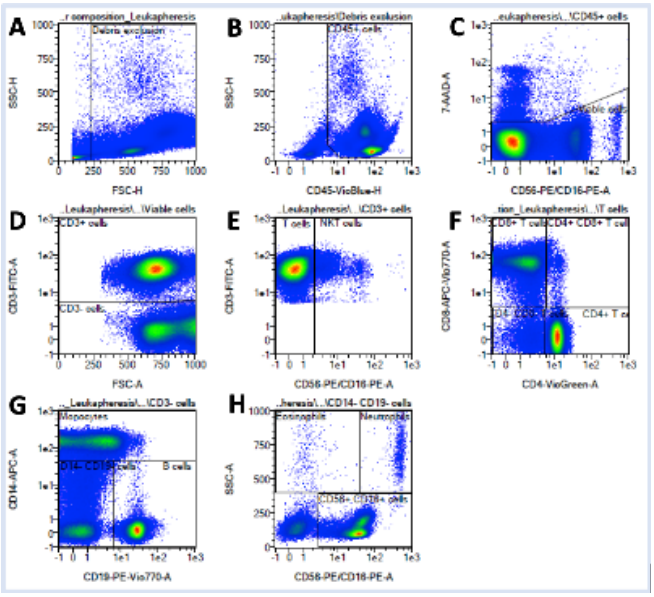


Figure 3: Gating strategy and populations that can be determined by using the Immune_Cell_Composition_h_02 or CART_Immune_Composition_h_03 Express Mode. Data from a healthy donor leukapheresis sample at day 0 of the CliniMACS Prodigy T Cell manufacturing process is depicted. After debris exclusion (A) 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 TCT process sample.

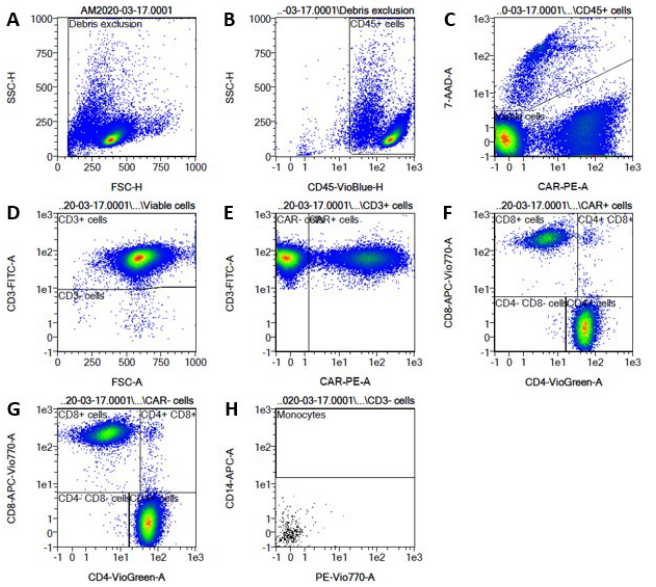


Figure 4: Gating strategy and populations that can be determined by using the CAR_T_Cell_Transduction_ h_02 or CART_Transduction_h_03 Express Mode. Data from an IPC sample at day 12 of the CliniMACS Prodigy T Cell manufacturing process is depicted. After debris exclusion (A) and gating for $CD45^+$ cells (leukocytes, B), the viable cells are detected by exclusion of 7-AAD (C). Subsequently, cells can be divided in $CD3^+$ and $CD3^-$ cells (D). With CD19 CAR Detection Reagent, $CD3^+$ cells can further be 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 (which is only available if the sample had to be adjusted for an appropriate concentration before staining), the total staining volume (e.g. 2 mL), and the sample volume (e.g. 100 µL) have to be considered.

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) =

Cell concentration $CD3^+$ cells (cells/µL) × 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) =

Cell concentration $CD3^+$ cells (cells/µL) × 1,000

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 with the observed frequency of CAR^+ cells among viable $CD3^+$ cells (TCT 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) =

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

Total CAR^+ cell count =

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

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com/local to find your nearest Miltenyi Biotec contact.