Miltenyi Biotec

Analysis of enriched viable IFN-γ-positive CD4+ and CD8+ T cells

Introduction

Virus-specific CD4+ and CD8+ T cells can be enriched based on their expression of IFN-γ after restimulation with the appropriate antigen by using, for example, the CliniMACS® Cytokine Capture System (IFN-gamma) (CCS). To determine the success of the enrichment, different cell fractions need to be analyzed with regard to the cellular composition, target cell count, and purity.

This protocol (research use only) outlines the required materials and procedures to analyze IFN-γ-positive T cells in the starting material, the non-target cell fraction as well as the target cell fraction after the CCS process. The analysis is performed by flow cytometry using the MACSQuant® Analyzer 10 in combination with Express Modes CCS_Immune_Cell_Composition_h_01 and CCS_Purity_h_01. These Express Modes are included together with the Express Mode RCI_CD4CD8_h_02, which is required for the automated analysis of the blood donor eligibility test in the Virus-Specific T Cell CCS Express Mode Package.

Materials

Buffers and solutions

Product		Order no.
CliniMACS PBS/EDTA Buffer containing 0.5% BSA	Dilute MACS BSA Stock Solution (# 130-091-376) 1:20 with CliniMACS PBS/EDTA Buffer. Keep buffer cold (2–8 °C).	PEB buffer
Double distilled water		
Red Blood Cell Lysis Solution (10×)	Prepare a 1×RBC Lysis Solution by diluting 10×RBC Lysis Solution 1:10 with double distilled water.	130-094-183

Fluorescent antibodies and solutions

Product	Clone	Order no.
CD45-VioBlue, human	REA747	130-110-637
CD4-VioGreen, human	REA623	130-113-230
CD3-FITC, human	REA613	130-113-138
CD16-PE, human	REA423	130-113-393
CD19-PEVio770, human	REA675	130-113-647
CD56-PE, human	REA196	130-113-312
CD14-APC, human	REA599	130-110-520
CD8-APC-Vio770, human	REA734	130-110-681
Anti-IFN-γ-PE, human	45-15	130-113-493
CD45RO-PEVio770, human	REA611	130-109-431
CD62L-APC, human	145/15	130-113-617
7-AAD Staining Solution		130-111-568

Equipment and disposables

Product	Order no.
MACSQuant Analyzer 10	130-096-343
MACSQuantify Software (Software Version 2.11 patch 2-4 or higher (required for the Virus-Specific T Cell CCS Express Mode Package))*	
Virus-Specific T Cell CCS Express Mode Package Contains: • RCI_CD4CD8_h_02 (analysis of the blood donor eligibility test) • CCS_Immune_Cell_Composition_h_01 (analysis of cell composition and cell count) • CCS_Purity_h_01 (analysis of target cell purity)	160-002-372
Chill 5 Rack	130-092-951
Pipette tips, appropriate sizes	130-092-951
Combitips, appropriate sizes	
12×75 mm FACS tubes	
Micropipettes	
Vortex mixer	
Refrigerator	

Methods

Target cell labeling and analysis is performed with flow cytometry, e.g., the MACSQuant® Analyzer 10 and the Express Modes CCS_Immune_Cell_Composition_h_01 and CCS_Purity_h_01.

The cell fractions to be analyzed are the starting material (from QC Bag (QCB)) and the final target cell fraction (from Target Cell Bag (TCB)). We also recommend to analyze the non-target cell fraction (from Non-Target Cell Bag (NTCB)), which can be useful for troubleshooting. For the analysis of the cell composition, cell count and the purity, two different staining protocols and antibody panels are applied. These are described in detail in the following section.

Before starting

Preparation of PEB buffer

Prepare a solution containing phosphate buffered saline (PBS), pH 7.2, 1 mM EDTA and 0.5% bovine serum albumin (BSA) by diluting MACS® BSA Stock Solution 1:20 with CliniMACS® PBS/EDTA Buffer (PEB buffer).

Note: Store the PEB buffer at 2–8 °C when not in use.
Note: Do not use PEB buffer after three months of storage.

Preparation of 1×RBC Lysis Solution

Prepare a 1×RBC Lysis Solution by diluting 10× RBC Lysis Solution 1:10 with double distilled water.

Note: Store the 1×RBC Lysis Solution at room temperature when not in use.
Note: Discard unused solution at the end of the day.

Sample preparations

Prepare the samples as follows:

1. Determine the volume of each cell fraction by weighing and subtracting the tare weight.
2. The following protocol describes two different flow cytometry analyses:
   Panel A: cell composition / cell count
   Panel B: purity

Therefore, for flow cytometry labeling and analysis take two samples from each fraction as given below in table 1 and store them at 4 °C.

Bag	Fraction	Panel A: cellular composition/ cell count	Panel B: purity
QCB	QC sample	100 µL	2 mL
TCB	Target cell fraction	100 µL	100 µL
NTCB	Non-target cell fraction	100 µL	500 µL

Table 1: Sampling overview.

Flow cytometry staining of the QC sample, the target cell fraction and the non-target cell fraction using two different antibody panels

For the flow cytometry QC analysis, two different flow cytometry procedures are required. They differ in the antibody panel, as well as in the staining protocol. An overview of the two antibody panels is shown in table 2.

Panel A: cellular composition / cell count	Panel B: purity
CD45-VioBlue®	CD45-VioBlue
CD4-VioGreen™	CD4-VioGreen
CD3-FITC	CD3-FITC
CD16-PE	Anti-IFN-γ-PE
CD56-PE	CD45RO-PEVio770
CD19-PEVio770	CD62L-APC
CD14-APC	CD8 APCVio770
CD8-APCVio770	7-AAD
7-AAD	

Table 2: Antibody panel overview.

Staining protocol for panel A: cellular composition / cell count

1. Prepare a master mix of fluorochrome-conjugated antibodies according to table 3.
2. Label one 12×75 mm FACS tube for each cell sample which is to be analyzed and transfer the sample volume for Panel A according to table 1.
3. Add 30 µL of the prepared master mix to all samples.
4. Mix cells by vortexing or pipetting up and down.
5. Incubate for 10 min at room temperature in the dark.
6. Add 470 µL of 1×RBC Lysis Solution to all tubes.
7. Vortex for 5 s and incubate for 10 min at room temperature in the dark.
8. Store the stained samples at 2–8 °C in the dark until acquisition, e.g., using the MACSQuant Analyzer 10 and the Express Mode CCS_Immune_Cell_Composition_h_01.

Note: Acquire the samples within 1 h of staining

Fluorochrome antibody conjugate	Volume per sample
CD45-VioBlue	2 µL
CD4-VioGreen	2 µL
CD3-FITC	2 µL
CD16-PE	2 µL
CD56-PE	2 µL
CD19-PEVio770	2 µL
CD14-APC	2 µL
CD8-APCVio770	2 µL
7-AAD Staining Solution	10 µL
PEB buffer	4 µL

Table 3: Preparation of master mix for panel A.

Staining protocol for panel B: purity

For the analysis of sample purity, a lyse-and-wash protocol is applied.

1. Prepare a master mix of fluorochrome-conjugated antibodies according to table 4.
2. Label one 12×75 mm FACS tube for each cell sample which is to be analyzed and transfer the sample volume for Panel B according to table 1.
3. Add 2 mL of PEB buffer to the tubes labeled as non-target cell bag and target cell bag.

4. Centrifuge all tubes at 300×g for 5 min.
5. Discard the supernatant.
6. Resuspend cells in 100 µL PEB buffer and add 24 µL of the prepared master mix to all samples.
7. Mix cells by vortexing or pipetting up and down.
8. Incubate for 10 min at room temperature in the dark.
9. Add 1 mL of 1×RBC Lysis Solution to each tube.
10. Vortex for 5 s and incubate for 10 min at room temperature in the dark.
11. Centrifuge at 300×g for 5 min and discard the supernatant.
12. Resuspend cells in 250 µL PEB buffer.
13. Store the stained samples at 2–8 °C in the dark, until acquisition on the MACSQuant® Analyzer 10 and the Express Mode CCS_Purity_h_01.

Note: Acquire the samples within 2 h of staining.

Fluorochrome antibody conjugate	Volume per sample
CD45-VioBlue	2 µL
CD4-VioGreen	2 µL
CD3-FITC	2 µL
Anti-IFN-γ-PE	2 µL
CD45RO-PEVio770	2 µL
CD62L-APC	2 µL
CD8-APCVio770	2 µL
7-AAD	10 µL

Table 4: Preparation of master mix for panel B.

Data acquisition, gating strategy and results

The following analysis is performed using the MACSQuant Analyzer 10 as well as the Express Modes CCS_Immune_Cell_Composition_h_01 and CCS_Purity_h_01. Detailed protocols on sample acquisition and analysis using the Express Modes is described in the following section. Table 5 summarizes the recommended uptake volumes to acquire per sample, which are already predefined in the respective Express Mode programs.

Fraction	Panel A: Uptake volume	Panel B: Uptake volume
QC sample	250 µL	200 µL
Target cell fraction	450 µL	200 µL
Non-target cell fraction	250 µL	200 µL

Table 5: Overview about predefined uptake volumes to acquire per sample.

Panel A

Fully automated flow acquisition of samples stained with panel A using the MACSQuant® Analyzer 10 and the Express Mode CCS_Immune_Cell_Composition_h_01

Note: Perform calibration and compensation (if necessary) of the MACSQuant Analyzer 10.
Note: Do not change any instrument settings during the entire experiment series.

1. Click on the **Open** icon and on the **Instrument setting** button. Choose the valid instrument setting from the **public** tab.
2. Verify that **Height** is switched on (click on the **Advanced** button in the **Channels** tab: **Height** must be selected under **Features**).
3. Select **Chill 5 rack** from the **rack** drop-down menu in the **Experiment** tab.
4. In the rack dialog box, the **Chill 5 rack** will be displayed. Select the appropriate number of sample positions to match the number of samples that will be used for Panel A analysis (full analysis = three samples per CliniMACS Prodigy® run ➞ QC Bag, Non-Target Cell Bag, Target Cell Bag; minimal analysis = two samples per CliniMACS Prodigy run ➞ QC Bag, Target Cell Bag). **Do not select more than three sample positions.**
5. Click on the **Group** button at the bottom of the window to group the corresponding samples (three wells at maximum). Grouped samples should now be labeled with the same number (e.g. "1" as depicted in figure 1). Use three wells for the full analysis (QC Bag, Non-Target Cell Bag, Target Cell Bag; figure 1 A) and two wells for the minimal analysis (QC Bag, Target Cell Bag; figure 1 B).

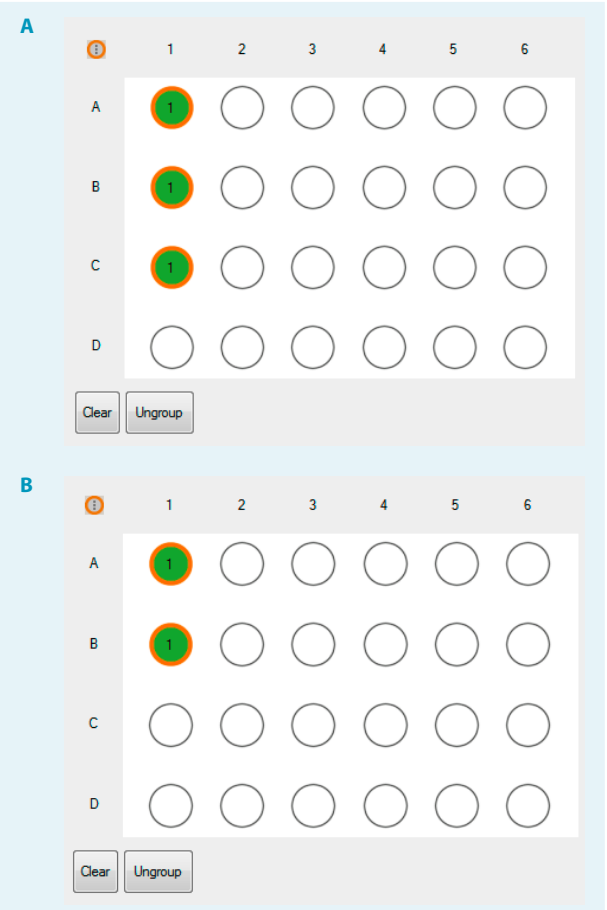

Figure 1: Grouped Chill 5 rack for three samples (QC Bag, Non-Target Cell Bag, Target Cell Bag; A) and for two samples (QC Bag, Target Cell Bag; B).

6. Select the Express Mode CCS_Immune_Cell_Composition_h_01: In the **Settings** tab, click on the **Express** button, then select **Analysis** from the **Type** drop-down menu and choose CCS_Immune_Cell_Composition_h_01 from the **Mode** drop-down list. All experiment settings are loaded automatically.

Note: If you just want to analyze two samples, you need to adjust the naming of the second sample by first selecting the single rack position (the well is activated when the orange ring is shown) followed by selecting **Target cell fraction** in the **Sample ID** drop down menu. Make sure to activate all samples by double-clicking on one of the samples after name adjustment.

7. The mixing can be chosen from the **Mix sample** drop-down menu at the left hand-side. Select **mix medium** from the **Mix sample** drop-down menu, as the Express Mode CCS_Immune_Cell_Composition_h_01 requires a mixing of samples.

Note: It is possible to have more grouped samples on the same Chill 5 rack. They are marked with consecutive numbers in the wells. If you select another well for an additional grouped measurement (consecutive number 2), a pop-up window will open. Confirm the pop-up window by clicking **OK** and continue with step 5. Repeat steps 5 to 8 until all samples are defined on your Chill 5 rack.

8. Fill in the **Description** for each well.
9. Mix the sample well, place it in the correct position of the Chill rack, e.g.,

   A1: QC sample

   A2: Non-Target Cell Bag

   A3: Target Cell Bag

Note: If you have more than one grouped analysis on one Chill 5 rack, more well positions are assigned.

10. Check in the experiment table that the **Sample IDs** match to your samples: Select **View** and **Experiment table…** and compare in the **Acquisition** tab the assigned Sample IDs with your samples in the rack. In addition to that, check in the **Settings** tab that the Express Mode CCS_Immune_cell_composition_h_01 is assigned. In case of inconsistencies, please correct.
11. Start the acquisition.

Analysis of data from samples stained with Panel A acquired with the Express Mode CCS_Immune_Cell_Composition_h_01

The analysis of the data files can be performed on the MACSQuant® Instrument itself or on a PC with installed MACSQuantify™ Software version 2.11 or higher.

Note: The MACSQuantify software version and the Express Mode package version must be the same on the MACSQuant instrument and on the PC.

1. Right click within the **Samples** tab and select **Add…** or **Open…** from the context menu to upload data files to the MACSQuantify Software.
2. Right click on the file name. Select **View with Analysis. CCS_Immune_Cell_Composition_h_01** for accessing the Express Mode analysis template. The appropriate analysis pages will be displayed after the analysis has been finished. During this step, the gates are created and individually adjusted to the selected sample.
3. After the automated analysis, a **Gate verification** pop-up appears. Verify the gating. If you accept the gating, confirm the pop-up window by selecting **Export results**. If you want to adjust the gates manually, move the pop-up window to the side, adjust the gates and confirm the pop-up window by selecting **Export results** afterwards.
4. **Note:** After adjusting a gate manually, a pop-up window will ask you if you want to **Apply change only to current samples or to all samples**. If you select **Current** the adjustment will only be applied to the selected sample. If you select **All**, the adjustment will be applied to all grouped samples.
5. **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 reanalysis is necessary, start the analysis of the data file again (Step 2) and export the data again.
6. The analysis on page 1 shows the pre-installed flow plot views and the information text field. The analysis on page 2 shows the pre-installed flow plot views, the information text field, as well as the statistical analysis of the respective sample.
7. The exported files can be found in the data file directory.
8. To print the analysis, select **File** from the menu tool bar and **Print**.

Gating strategy and results

The following flow cytometric analysis was performed with the Express Mode CCS_Immune_Cell_Composition_h_01 on CMV-, AdV- and EBV-multispecific T cells separated with the CCS. The following analysis allows to determine the cell count (e.g. viable $CD3^+$ T cells) and frequency of different immune cell populations in the analyzed sample.
A hierarchical gating strategy is depicted in figure 3.

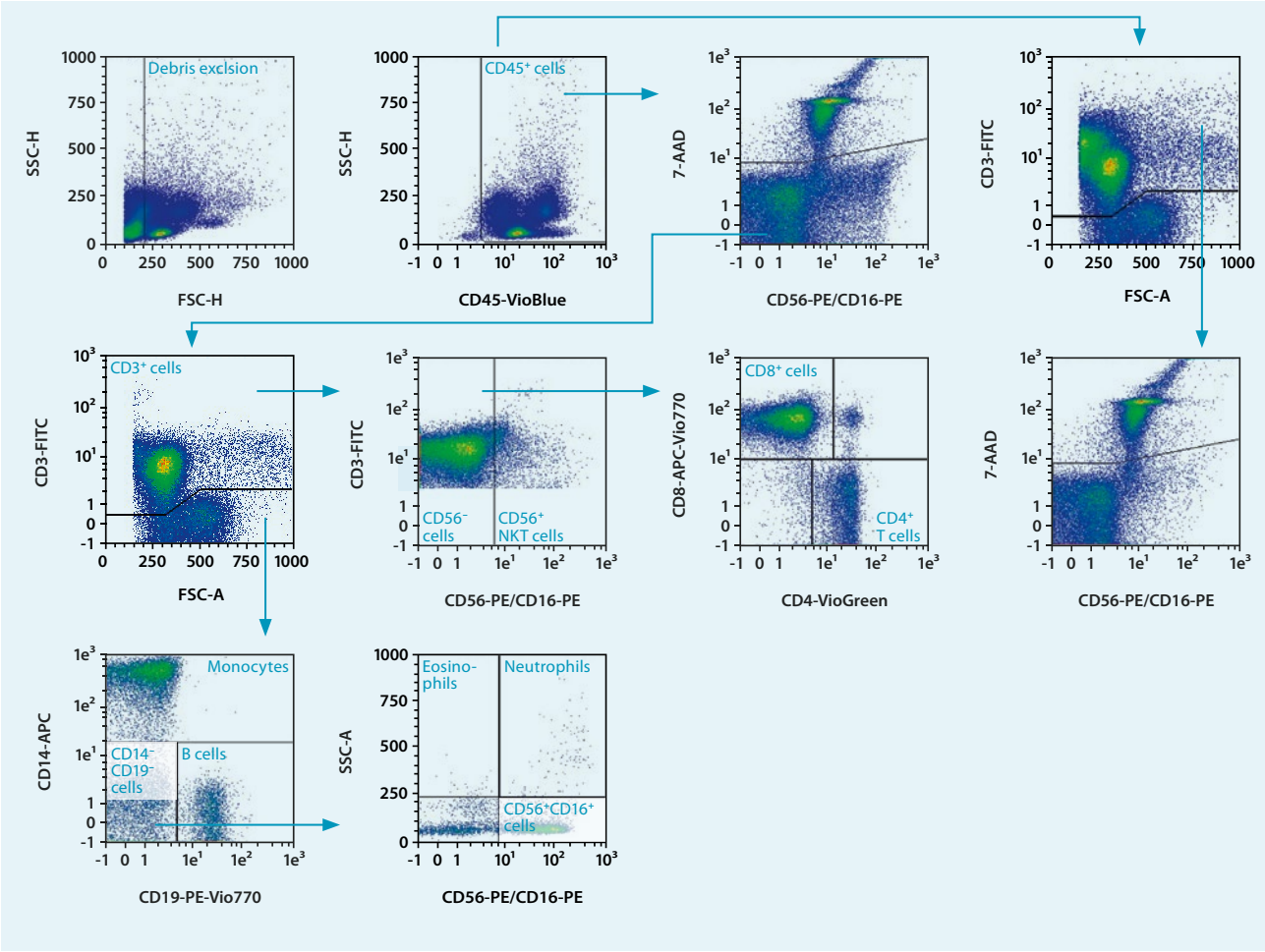

Figure 2: Gating strategy performed by the Express Mode CCS_Immune_Cell_Composition_h_01. CMV-, AdV-, and EBV-multispecific T cells were separated with the CCS. The cell composition and cell count of the final cell product was determined by flow cytometry using the MACSQuant® Analyzer 10 in combination with the Express Mode CCS_Immune_Cell_Composition_h_01. Shown here is the analysis of the target cell fraction obtained from the TCB.

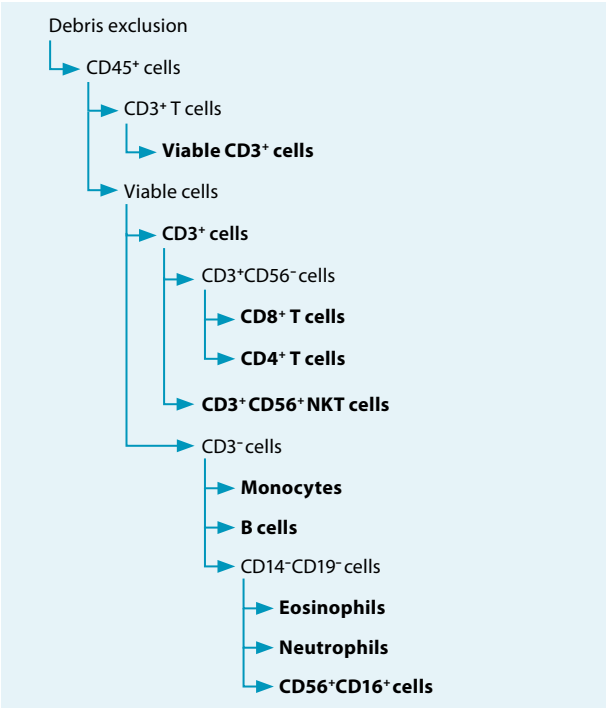

Figure 3: Hierarchical gating strategy according to figure 2.

Automated analysis

The automated analysis provides the cell number per µL (cells/µL), the total number (count) and frequency of target cell populations among viable CD45⁺ cells ([%] among viable CD45⁺), here exemplary shown for the target cell fraction. A detailed statistical analysis can be exported as Excel format.

Cell type	Defined population	Cells/µL	Count	% among viable CD45$^+$
Target cell fraction	All acquired events	321.99	144,096	
Debris exclusion	FSC small events excluded	167.38	74,908	
CD45$^+$ cells	CD45$^+$	166.28	74,412	
Viable cells	CD45$^+$ 7-AAD$^-$	124.46	55,697	
CD3$^+$ cells	CD45$^+$ 7-AAD$^-$ CD3$^+$	83.52	37,377	67.11
CD3$^-$ cells	CD45$^+$ 7-AAD$^-$ CD3$^-$	40.94	18,320	32.89
CD56$^-$ T cells	CD45$^+$ 7-AAD$^-$ CD3$^+$ CD56$^-$ CD16$^-$	76.64	34,297	61.58
CD56$^+$ NKT cells	CD45$^+$ 7-AAD$^-$ CD3$^+$ CD56$^+$ CD16$^+$	6.88	3,080	5.53
CD4$^+$ T cells	CD45$^+$ 7-AAD$^-$ CD3$^+$ CD56$^-$ CD16$^-$ CD4$^+$ CD8$^-$	19.41	8,686	15.60
CD8$^+$ T cells	CD45$^+$ 7-AAD$^-$ CD3$^+$ CD56$^-$ CD16$^-$ CD4$^+$ CD8$^+$	53.58	23,979	43.05
CD4$^+$CD8$^+$ T cells	CD45$^+$ 7-AAD$^-$ CD3$^+$ CD56$^-$ CD16$^-$ CD4$^+$ CD8$^+$	1.87	838	1.50
CD4$^-$CD8$^-$ T cells	CD45$^+$ 7-AAD$^-$ CD3$^+$ CD56$^-$ CD16$^-$ CD4$^-$ CD8$^-$	1.77	794	1.43
Monocytes	CD45$^+$ 7-AAD$^-$ CD3$^-$ CD14$^+$	22.23	9,950	17.86
B cells	CD45$^+$ 7-AAD$^-$ CD3$^-$ CD19$^+$	6.97	3,120	5.60
Neutrophils	CD45$^+$ 7-AAD$^-$ CD3$^-$ CD14$^-$ CD19$^-$ CD56$^+$ CD16$^+$ SSC^{hi}	0.25	111	0.20
Eosinophils	CD45$^+$ 7-AAD$^-$ CD3$^-$ CD14$^-$ CD19$^-$ CD56$^-$ CD16$^-$ SSC^{hi}	0.06	29	0.05
CD56$^+$CD16$^+$ cells	CD45$^+$ 7-AAD$^-$ CD3$^-$ CD14$^-$ CD19$^-$ CD56$^+$ CD16$^+$ SSC^{lo}	7.74	3,463	6.22
Viability of CD3$^+$ cells	CD45$^+$ CD3v 7-AAD$^-$ among CD45$^+$ CD3$^+$	83.52	37,377	66.78
% CD3$^+$ in viable CD45$^+$	CD45$^+$ 7-AAD$^-$ CD3$^+$ among CD45$^+$ 7-AAD$^-$	83.52	37,377	67.11

Table 6: Number and frequency of target cell populations.

Example for cell count determination
To determine the total number of viable CD3$^+$ T cells in the cell fraction of interest (e.g. target cell fraction), the following equation can be used:

Total viable CD3$^+$ T cells =
viable CD3$^+$ T cells/mL × dilution factor × bag volume

Viable CD3$^+$ T cells/µL (taken from table 6) = 83.52×1000 = 8.35×10^4 viable CD3$^+$ T cells/mL

Dilution factor = 6 (due to the 1:6 dilution in the staining tube)

Bag volume = to be determined by weighing or measuring (7 mL in this example)

Total viable CD3$^+$ T cells =
8.35×10^4/mL ×6 × 7 mL = 3.5×10^6

Following this protocol, the dilution factor of the QC sample is 60 (1:10 in QC Bag, 1:6 in staining tube) and the total volume is 100 mL (CliniMACS Prodigy® chamber volume). The dilution factor of the NTCB sample is 6 (1:6 dilution in the staining tube).

Panel B

Fully automated flow acquisition of samples stained with panel B (purity) using the Express Mode CCS_Purity_h_01

Note: Perform calibration and compensation (if necessary) of the MACSQuant® Analyzer 10.
Note: Do not change any instrument settings during the entire experiment series

1. Click on the **Open** icon and on the **Instrument setting** button. Choose the currently valid instrument setting from the **public** tab.
2. Verify that **Height** is switched on (click on the **Advanced** button in the **Channels** tab: **Height** must be selected under **Features**).
3. Select **Chill 5 rack** from the **Rack** drop-down menu in the **Experiment** tab.
4. In the Rack dialog box, the **Chill 5 rack** will be displayed. Select the appropriate number of sample positions to match the number of samples that will be used for panel B analysis (full analysis = three samples per CliniMACS Prodigy® run → QC Bag, Non-Target Cell Bag, Target Cell Bag; minimal analysis = two samples per CliniMACS Prodigy run → QC Bag, Target Cell Bag).
   Do not select more than three sample positions.
5. Click on the **Group** button at the bottom of the window to group the corresponding samples (three wells at maximum). Group samples should now be labeled with the same number (e.g. "1" as depicted in Figure 1). Use three wells for the full analysis (QC Bag, Non-Target Cell Bag, Target Cell Bag; Figure 1 A) and two wells for the minimal analysis (QC Bag, Target Cell Bag; Figure 1 B).
6. Selection of the Express Mode CCS_Purity_h_01:
   In the **Settings** tab, select the **Express** button, then select **Analysis** from the **Type** drop-down menu and choose CCS_Purity_h_01 from the **Mode** drop-down list. All experiment settings, except sample mixing, are loaded automatically.

   Note: If you want to analyze just two samples, you will need to adjust the naming of the second sample by first selecting the single rack position (the well is activated when the orange ring is shown), followed by selecting **Target cell fraction** in the **Sample ID** drop down menu. Make sure to activate all samples by double-clicking on one of the samples after name adjustment.
7. The mixing can be chosen from the **Mix sample** drop-down menu at the left hand-side. Select **mix medium** from the **Mix sample** drop-down menu, as the Express Mode CCS_Immune_cell_composition_h_01 requires a mixing of samples.

Note: It is possible to have more grouped samples on the same Chill 5 rack. They are marked with consecutive numbers in the wells. If you select another well for an additional grouped measurement (consecutive number 2),
a pop-up window will open. Confirm the pop-up window by clicking **OK** and continue with step 5. Repeat steps 5 to 8 until all samples are defined on your Chill 5 rack.

8. Fill in the **Description** for each well.
9. Mix the sample well, place it in the correct position of the Chill 5 rack, e.g.:
   A1: QC sample
   A2: Non-Target Cell Bag
   A3: Target Cell Bag

Note: If you have more than one grouped analysis on one Chill 5 rack, more well positions are assigned.

10. Check in the experiment table that the **Sample IDs** match to your samples: Select **View** and **Experiment table…** and compare in the **Acquisition** tab the assigned **Sample IDs** with your samples in the rack. In addition to that, check in the **Settings** tab that the Express Mode CCS_Purity_h_01 is assigned. In case of inconsistencies, please correct.
11. Start the acquisition.

Analysis of data from samples stained with panel B (purity) using the Express Mode CCS_Purity_h_01
The analysis of the data files can be performed on the MACSQuant Instrument itself or on a PC with installed MACSQuantify™ Software version 2.11 or higher.

Note: The MACSQuantify software version and the Express Mode package version must be the same on the MACSQuant instrument and on the PC.

1. Right click within the **Samples** tab and select **Add...** or **Open...** from the context menu to upload data files to the MACSQuantify Software.
2. Right click on the file name. Select **View with Analysis. CCS_Purity_h_01** for accessing the Express Mode analysis template. The appropriate analysis pages will be displayed after analysis has been finished. During this step, the gates are created and individually adjusted to the selected sample.
3. After the automated analysis, a **Gate verification** pop-up appears. Verify the gating. If you accept the gating, confirm the pop-up window by selecting **Export results**. If you want to adjust the gates manually, move the pop-up window to the side, adjust the gates and confirm the pop-up window by selecting **Export results** afterwards.

Note: After adjusting a gate manually, a pop-up window will ask you if you want to **Apply change only to current samples or to all samples**. If you select **Current** the adjustment will only be applied to the selected sample. If you select **All**, the adjustment will be applied to all grouped samples.

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 reanalysis is necessary, start the analysis of the data file again (Step 2) and export again.

4. The analysis page 1 shows the pre-installed flow plot views and the info text field. The analysis page 2 shows the info text field as well as the statistical analysis of the respective sample. The analysis page 3 shows the pre-installed flow plot views and the info text field and page 4 shows the corresponding info text field as well as the statistical analysis of the respective sample.
5. The exported files can be found in the data file directory.
6. To print the analysis, select **File** from the menu tool bar and **Print**.

Gating strategy and results

The following flow cytometric analysis was performed with the Express Mode CCS_Purity_h_01 on CMV-, AdV-, and EBV-multispecific T cells separated with the CCS. The following analysis allows to determine the frequency and phenotype of IFN-γ⁺CD3⁺, IFN-γ⁺CD4⁺ and IFN-γ⁺CD8⁺ T cells in the analyzed sample. A hierarchical gating strategy is depicted in figure 5.

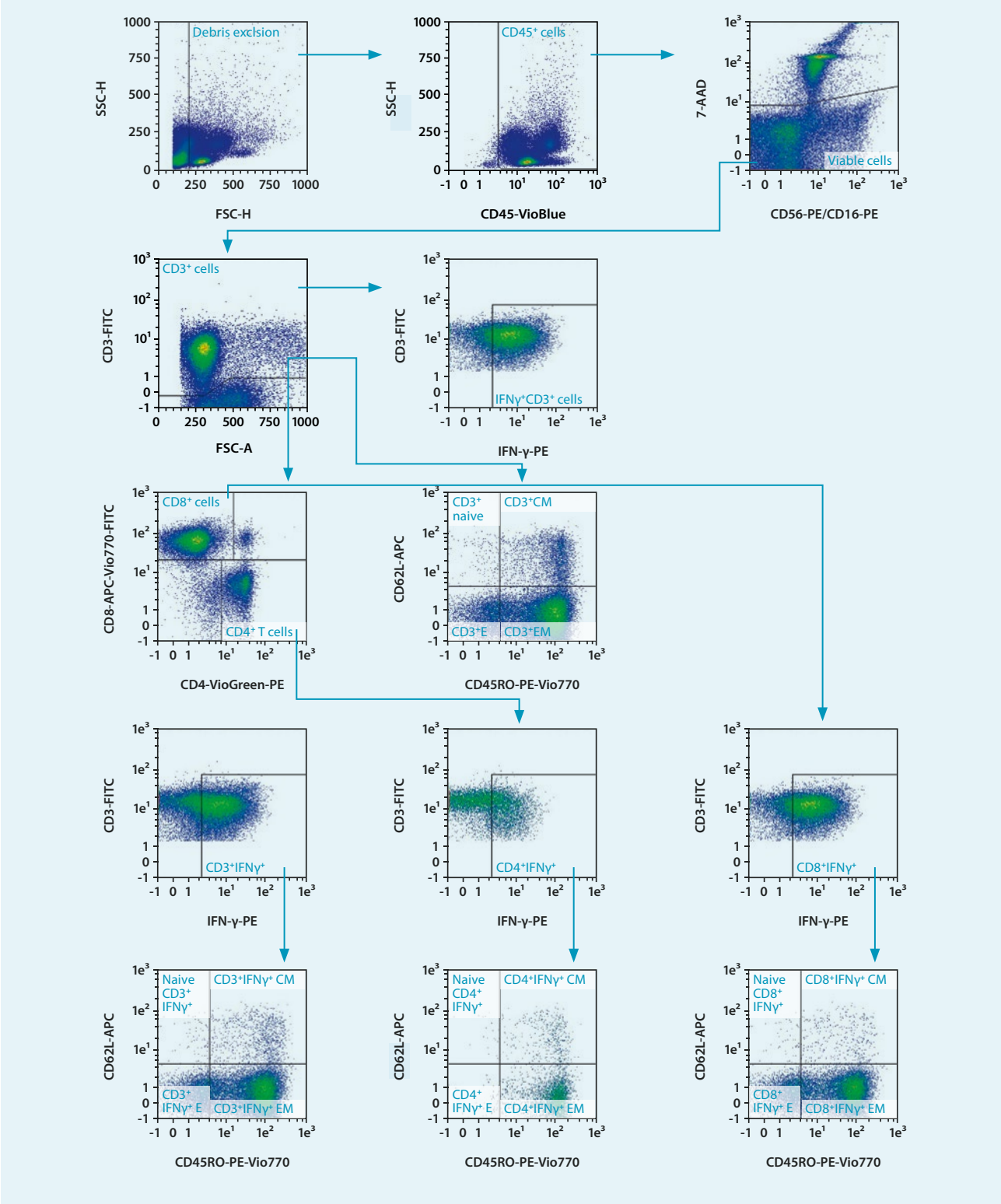

Figure 4: Gating strategy performed by the Express Mode CCS_Purity_h_01. CMV-, AdV-, and EBV-, multispecific T cells were separated with the CCS. The frequency and phenotype of IFN-γ⁺CD3⁺, IFN-γ⁺CD4⁺ and IFN-γ⁺ CD8⁺ T cells of the final cell product was determined by flow cytometry using the MACSQuant® Analyzer 10 in combination with the Express Mode CCS_Immune_Purity_h_01. Shown here is the analysis on the target cell fraction obtained from TCB (CM = central-memory, E = effector, EM = effector memory).

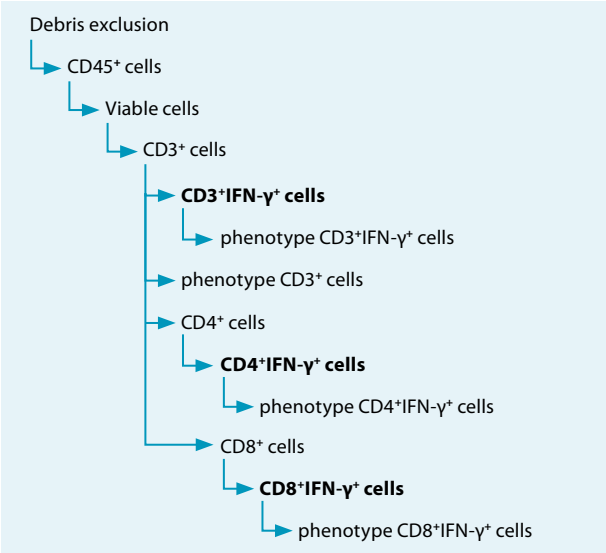

Figure 5: Hierarchical gating strategy according to figure 4.

Automated analysis

The automated analysis provides the cell number per µl (Cells/µl), the total cell count (Count) and the frequency of target cell populations among parent cell population ([%] among viable $CD45^+$), here exemplary shown for the target cell fraction. A detailed statistical analysis can be exported as an Excel format.

Note: This analysis cannot be used to determine the cell count since the staining protocol includes a washing step. If the cell count of, e.g., $CD3^+IFN\text{-}\gamma^+$ cells needs to be determined, it can be calculated by using the number of viable $CD3^+$ cells obtained with the Express Mode CCS_Immune_Cell_Composition_h_01 and the frequency of $CD3^+IFN\text{-}\gamma^+$ obtained with the Express Mode CCS_Immune_Purity_h_01.

Cell type	Defined population	Cells/µL	Count	% among viable $CD45^+$
Target cell fraction	All acquired events	649.07	128084	
Debris exclusion	FSC small events excluded	368.09	72637	56.71
$CD45^+$ cells	$CD45^+$	363.00	71632	98.62
Viable cells	$CD45^+$ 7-AAD$^-$	273.45	53960	75.33
$CD3^+$ cells	$CD45^+$ 7-AAD$^-$ $CD3^+$	187.19	36938	68.45
IFN-γ^+ cells among viable $CD3^+$	$CD45^+$ 7-AAD$^-$ $CD3^+$ IFN-γ^+ among $CD45^+$ 7-AAD$^-$ $CD3^+$	127.35	25130	68.03
$CD3^+$ cells	$CD45^+$ 7-AAD$^-$ $CD3^+$	187.19	36938	68.45
$CD4^+$ cells	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^+$ $CD8^-$	46.10	9098	24.63
$CD8^+$ cells	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^-$ $CD8^+$	124.94	24655	66.75
$CD3^+$ N	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD45RO^-$ $CD62L^+$	0.88	174	0.47
$CD3^+$ CM	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD45RO^+$ $CD62L^+$	18.70	3690	9.99
$CD3^+$ EM	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD45RO^+$ $CD62L^-$	145.44	28701	77.70
$CD3^+$ E	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD45RO^-$ C062L$^-$	22.16	4373	11.84
$CD3^+IFN\text{-}\gamma^+$	$CD45^+$ 7-AAD$^-$ $CD3^+$ IFN-γ^+	127.35	25130	68.03
$CD3^+IFN\text{-}\gamma^+$ N	$CD45^+$ 7-AAD$^-$ $CD3^+$ IFN-γ^+ CD45R0$^-$ $CD62L^+$	0.41	80	0.32
$CD3^+IFN\text{-}\gamma^+$ CM	$CD45^+$ 7-AAD$^-$ $CD3^+$ IFN-γ^+ $CD45RO^+$ $CD62L^+$	5.77	1139	4.53
$CD3^+IFN\text{-}\gamma^+$ EM	$CD45^+$ 7-AAD$^-$ $CD3^+$ IFN-γ^+ $CD45RO^+$ $CD62L^-$	101.35	19999	79.58
$CD3^+IFN\text{-}\gamma^+$ E	$CD45^+$ 7-AAD$^-$ $CD3^+$ IFN-γ^+ $CD45RO^-$ $CD62L^-$	19.82	3912	15.57
$CD4^+IFN\text{-}\gamma^+$	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^+CD8^-$ IFN-γ^+	19.67	3881	42.66
$CD4^+IFN\text{-}\gamma^+$ N	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^+$ $CD8^-$ IFN-γ^+ $CD45RO^-$ $CD62L^+$	0.11	21	0.54
$CD4^+IFN\text{-}\gamma^+$ CM	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^+$ $CD8^-$ IFN-γ^+ $CD45RO^+$ $CD62L^+$	2.24	442	11.39
$CD4^+IFN\text{-}\gamma^+$ EM	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^+$ $CD8^-$ IFN-γ^+ $CD45RO^+$ $CD62L^-$	17.09	3373	86.91
$CD4^+IFN\text{-}\gamma^+$ E	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^+$ $CD8^-$ IFN-$\gamma^+CD45RO^-$ $CD62L^-$	0.23	45	1.16
$CD8^+IFN\text{-}\gamma^+$	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^-$ $CD8^+$ IFN-γ^+	98.10	19359	78.52
$CD8^+IFN\text{-}\gamma^+$ N	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^-$ $CD8^+$ IFN-γ^+ $CD45RO^-$ $CD62L^+$	0.28	55	0.28
$CD8^+IFN\text{-}\gamma^+$ CM	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^-$ $CD8^+$ IFN-γ^+ $CD45RO^+$ $CD62L^+$	2.09	412	2.13
$CD8^+IFN\text{-}\gamma^+$ EM	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^-$ $CD8^+$ IFN-γ^+ $CD45RO^+$ $CD62L^-$	77.69	15330	79.19
$CD8^+IFN\text{-}\gamma^+$ E	$CD45^+$ 7-AAD$^-$ $CD3^+$ $CD4^-$ $CD8^+$ IFN-γ^+ $CD45RO^-$ $CD62L^-$	18.05	3562	18.40

Table 7: Number and frequency of target cell populations of the target cell fraction (N = naive, CM = central memory, E = effector, EM = effector memory).

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