![MACS Miltenyi Biotec logo]

Miltenyi Biotec

Blood donor eligibility test for IFN-γ secreting cell enrichment

Introduction

Virus-specific T cells can be enriched based on their production of IFN-γ after restimulation with the appropriate antigen by using, for example, the CliniMACS® Cytokine Capture System (IFN-gamma). Since this technology targets existing virus- specific T cells from donor blood materials (e.g. leukapheresis), it is recommended to validate the eligibility of each blood donor. This can be accomplished by the antigen-specific induction of IFN-γ expression using, for example, PepTivator® HT Peptide Pools followed by a subsequent flow cytometry analysis using the Rapid Cytokine Inspector (RCI) (CD8/CD4) Kit in combination with the RCI Anti-IFN-γ-PE. The following protocol (for research use only) describes the whole experimental workflow starting from convenient PBMC isolation from whole blood (day 1), restimulation of T cells and final flow cytometry analysis using the MACSQuant® Analyzer 10 in combination with the Express Mode RCI_CD4CD8_h_02 (day 2). This protocol describes the test for three different viral antigens, but can easily be scaled up or down if more or less viral antigens are of interest.

Materials

Buffers, cell culture media, and reagents

Product	Comments	Referred to as
PBS (pH 7.2) containing 0.5% BSA and 2 mM EDTA	Dilute MACS® BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C).	PBS/BSA/EDTA buffer
• Human AB (hAB) serum • RPMI 1640 • L-Glutamine	Add 2 mM L-Glutamine and the hAB serum to a final concentration of 5% (w/v) to the RPMI 1640 medium.	RPMI + 2 mM L-Glutamine + 5% AB serum

Material for PBMC preparation

Product	Order no.
10 mL EDTA-whole blood*	
MACSprep PBMC Isolation Kit	130-115-169

* Note: EDTA as anticoagulant is recommended. The use of other anticoagulants, e.g. heparin or sodium citrate may decrease the yield and purity of target cells.

PepTivator high-throughput (HT) plates

Product	Order no.
PepTivator CMV pp65 (HT) – premium grade, human	130-097-727
PepTivator AdV5 Hexon (HT) – premium grade, human	130-098-237
PepTivator EBV consensus (HT) – premium grade, human	130-119-472
Control plate (8×12)	130-098-235

Fluorescent antibody kits

Product	Components	Order no.
Rapid Cytokine Inspector (CD4/CD8) Kit	1 mL CD4/CD8 T Cell Detection Cocktail, human: • CD3-VioBlue® (clone: BW264-56) • CD4-APC (clone: VIT4) • CD8-FITC (clone BW135/80) • CD14-PerCP (clone TÜK4) • CD20-PerCP (clone LT20) • FcR Blocking Reagent 2×200 µL Brefeldin A (100 µg/mL) 2 mL Inside Fix 2 mL Inside Perm	130-097-343
Rapid Cytokine Inspector Anti-IFN-γ-PE	1 mL monoclonal Anti-IFN-γ-PE antibody (clone 45-15)	130-097-600

Equipment and disposables

Material	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 or Express Mode RCI_CD4CD8_h_02	160-002-372
Chill 96 Rack	130-094-459
Pipette tips, appropriate sizes	
Combitips, appropriate sizes	
12×75 mm FACS tubes	
15 mL conical tubes	
24-well plate	
Micropipettes	
Vortex mixer	
Refrigerator	
CO_2 incubator	
Orbital shaker	

Methods

In the first step (day 1) of the test, PBMCs are isolated from 8–10 mL of whole blood and stored under culture conditions at 37 °C and 5% CO_2 overnight. On the following day (day 2) the PBMCs are stimulated with the desired antigen(s), fluorescently labeled and analyzed by flow cytometry using the MACSQuant® 10 Analyzer and the ExpressMode RCI_CD4CD8_h_02 as described in detail in the following sections.

Day 1

PBMC preparation using the MACSprep™ PBMC Isolation Kit, human

1. Pipet 4 mL of PBMC Isolation Buffer into a 15 mL tube.
2. Add 200 µL of MACSprep™ PBMC Isolation Cocktail.
3. Add 400 µL of MACSprep Anti-Biotin MicroBeads and mix by vortexing.
4. Add up to 10 mL EDTA-whole blood to the 15 mL tube.
5. Close tube tightly and invert gently three times.
6. Place the tube in an upright position in, e.g., a tube rack and incubate for 3 min at room temperature.
7. Centrifuge for 3 min at 50×g for erythrocyte sedimentation.
8. Prepare LS Column by rinsing with 2 mL of buffer. Discard effluent and change collection tube. For details refer to the LS Column data sheet.
9. After erythrocytes have sedimented, carefully collect the supernatant and apply instantly onto the prepared LS Column. Collect flow-through containing unlabeled target cells.

Note: Leave a residual volume of supernatant (approx. 1–2 mm above erythrocyte pellet) to avoid unintended aspiration of erythrocytes.

10. Wash column with 1×3 mL of PBS/BSA/EDTA buffer. Collect unlabeled cells that pass through and combine with the effluent from step 9.

Note: Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.

11. Centrifuge flow through for 10 min at 300×g and discard the supernatant.
12. Resuspend cells in 10 mL PBS/BSA/EDTA buffer and centrifuge for 10 min at 200×g.
13. Discard supernatant and resuspend PBMCs in 1 mL PBS/BSA/EDTA buffer.
14. Determine cell count.
15. Take a sample of at least $4×10^6$ PBMCs. Wash cells with RPMI + 2 mM L-Glutamine + 5% AB serum and adjust to a concentration of $1×10^7$/mL. This sample will be used for the stimulation with viral antigens. A minimum total cell number of $4×10^6$ PBMCs is required when analysis of T cell reactivity against three virus antigens + negative control is desired (four samples each with $1×10^6$ PBMCs). Please stick to the cell number to guarantee the sensitivity of the assay!

Seeding of PBMCs

1. Seed $1×10^7$/mL PBMCs in RPMI + 2 mM L-Glutamine + 5% AB serum in an appropriate cell culture plate, e.g. 1 mL in one well of a 24 well plate.
2. Incubate cells over night at 37 °C and 5% CO_2 until stimulation on the next morning.

Day 2

Stimulation of PBMCs using PepTivator® HT Peptide Pools

PepTivator® HT Peptide Pools are lyophilized peptide pools in the wells of a 96-well plate, allowing stimulation and analysis of cells directly on the plate. The convenient 96-well format, composed of 12 individually removable strips of eight wells each allows for easy and flexible experimental set-up. Different antigen can be combined by assembling different strips. A control can also be added easily by using strips from the control plate (12×8). The capacity is 0.06 nmol (0.01 µg) per peptide, per well. One well is for stimulation of up to 10^6 total cells in 100 µL.

1. Prepare a PepTivator HT plate with one strip of
   - PepTivator HT CMV pp65
   - PepTivator HT AdV5 Hexon
   - PepTivator HT EBV Consensus
   - PepTivator HT Control plate

Before removing the plate from the aluminum pouch, warm it up to room temperature. Remove the necessary amount of strips. Store the remaining ones at –20 °C in the tightly sealed aluminum pouch protected from humidity.

2. Carefully resuspend the overnight stored PBMC suspension by pitpetting up and down and transfer 100 µl of the cell suspension ($1×10^6$ cells) into one well of each of the strips. Perform duplicates or triplicates in case of enough PBMC material.
3. Incubate the cells in an incubator for 2 h at 37 °C and 5% CO_2.
4. Prepare a 1:10 dilution of the Brefeldin A solution (taken from the Rapid Cytokine Inspector (CD4/CD8) T cell Kit).
5. Add 10 µL of the diluted Brefeldin A solution to each of the wells and mix gently.
6. Incubate the cells in an incubator for additional 4 h.

Flow cytometry staining of T cells using the Rapid Cytokine Inspector (RCI) (CD4/CD8 T cell) Kit and the RCI Anti-IFN-γ-PE

1. Remove 80 µL of cell culture supernatant from each well.

2. Prepare a fluorescent antibody staining mix. You need 50 µL of the staining mix for each well: add 10 µL of CD4/CD8 T Cell Detection Cocktail and 10 µL of RCI Anti-IFN-γ-PE antibody to 30 µL of PBS/BSA/EDTA buffer (e. g. for four wells: 40 µL CD4/CD8 Detection Cocktail + 40 µL Anti-IFN-γ-PE + 120 µL buffer).

3. Add 50 µL of staining mix to each well.

4. Mix well for 2 min on orbital shaker.

5. Incubate for 8 min in the dark at RT.

6. Add 25 µL Inside Fix to each well.

7. Mix well for 2 min on orbital shaker.

8. Incubate for 18 min in the dark at RT.

9. Add 25 µL of 10× Inside Perm.

10. Mix well for 2 min on orbital shaker.

11. Incubate for 8 min in the dark at RT.

12. Add 100 µL of PBS/BSA/EDTA buffer.

13. Centrifuge cells at 300×g for 5 min and aspirate supernatant.

14. Resuspend cells in 200 µL PBS/BSA/EDTA buffer.

15. Analyze cells using the MACSQuant® Analyzer 10.

Data acquisition, gating strategy and results

The following analysis is performed using the MACSQuant Analyzer 10 and the Express Mode RCI_CD4CD8_h_02. A detailed protocol on sample acquisition and analysis using the Express Mode is described in the following section.

Fully automated flow acquisition with the MACSQuant® Analyzer 10 using the Express Mode RCI_CD4CD8_h_02

Note: Perform calibration and compensation (if necessary) of the MACSQuant Analyzer 10.

Note: Do not change any instrument settings during acquisition of one 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 96 rack** from the **rack** drop-down menu in the **Experiment** tab.

4. In the rack dialog box, the **Chill 96 rack** will be displayed. Select the appropriate number of sample positions to match the number of samples that will be used.

Note: For adaptation of experiment settings make sure that samples are activated for editing (indicated by orange rim).

5. Select the Express Mode RCI_CD4CD8_h_02: In the **Settings** tab, click on the **Express** button, then select **Analysis** from the **Type** drop-down menu and choose RCI_CD4CD8_h_02 from the **Mode** drop-down list. All experiment settings except **sample mixing** are loaded automatically. The loaded values are shown in the respective fields in the **Experiment** tab. These can be adapted manually if needed, for example, uptake volume. An information pop-up reminds you to select an appropriate mixing of your samples. 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 RCI_CD4CD8_h_02 requires a mixing of samples.

6. In the **Annotations** tab, replace the placeholder annotation Anti-Cyt-PE by the correct annotation Anti-IFN-g-PE in the B2 channel.

7. Fill in the **Description** and **Sample ID** for each well.

8. Check in the experiment table that the correct **Sample ID** matches to your samples: Select **View** and **Experiment table…** and check in the **Acquisition** tab if the assigned **Sample IDs** fit to your samples in the rack. In addition to that, check in the **Settings** tab that the Express Mode RCI_CD4CD8_h_02 is assigned. In case of inconsistencies, please correct.

9. Start the acquisition.

Analysis of data from samples acquired with the Express Mode RCI_CD4CD8_h_02

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 analysis 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 of the unstimulated control sample, which is used as template for the automated analysis. Select **View with Analysis.RCI_CD4CD8_h_02** 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. At this point it is possible to adjust the gates manually if necessary.

3. To apply the analysis to all acquired samples, select the **Analysis mode** button **"A"** on the tool bar and scroll through your samples by pressing **Next sample** or **Previous sample** (tube icon forward and back).

4. The analysis page 1 shows the pre-installed flow plot views and the info text. The info text informs about the control sample which was used as template for the automated analysis. By clicking on **Previous window** and **Next window** (window icon previous and next) you can chose between the different analysis pages. The analysis page 2 shows details of the analyzed sample as well as the statistical analysis of the respective sample.

5. To print the analysis, select each sample individually by using the **Next sample** or **Previous sample** (tube icon forward and back) and select **File** from the menu tool bar and **Print**.

6. If you want to export your results, use the **Export sample list…** feature of the MACSQuantify software.

Gating strategy and results

The following flow cytometric analysis was performed with the Express Mode RCI_CD4CD8_h_02 on PBMCs of a healthy donor stimulated with PepTivator® CMV pp65 (A), PepTivator AdV5 Hexon (B), PepTivator EBV consensus (C) and control (D). A gating hierarchy is depicted in figure 2.
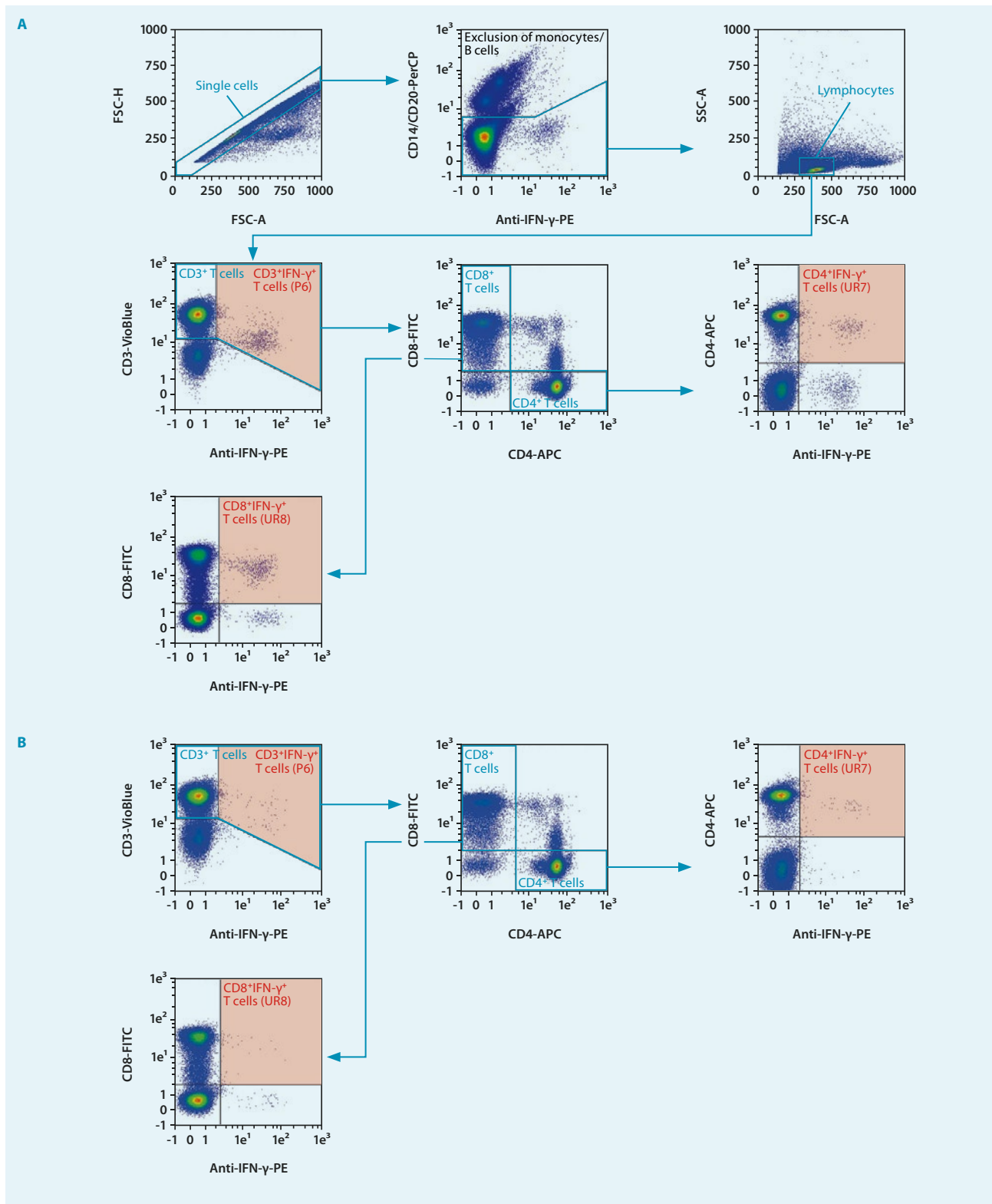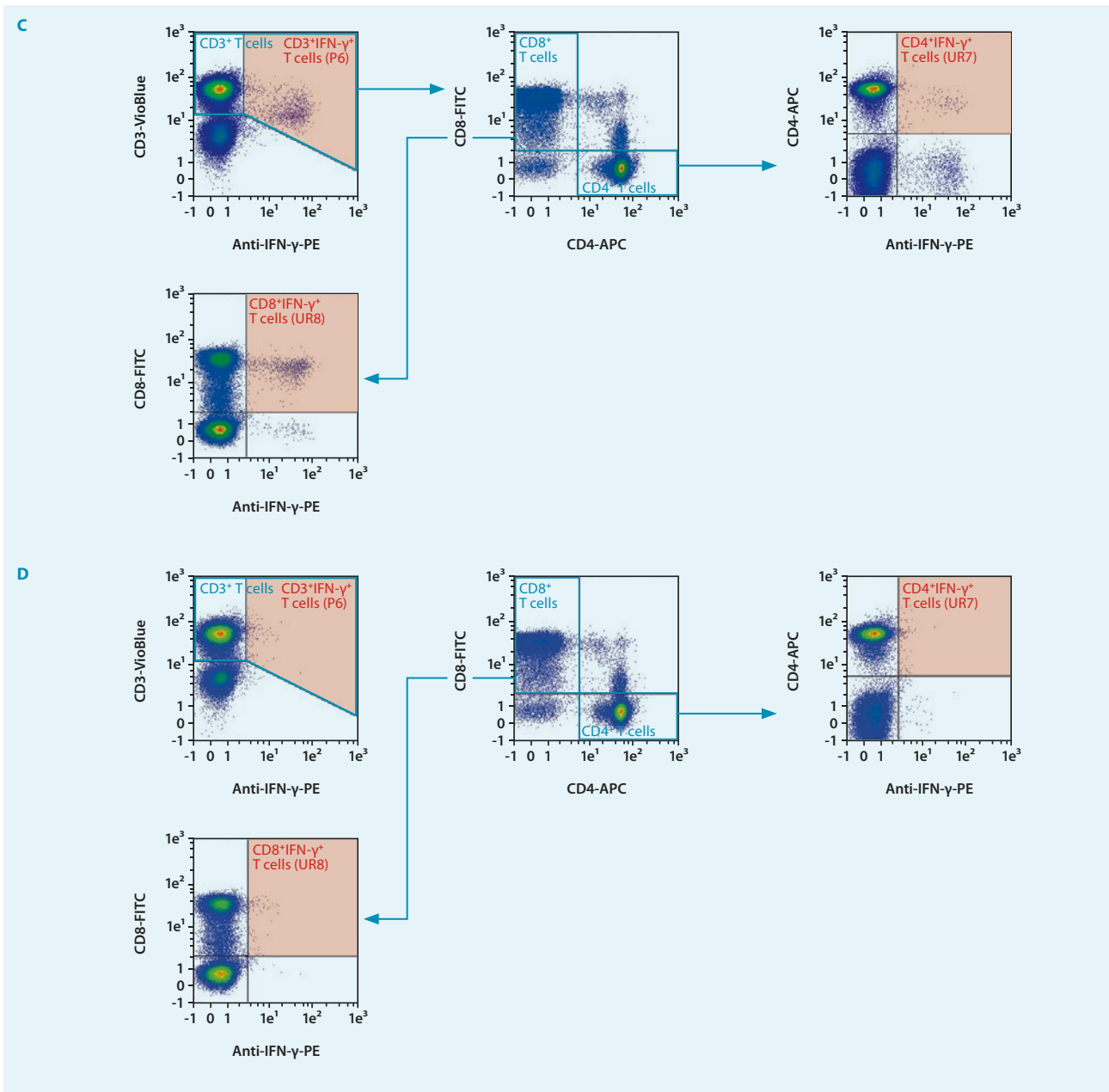

Figure 1: Gating strategy performed by the Express Mode RCI_CD4CD8_h_02. IFN-γ expression of T cells after antigen stimulation. PBMCs were isolated from whole blood and IFN-γ expression was determined after stimulation with A) CMV pp65, B) AdV5 hexon, C) EBV consensus and D) for control without antigen using PepTivator HT Peptide Pools. The whole gating strategy of the flow cytometric analysis is depicted in A), whereas B)–D) only show parts of the analysis. The red boxes highlight the read-out area of interest (also see table 6).

Figure 1 (continued): Gating strategy performed by the Express Mode RCI_CD4CD8_h_02. IFN-γ expression of T cells after antigen stimulation. PBMCs were isolated from whole blood and IFN-γ expression was determined after stimulation with A) CMV pp65, B) AdV5 hexon, C) EBV consensus and D) for control without antigen using PepTivator HT Peptide Pools. The whole gating strategy of the flow cytometric analysis is depicted in A), whereas B)–D) only show parts of the analysis. The red boxes highlight the read-out area of interest (also see table 6).

Single cells
↳ Exclusion of monocytes and B cells
  ↳ Lymphocytes
    ↳ CD3⁺ T cells
      ↳ CD3⁺IFN-γ⁺ T cells (P6) *readout*
      ↳ CD4⁺ T cells
        ↳ CD4⁺IFN-γ⁺ T cells (UR7) *readout*
      ↳ CD8⁺ T cells
        ↳ CD8⁺IFN-γ⁺ T cells (UR8) *readout*
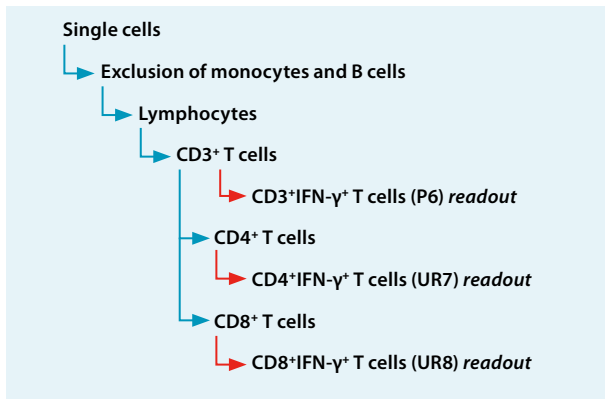
Figure 1 (continued): Gating strategy performed by the Express Mode RCI_CD4CD8_h_02. The blue arrows indicate the gated populations of interest. The red arrows indicate the readout populations of interest. Note that the gated CD3⁺ T cell population is used for further definition and analysis of CD4⁺ and CD8⁺ T cells as well as for the analysis of CD3⁺ IFN-γ⁺ T cells among CD3⁺ T cells.

Automated analysis

The automated analysis provides the number (Count) and frequency of target cell populations among total (Freq. [%-T]) and parent cell population (Freq. [%-#]), here exemplary shown for CMV. A detailed statistical analysis can be exported as Excel format.

Population	Definition	Count	Freq. [%-T]	Freq. [%-#]
Single cells	P1	234359	95.170	95.170
CD14⁻CD20⁻ cells	P2	195501	79.391	83.419
Lymphocytes	P3	168336	68.359	86.105
CD3⁺ T cells	P4	134324	54.547	79.795
CD4⁺ T cells	LR5	78329	31.808	58.313
CD8⁺ T cells	UL5	49253	20.001	36.667
Anti-IFN-γ-PE⁺ among CD3⁺ T cells	P6	780	0.317	0.581
Anti-IFN-γ-PE⁺ among CD4⁺ T cells	UR7	191	0.078	0.244
Anti-IFN-γ-PE⁺ among CD8⁺ T cells	UR8	510	0.207	1.035

Table 1: Number and frequency of target cell populations.

MACS
Miltenyi Biotec