Miltenyi Biotec

Determination of immune cell composition and CAR NK cells from a CliniMACS Prodigy® CAR NK cell manufacturing process

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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 NK Cell Transduction enables the automated production of chimeric antigen receptor (CAR) NK cells. This workflow results from the combination of two processes, CliniMACS Prodigy LP-3-56 System and CliniMACS Prodigy PD-56 Engineering System. During the workflow, cell samples are collected and monitored by flow cytometry to quantify and analyze the immune cell composition and presence of CAR NK cells. The MACSQuant® Analyzer 10 and MACSQuant Analyzer 16 in conjunction with the NK Cell Express Mode Package can be used for automated analysis of immune cell composition and detection of CAR NK cells at all stages required during the manufacturing process. The number and frequencies of cell populations can be determined from the flow cytometric statistics generated at the end of the NK Cell Express Mode analysis. This protocol describes the preparation of samples, setup of the instrument, and analysis of data using the NK 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 2.13.3 or 3.0.1 and NK Cell Express Mode Package (# 160-003-341) or Engineered Immune Cell Express Mode Package (# 160-003-349).

  ▲ **Note:** It is recommended to verify the compatibility of the MACSQuantify Software and the respective 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.

- Micropipettes with disposable tips.
- 12×75 mm flow cytometry tubes with cap.
- Vortex mixer.
- Refrigerator.
- Centrifuge suitable for 12×75 mm tubes with cap.
- 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.

- 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. Follow local regulations.

- Double-distilled water (ddH_2O).
- Antibodies and reagents as listed below in tables 1 and 2.
- FcR Blocking Reagent, human (# 130-059-901).
- (Optional) MACS Comp Bead Kit, anti-REA (# 130-104-693) for optimal compensation of the fluorescence spillover from fluorochrome-conjugated antibodies.

CL_SP80.01

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Product	Clone
CD45 Antibody, anti-human, VioBlue®, REAfinity™	REA747
CD14 Antibody, anti-human, VioGreen™, REAfinity	REA599
TCRα/β Antibody, anti-human, FITC, REAfinity	REA652
CAR Detection Reagent, human, Biotin*	
Biotin Antibody, Vio® Bright B515 REAfinity	REA746
CD3 Antibody, anti-human, PE (BioLegend)	SK7
7-AAD Staining Solution	
CD56 Antibody, anti-human, APC	REA196
CD19 Antibody, anti-human, APC-Vio® 770, REAfinity	REA675
FcR Blocking Reagent, human	

* Choose one CAR Detection Reagent mentioned in table 2.

Table 1: Overview of antibodies needed for the panels included in the NK Cell Express Mode Package.

Product	Order no.
CD19 CAR FMC63 Idiotype Antibody, Biotin, REAfinity	130-127-345
CD19 CAR Detection Reagent, human, Biotin	130-129-550
CD22 CAR Detection Reagent, human, Biotin	130-126-727
CD33 CAR Detection Reagent, human, Biotin	130-127-642
CD123 CAR Detection Reagent, human, Biotin	130-133-273
BCMA CAR Detection Reagent, human, Biotin	130-126-090

Table 2: Recommended CAR Detection Reagents. Refer to www.miltenyibiotec.com for additional CAR Detection Reagents. For each of these reagents use the volume, incubation time, and dilution indicated in the respective data sheet.

1.3 Preparation of solutions

▲ Prepare solutions freshly. Discard unused solutions at the end of the day.

1. Red Blood Cell Lysis Solution (1×): Prepare a 1:10 dilution of Red Blood Cell Lysis Solution (10×) using ddH_2O. Adjust volumes for multiple samples accordingly. Store and use at room temperature (+19 to +25 °C). Discard after use.
2. Buffer: Prepare PBS/EDTA buffer supplemented with 0.5% HSA or BSA.

2. Protocol

2.1 Sample preparation

1. Collect the respective samples for cell count (CC), analysis of immune cell composition (ICC), and CAR NK cell transduction efficiency (CAR NK). An exemplary sampling during a 14-day manufacturing process is described in table 3. CliniMACS Prodigy Process Samples are obtained using the sampling adapter. 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 count calculations at the end of the analysis.

   ▲ **Note:** For analysis purpose take aliquots (at least 10^6 cells to ensure analysis of rare T cells and their CAR expression within the culture) from the culture, e.g., from day 7 and 9, and of the harvested cells.

Sampling date	Sample type	Analysis
Day 0	Starting material and enriched NK cells	CC, ICC
Day 7	CliniMACS Prodigy sample	CC, ICC, CAR NK
Day 9-10 (optional)	CliniMACS Prodigy sample	CC, ICC, CAR NK
Day 14	CliniMACS Prodigy sample and final cell product	CC, ICC, CAR NK

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

2. Label the required number of tubes with the panel identification (e.g. ICC or CAR NK) and suitable sample ID.

2.2 Staining protocol

2.2.1 Staining protocol for cell count

▲ The sample should not be older than 24 hours.

1. Prepare a staining cocktail according to table 4.
2. Label 12×75 mm flow cytometry tubes for each cell sample.
3. Transfer 20 µL of each cellular sample into the corresponding tube.

   ▲ **Note:** If the cell count of a sample exceeds 5×10^7 cells/mL, dilute the sample 1:10 and take 20 µL of the diluted sample for staining.
4. Add 80 µL of the prepared staining cocktail for the CC panel into each tube. The final volume including the fluorochrome-conjugated antibodies should be 100 µL.
5. Mix well and incubate the tubes for 10 minutes in the dark in the refrigerator (+ 2 to +8 °C).

Component	Dilution	Volume per sample
CD45 Antibody, anti-human, VioBlue REAfinity	1:50	2 µL
7-AAD Staining Solution	1:10	10 µL
Buffer	–	68 µL

Table 4: Preparation of the staining cocktail for the CC panel.

6. If the sample does not require red blood cell lysis or fixation, add 700 µL of buffer for analysis by flow cytometry.

   or

   If the sample requires red blood cell lysis but no fixation, add 700 µL of 1× Red Blood Cell Lysis Solution (for details refer to section 1.3). Close the tube and mix immediately. The final volume is 800 µL (dilution factor: 40). Incubate for at least 10 minutes in the dark at room temperature.

   ▲ **Note:** If red blood cell lysis is applied and the sample is not light red after 10 minutes, incubate for additional 2 minutes.

   or

   If the sample requires fixation, follow the user instructions 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. Follow local regulations.

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

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7. Analyze the samples by flow cytometry. Perform analysis within one hour after staining.

2.2.2 Staining protocol for immune cell composition

1. Prepare a staining cocktail according to table 5.

Component	Dilution	Volume per sample
CD45 Antibody, anti-human, VioBlue, REAfinity	1:50	2 µL
CD14 Antibody, anti-human, VioGreen, REAfinity	1:50	2 µL
TCRα/β Antibody, anti-human, FITC, REAfinity	1:50	2 µL
CD3 Antibody, anti-human, PE (BioLegend)	1:20	5 µL
CD56 Antibody, anti-human, APC	1:50	2 µL
CD19 Antibody, anti-human APC-Vio 770 REAfinity	1:50	2 µL
7-AAD Staining Solution	1:10	10 µL
FcR Blocking Reagent, human	1:5	20 µL
Buffer	–	55 µL

Table 5: Preparation of the staining cocktail for the ICC panel.

2. Label 12×75 mm flow cytometry tubes for each cell sample.

   ▲ **Note:** For the correct determination of remaining T cells after NK cell enrichment, at least 10^6 white blood cell (WBC) events have to be acquired from the enriched fraction (target cells) and optionally 10^5 WBC events for start and non-target cell fractions.

3. Transfer the required volume for 2×10^6 cells of each cellular sample into the corresponding tube.
4. Wash the cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes at room temperature. Aspirate the supernatant completely.
5. Resuspend the cells in 100 µL staining cocktail for the ICC panel per tube.
6. Mix well and incubate for 10 minutes in the dark in the refrigerator (+ 2 to +8 °C).
7. 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 requires red blood cell lysis but no fixation, add 1900 µL of 1× Red Blood Cell Lysis Solution (for details refer to section 1.3). Close the tube and mix immediately. Incubate for at least 10 minutes in the dark at room temperature.

   ▲ **Note:** If the sample is not light red after 10 minutes, incubate for additional 2 minutes.

   or

   If the sample requires fixation, follow the user instructions 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.
8. Centrifuge the cells at 300×g for 5 minutes at room temperature. Aspirate supernatant completely.
9. Resuspend the cells in 500 µL of buffer and analyze by flow cytometry.

2.2.2 Staining protocol for CAR NK cell transduction efficiency

1. Prepare staining cocktail A of the CAR NK panel according to table 6.
2. Label 12×75 mm flow cytometry tubes for each cell sample.

   ▲ **Note:** For correct determination of remaining T cells and their CAR expression at least 10^6 WBC (leukocytes) events have to be acquired.

3. Transfer the required volume for 2×10^6 cells of each cellular sample into the corresponding tube.
4. Wash the cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes at room temperature. Aspirate supernatant completely.
5. Resuspend the cells in 100 µL of the prepared staining cocktail A for the CAR NK panel (table 6) per tube.
6. Mix well and incubate for 10 minutes in the dark in the refrigerator (+2 to +8 °C).

Component	Dilution	Volume per sample
CAR Detection Reagent, human, Biotin*	1:50	2 µL
FcR Blocking Reagent, human	1:5	20 µL
Buffer	–	78 µL

* Choose one CAR Detection Reagent mentioned in table 2.

Table 6: Preparation of the staining cocktail A for the CAR NK panel.

7. Wash cells by adding 1 mL of buffer. Close the tube and mix immediately.
8. Centrifuge the cells at 300×g for 5 minutes at room temperature. Aspirate supernatant completely.
9. Wash cells a second time by repeating steps 7 and 8.
10. Prepare staining cocktail B according to table 7.
11. Resuspend the cells in 100 µL of the prepared staining cocktail B for the CAR NK panel per tube.

Component	Dilution	Volume per sample
CD45 Antibody, anti-human, VioBlue, REAfinity	1:50	2 µL
CD14 Antibody, anti-human, VioGreen, REAfinity	1:50	2 µL
Biotin Antibody, Vio Bright B515 REAfinity	1:50	2 µL
CD3 Antibody, anti-human, PE (BioLegend)	1:20	5 µL
CD56 Antibody, anti-human, APC	1:50	2 µL
7-AAD Staining Solution	1:10	10 µL
Buffer	–	97 µL

Table 7: Preparation of the staining cocktail B for the CAR NK panel.

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12. Mix well and incubate the tubes for 10 minutes in the dark in the refrigerator (+2 to +8 °C).
13. Wash cells by adding 1 mL of buffer. Close the tube and mix immediately.
14. Centrifuge the cells at 300×g for 5 minutes at room temperature. Aspirate supernatant completely.
15. If the sample does not require fixation, resuspend cell pellet in 500 µL of buffer for analysis by flow cytometry.

    or

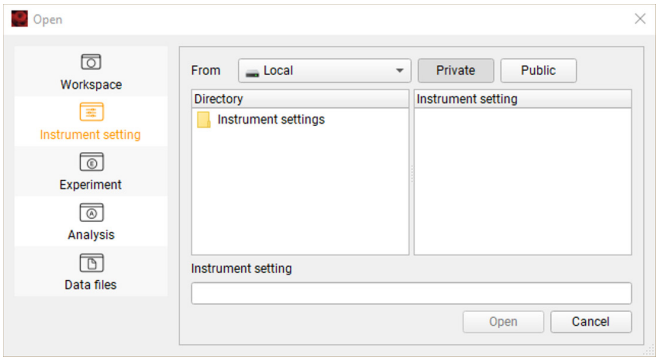
    If the sample requires fixation, follow the user instruction of an appropriate fixative reagent, e.g., Inside Fix, a component of the Inside Stain Kit. In case Inside Fix is used, it is recommended to adjust the sample volume to 250 µL with buffer prior to adding 250 µL of Inside Fix. Mix well and incubate for 20 min at room temperature in the dark. Add 1500 µL of buffer for analysis.
16. Store samples at +2 to +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

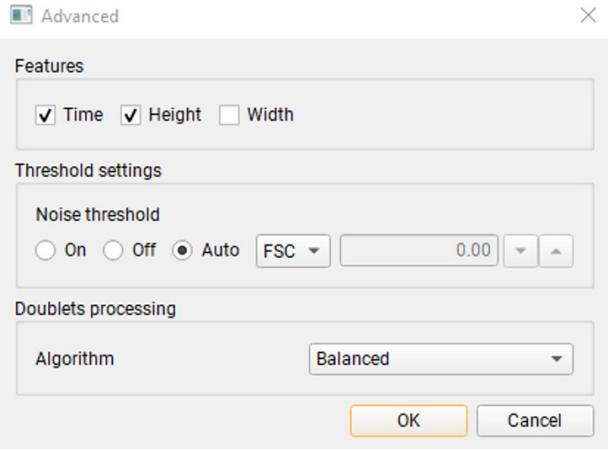
3.1 Data acquisition

The MACSQuant Analyzers in conjunction with the respective NK Cell Express Mode Package or the Engineered Immune 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 **NK_Cell_Composition_h_01**, **NK_Cell_Transduction_h_01**, and **NK_Cell_Count_h_01** 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.

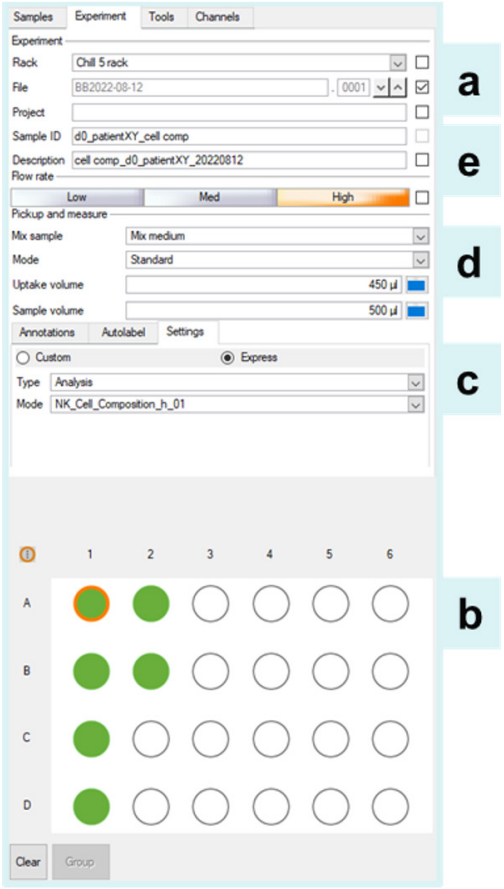
c. Select the respective Express Mode (**NK_Cell_Composition_h_01**, **NK_Cell_Transduction_h_01**, or **NK_Cell_Count_h_01**) from the drop-down list within the **Settings** tab. All pre-defined experiment settings (flow rate, annotations, sample, and uptake volume) are loaded automatically, except for the sample mixing which has to be selected manually.

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

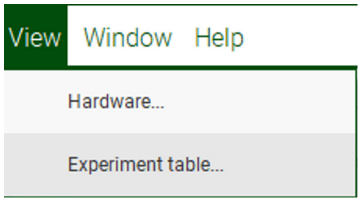
▲ **Note:** If no sample mixing is chosen, the cells might sediment before sample uptake. Sedimentation will influence the results.

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.

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



5. Mix the 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.

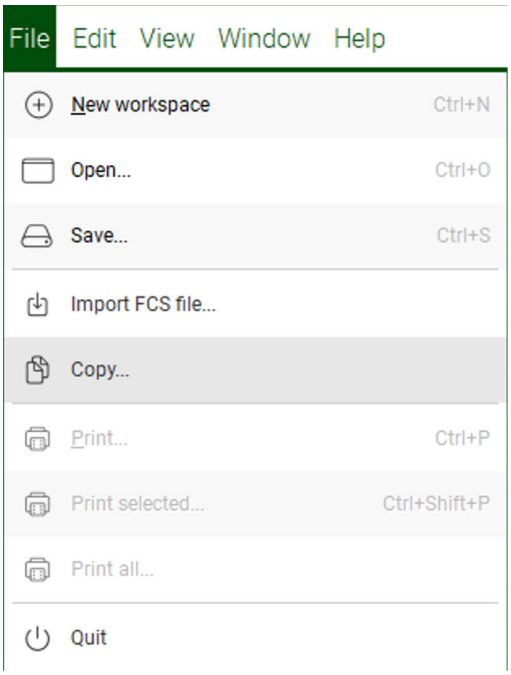
6. Check for sufficient amount of buffers and ensure that the waste bottle is empty.

7. Start the acquisition.

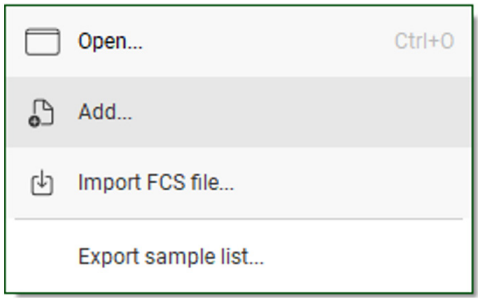
3.2 Data analysis

▲ 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 NK Cell Express Mode Package or Engineered Immune 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 PC for analysis. Go to **File**, click on **Copy,** and select the respective data files and directory for copying.

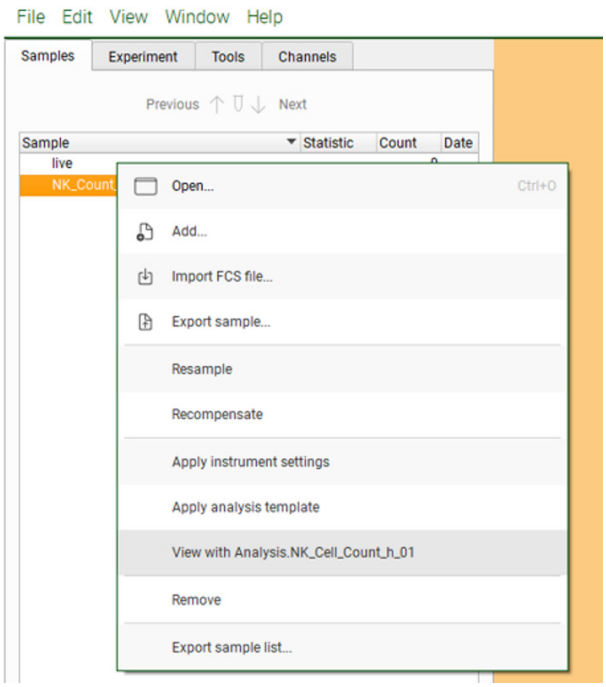


2. On the analysis PC, right click within the **Samples** tab and select **Add...** to upload data files to the MACSQuantify Software.

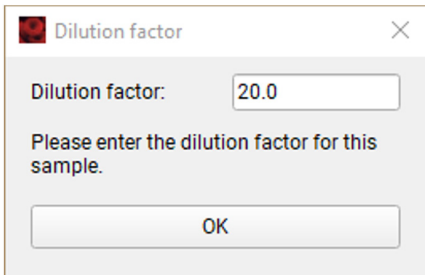


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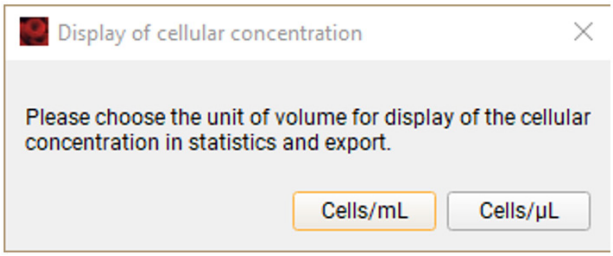
3. Right click on the first data file and select **View with Analysis. NK_Cell_Composition_h_01** or **View with Analysis.NK_Cell_Count_h_01**, respectively, for accessing the Express Mode analysis.



4. For calculation of the cell numbers, enter the dilution factor.

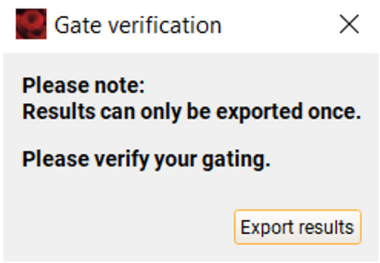


5. For display of the cellular concentration, choose the unit of the volume.



6. 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. Figures 1, 2, and 3 show the gating strategy applied.
7. 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, the **Gate verification** window is not shown and therefore data export cannot be performed.



8. By selecting **Export results**, the Express Mode software version, 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.
9. To print the analysis, go to **File** and select **Print**.
10. 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.

As shown in figures 1 and 4 the major lymphocyte subtypes, including T cells, B cells, NK cells, and NKT cells ($CD56^+$ T cells) as well as monocytes can be determined with the antibodies included in the ICC panel.

▲ **Note:** Keep in mind that populations other than NK cells might be absent when analyzing the enriched NK cell sample at day 0 or the CliniMACS Prodigy PD-56 Engineering process samples.

CliniMACS Prodigy PD-56 Engineering process samples are stained with the respective antibodies for the CAR NK panel to detect CAR^+ and CAR^- NK cells, as well as potential residual T cells that may have been transduced (see figures 3 and 5).

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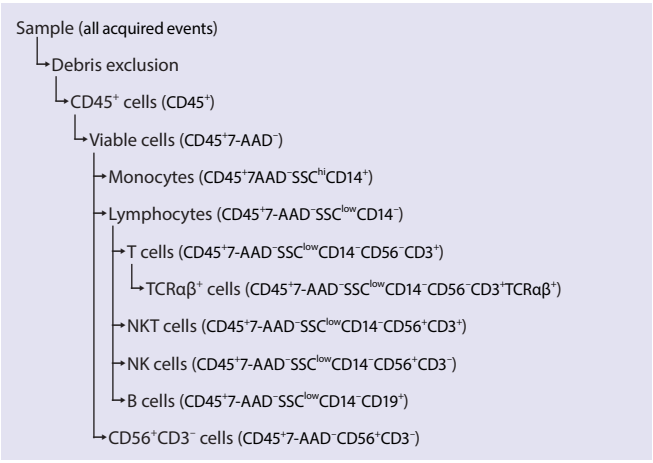


Figure 1: Hierarchical gating strategy for the NK cell composition analysis.

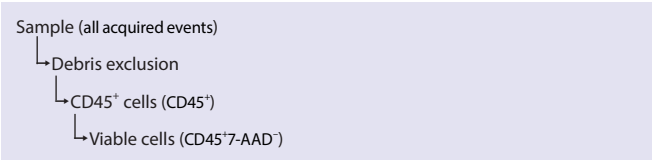


Figure 2: Hierarchical gating strategy for the NK cell count analysis.

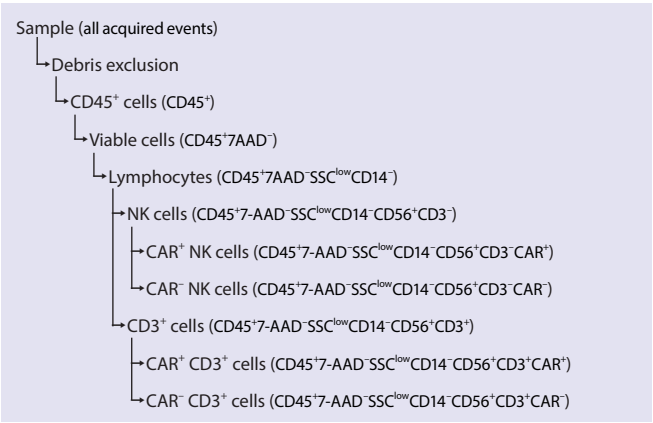


Figure 3: Hierarchical gating strategy for the CAR NK cell transduction efficiency analysis.

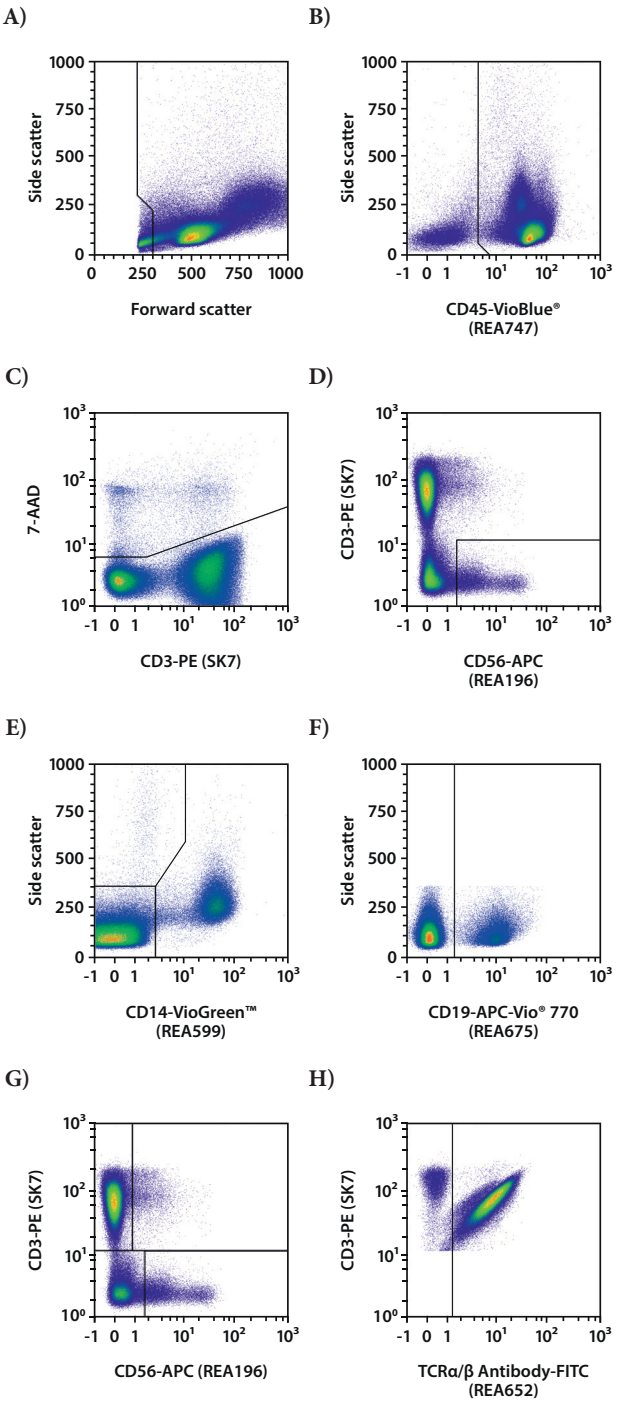


Figure 4: Gating strategy and populations that can be determined by using the **NK_Cell_Composition_h_01** Express Mode. Data shown correspond to a healthy donor fresh leukapheresis sample at day 0 prior to starting the CliniMACS Prodigy NK Cell Transduction process. After debris exclusion (A) and gating for $CD45^+$ cells (leukocytes) (B), the viable cells were selected by exclusion of $7\text{-}AAD^+$ events (C). Subsequently, $CD56^+$ and $CD3^-$ cells (D), $CD14^+$ cells (E) and $CD19^+$ cells (F) are defined. NK cells and αβ T cells are further distinguished by their differential expression of CD3, CD56, and αβ T cell receptor (TCR) (G and H).

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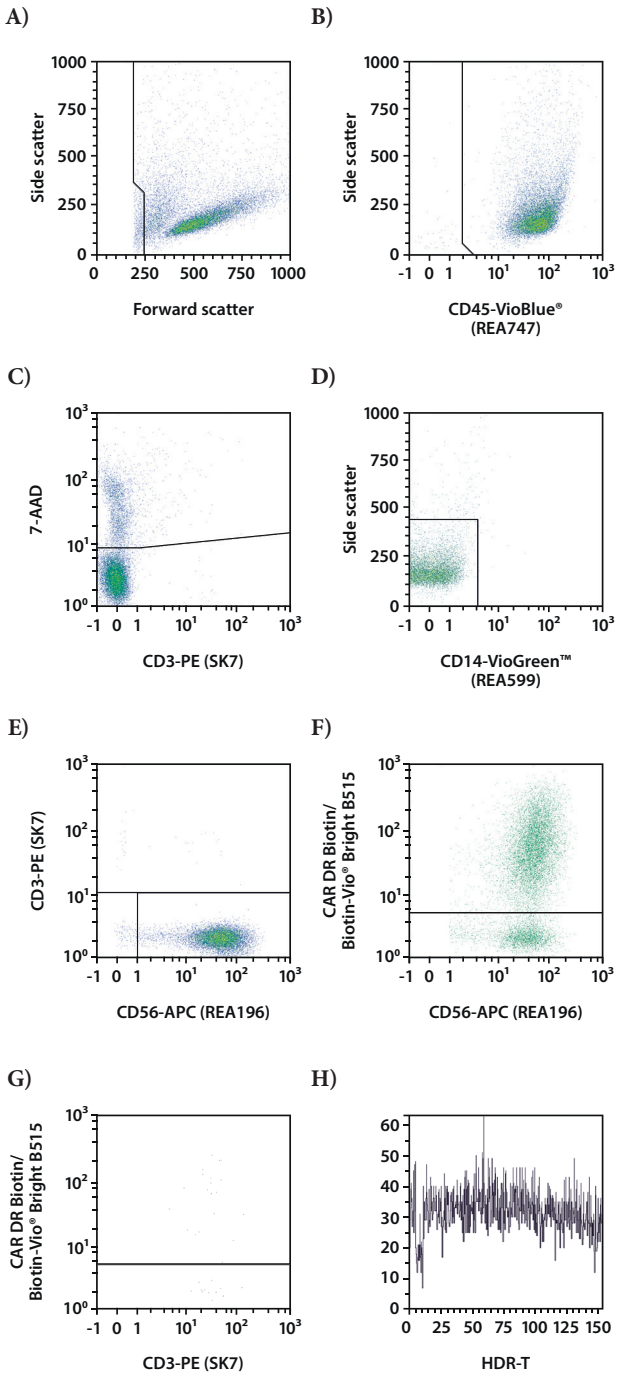


Figure 5: Gating strategy and populations that can be determined by using the **NK_Cell_Transduction_h_01** Express Mode. Data shown correspond to primary human NK cells isolated from fresh leukapheresis by CD56 enrichment and CD3 depletion, transduced with a CAR construct and expanded. After debris exclusion (A) and gating for $CD45^+$ cells (leukocytes) (B), the viable cells were selected by exclusion of $7\text{-}AAD^+$ events (C). Subsequently, $CD14^+$ cells are excluded from the analysis to define the lymphocyte populations (D). Next $CD56^+$ $CD3^-$ cells and $CD3^+$ cells are gated (E) and the expression of CAR is analyzed in those cells (F and G). HDR-T histogram is shown (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 to 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 $CD56^+CD3^-$ cells) is calculated using the MACSQuant ICC Analysis Report for the $CD56^+CD3^-$ frequency and it is suitable for the determination of rare cell populations (e.g. $TCR\alpha\beta^+$ T cells) in the final cell product. In addition, the MACSQuant NK CC Analysis Report in combination with the dilution factor enables an accurate determination of the WBC concentration.

▲ **Note:** If a dilution factor was entered during the Express Mode analysis and the unit of cell concentration cells/mL was selected, the dilution factor can be excluded from the calculation.

Final $CD56^+CD3^-$ cell concentration (cells/µL) =

WBC concentration (cells/µL) × Total dilution factor × $CD56^+CD3^-$ cell frequency (%)

To determine the final cell concentration of $CD56^+CD3^-$ per mL (cells/mL), multiply the final cell concentration of $CD56^+CD3^-$ cells (cells/µL) by 1000.

Final $CD56^+CD3^-$ cell concentration (cells/mL) =

Concentration $CD56^+CD3^-$ cells (cells/µL) × 1,000

Calculation of total cell count

The total cell count (exemplary for $CD56^+CD3^-$ cells) is calculated using the final cell concentration and the cell product volume.

Total $CD56^+CD3^-$ cell count =

Final $CD56^+CD3^-$ cell concentration (cells/mL) × Cell product volume (mL)

Calculation of CAR^+ NK cell count

The final cell concentration of CAR^+ NK cells is calculated using the MACSQuant CAR NK Analysis Report for the CAR^+ NK cell frequency and enables the determination of rare cell populations potentially transduced (e.g. CAR^+ T cells) in the final cell product. In addition, the MACSQuant NK CC Analysis Report in combination with the dilution factor enables an accurate determination of the WBC concentration.

Final CAR^+ NK cell concentration (cells/mL) =

WBC concentration (cells/µL) × Total dilution factor × CAR^+ NK cell frequency (%)

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To determine the final cell concentration of CAR^+ NK cells (cells/mL), multiply the final cell concentration of cells (cells/µL) by 1000.

Final CAR^+ NK cell concentration (cells/mL) =

Concentration CAR^+ NK cells (cells/µL) × 1,000

The total cell count is calculated using the final cell concentration and the cell product volume.

Total CAR^+ NK cell count =

Final CAR^+ NK cell concentration (cells/mL) × Cell product volume (mL)

Calculation of CAR^- NK cell count

The total cell count of CAR^- NK cells is calculated using the MACSQuant CAR NK Analysis Report for the CAR^- NK cell frequency and enables the determination of rare cell populations (e.g. CAR^- T cells) in the final cell product. In addition, the MACSQuant NK CC Analysis Report in combination with the dilution factor enables an accurate determination of the WBC concentration.

Final CAR^- NK cell concentration (cells/mL) =

WBC concentration (cells/µL) × Total dilution factor × CAR^- NK cell frequency (%)

To determine the final cell concentration of CAR^- NK cells per mL (cells/mL), multiply the final cell concentration of cells (cells/µL) by 1000.

Final CAR^- NK cell concentration (cells/mL) =

Concentration CAR^- NK cells (cells/µL) × 1,000

The total cell count is calculated using the final cell concentration and the cell product volume.

Total CAR^- NK cell count =

Final CAR^- NK cell concentration (cells/mL) × Cell product volume (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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