Miltenyi Biotec

Proposal for NK, NKT, T, B, and CD14$^+$ cell determination by flow cytometry after CD3$^+$ cell depletion and/or CD56$^+$ cell enrichment

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 Purpose

The CliniMACS® Prodigy LP-3-56 System and CliniMACS Prodigy® NK Cell Transduction are used for isolation of NK cells by depletion of CD3$^+$ cells and enrichment of CD56$^+$ cells.

For the analysis of CD56$^+$CD3$^-$ NK cells before and after automated cell separation on the CliniMACS Prodigy Platform, we have developed two staining panels.

The cell count panel (panel CC) is used for determining cell concentration, and viability of leukocytes of the final cellular product.

The frequency panel (panel A) is used for determining the frequency of NK, NKT, T, B, and CD14$^+$ cells in the sample. Together, both panels are used to determine the total amount of NK, NKT, T, B, and CD14$^+$ cells in the cellular product. The described staining and analysis strategy was optimized for leukapheresis product as starting material and is suitable for determination of a very low percentage of remaining T and B cells in the target cell fraction.

2. Reagent and instrument requirements

2.1 Fluorescent antibodies and solutions

Product	Clone
CD45 Antibody, anti-human, VioBlue®, REAfinity™	REA747
CD14 Antibody, anti-human, VioGreen™, REAfinity	REA599
TCRα/β Antibody, anti-human, FITC, REAfinity	REA652
CD56 Antibody, anti-human, APC, REAfinity	REA196
CD19 Antibody, anti-human, APC-Vio® 770, REAfinity	REA675
CD3 Antibody, anti-human, PE (BioLegend)	SK7
7-AAD Staining Solution (# 130-111-568)	
FcR Blocking Reagent, human (# 130-059-901)	

Table 1: Reagent overview.

Important information when working with REAfinity Antibodies

REAfinity Antibodies are recombinant antibodies that provide superior lot-to-lot consistency and purity compared to mouse or rat monoclonal antibodies. They have been recombinantly engineered to produce highly specific antibodies that do not require an FcR blocking step. REAfinity™ Antibodies have human IgG1 as their isotype. The result is that you need only one type of isotype control per fluorescent color for all REA clones, named REA Control Antibodies or clone REA293. This control is available in two formats – one for surface and one for intracellular-expressed antigens.

In case REA Control Antibodies are not at hand, performance of the FMO (fluorescence minus 1) test method is also a valuable option. Please get into contact with our flow specialists at macstec@miltenyibiotec.de to get more information in case you would like to learn more about FMO.

For compensation measurements: The MACS® Comp Bead Kit, anti-REA has been developed for optimal compensation of fluorescence spillover of fluorochrome-conjugated REAfinity Antibodies. After staining with fluorochrome-conjugated REAfinity Antibodies, the MACS Comp Beads – anti-REA can be used for automated or manual compensation along with the MACS Comp Beads – blank for the control of the negative population.

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Miltenyi Biotec B.V. & Co. KG
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macs@miltenyibiotec.de
www.miltenyibiotec.com

2.2 Buffer and solutions

Product	Order no.
CliniMACS® PBS/EDTA Buffer or autoMACS® Rinsing Solution	200-070-025 or 130-091-222
MACS® BSA Stock Solution or Human serum albumin (HSA)	130-091-376
Red Blood Cell Lysis Solution (10×)	130-094-183
Double-distilled water (ddH$_2$O)	

Table 2: Buffers and solutions overview.

2.3 Equipment and disposables

Product	Order no.
MACSQuant® Analyzer 10 or MACSQuant Analyzer 16	130-096-343 130-109-803
Pipette tips, appropriate sizes	
12×75 mm FACS tubes	
Vortex mixer	
Refrigerator	
Automated hematology analyzer	
Table top centrifuge	

Table 3: Equipment and disposables overview.

2.4 Preparation of solutions

- 1× Red Blood Cell Lysis Solution
  To prepare 1× Red Blood Cell Lysis Solution, add 2.5 mL Red Blood Cell Lysis Solution (10×) to 22.5 mL of double-distilled water, and mix well. Store at room temperature (19–25 °C). Discard after use.
- Buffer: PBS/EDTA Buffer or autoMACS® Rinsing Solution supplemented with HSA or BSA, final concentration 0.5%.

3. Staining protocol

An analysis of NK, NKT, T, B, and CD14$^+$ cells is performed by fluorochrome-conjugated monoclonal antibody labeling of the target cells followed by determination using a flow cytometer.

For analysis purpose take aliquots (approximately 0.5 mL of starting material and original fraction and at least 1.5 mL of all separated fractions) from the following samples:

Sample	Referred to as
Leukapheresis product: before labeling and washing and prior separation	Starting material (start)
Target cells, e.g. after CD3 depletion with or without subsequent CD56 enrichment	Target cell fraction: → analyze triplicates
(Optional) Non-target cells	Non–target cell fraction
(Optional) Waste fraction	Waste fraction

Table 4: Samples overview.

For the flow cytometry 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 5.

Panel CC Cell count	Panel A Frequency
CD45 Antibody, anti-human, VioBlue®, REAfinity™	CD45 Antibody, anti-human, VioBlue, REAfinity
	CD14 Antibody, anti-human, VioGreen™, REAfinity
	TCRα/β Antibody, anti-human, FITC, REAfinity
	CD56 Antibody, anti-human, APC, REAfinity
	CD19 Antibody, anti-human, APC-Vio® 770, REAfinity
	CD3 Antibody, anti-human, PE (BioLegend)
7-AAD Staining Solution	7-AAD Staining Solution
	FcR Blocking Reagent

Table 5: Antibody panels overview.

3.1 Staining with panel "CC" to determine the cell count

▲ The sample should not be older than 24 hours.

1. Prepare a master mix of fluorochrome-conjugated antibodies according to table 6.
2. Label one 12×75 mm FACS tube for each cell sample which is to be analyzed as follows:
   (1) Starting material
   (2) Target cell fraction → **triplicates**
   (Optional)
   (3) Non-target cell fraction
   (4) Waste fraction
3. Transfer 20 µL of each fraction into the tubes.
   ▲ **Note:** If the cell count of a sample exceeds 5×10^7/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 panel CC 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 (2–8 °C).
6. Lyse red blood cells by adding 700 µL of 1× Red Blood Cell Lysis Solution (for details refer to section 2.4). Close the tube and mix immediately. The final volume is 800 µL (dilution factor: 40).
7. 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.
8. Analyze the samples by flow cytometry. The analysis should be performed within one hour after staining.

Fluorochrome-conjugated antibody	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 6: Preparation of master mix for panel CC.

3.2 Staining with panel "A" to determine the frequency of NK, NKT, T, B, and CD14$^+$ cells

1. Prepare a master mix of fluorochrome-conjugated antibodies according to table 7.
2. Label one 12×75 mm FACS tube for each cell sample which is to be analyzed as follows:
   (1) Starting material
   (2) Target cell fraction: → **triplicates**

   (Optional)

   (3) Non-target cell fraction
   (4) Waste fraction

   ▲ Note: For correct determination of remaining T cells in the target cell fraction at least 10^6 WBC events have to be acquired (10^5 WBC events for cell fractions: start and non-target cell fraction).
3. Transfer the required volume for 2×10^6 cells of each fraction into the tubes and wash the cells by adding 1 mL of buffer.
4. Centrifuge the cells at 300×g for 5 minutes at room temperature. Aspirate the supernatants completely.
5. Resuspend the cells in 100 µL of the prepared staining cocktail for the panel A into each tube.
6. Mix well and incubate the tubes for 10 minutes in the dark in the refrigerator (2–8 °C).
7. Lyse red blood cells by adding 2 mL of 1× Red Blood Cell Lysis Solution (for details refer to section 2.4). Close the tube and mix immediately.
8. 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.
9. Centrifuge the cells at 300×g for 5 minutes at room temperature. Aspirate supernatant completely.
10. Resuspend the cells in 0.5 mL of buffer and analyze the samples by flow cytometry.

Fluorochrome-conjugated antibody	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
CD56 Antibody, anti-human, APC, REAfinity	1:50	2 µL
CD19 Antibody, anti-human, APC-Vio® 770, REAfinity	1:50	2 µL
CD3 Antibody, anti-human, PE (BioLegend)	1:20	5 µL
7-AAD Staining Solution	1:10	10 µL
FcR Blocking Reagent	1:5	20 µL
Buffer	–	55 µL

Table 7: Preparation of master mix for panel A.

4. Example of a flow cytometric data acquisition and analysis

The following protocol is designed for data acquisition and for analysis of all samples.

4.1 Acquisition and analysis software

Commercially available software can be used for data acquisition and data analysis, e.g., MACSQuantify™ Software.

4.2 Instrument settings for the staining panels

Choose an appropriate setting for the analysis of human leukocytes with a 7-color-panel. An appropriate instrument setting can be achieved by automatic compensation with VioBlue®-, VioGreen™-, FITC-, PE-, PerCP-, APC-, and APC-Vio® 770-conjugated antibodies (e.g. CD4-conjugated antibodies) using the MACSQuant® Analyzer, or by using commercially available fluorescence reference standards.

- Exclude debris from data acquisition by setting the threshold (trigger) on the FSC channel.
- Adjust scatter settings to the sample and make sure that no leukocyte population is excluded from the analysis.
- Accurate fluorescence compensation is important to obtain accurate results.

4.3 Data acquisition for the panels "CC" and "A"

The samples are analyzed using a MACSQuant Analyzer according to the following table:

	Sample	Panel CC		Panel A	
		Uptake volume [µL]	Measure rate [cells/s]	Uptake volume [µL]	Measure rate [cells/s]
1	Starting material	200	High	100	2000
–	ddH_20	–	–	200	High*
2	Target cell fraction –> triplicates	200	High	450	2000
	optional				
3	Non–target cell fraction	200	High	100	2000
4	Waste fraction	200	High	450	2000

Table 8: Acquisition overview.

*To avoid determination of false CD3$^+$ events, rinsing of the MACSQuantAnalyzer and the measuring of ddH_2O prior to the measurement of the target cell fraction is recommended. Rinsing of the MACSQuant Analyzer by using the clean button or changing from standard to extended washing mode.

4.4 Creation of dot plots

4.4.1 Creation of dot plots for panel "CC"

For data acquisition create the following dot plots (the regions are set according to the figures shown below). The x-axis is mentioned first.

A Plot: Forward scatter versus side scatter

B Plot: CD45 Antibody, anti-human, VioBlue® versus side scatter

C Plot: PE channel versus 7-AAD Staining Solution

Definition of logical gates

Define and label the gates as described in the following:

Gate	Label	Defined population
G1	Debris exclusion	FSC small events excluded
G2	CD45$^+$ cells	FSC small events excluded and CD45$^+$ cells
G3	Viable CD45$^+$ cells	CD45$^+$ and 7-AAD$^-$ cells

Table 9: Gate definition for panel CC.

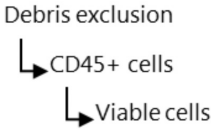


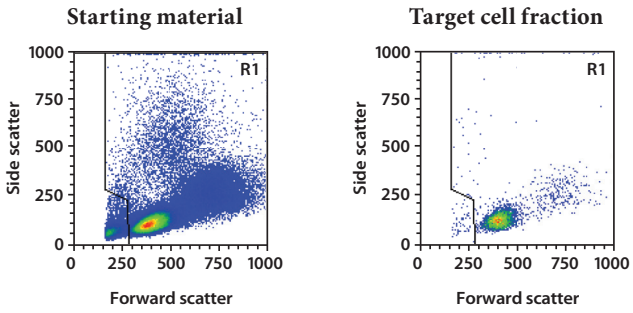
Figure 1: Hierarchical gating strategy for panel CC according to table 9.

Description of the detailed gating strategy

A Plot: Forward scatter (FSC) versus side scatter (SSC) – Exclusion of FSC small events

Activated gate: no gate.

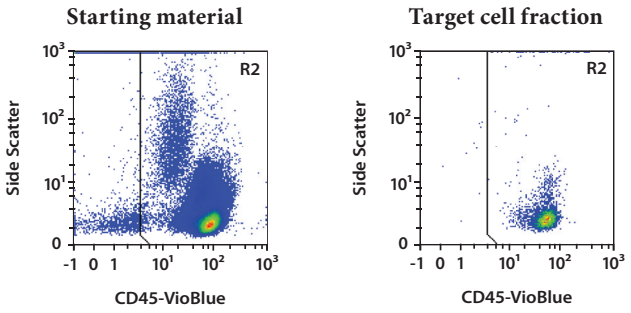
Set region "debris exclusion" (R1) including all leukocytes (WBCs) while excluding debris.



B Plot: CD45 Antibody, anti-human, VioBlue® versus side scatter (SSC) – Identification of CD45$^+$ cells (leukocytes)

Activated gate: G1= FSC small events excluded.

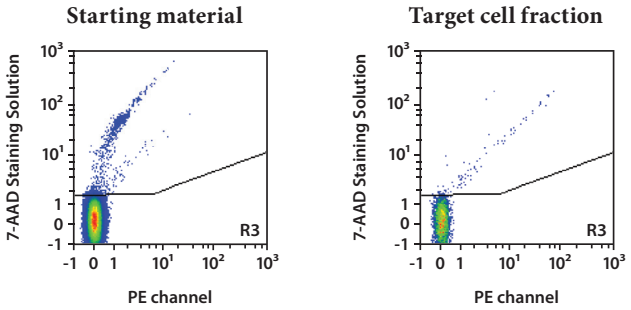
Set region "CD45$^+$ cells" (R2) to exclude CD45$^-$ events and include all CD45$^+$ cells.



C Plot: PE channel versus 7-AAD Staining Solution – Identification of viable leukocytes

Activated gate: G2=FSC small events excluded and CD45$^+$ cells included.

Define region "viable cells" (R3) thereby including all 7-AAD$^-$ viable leukocytes.



4.4.2 Creation of dot plots for panel "A"

For data acquisition create the following dot plots (the regions are set according to the figures shown below). The x-axis is mentioned first.

A Plot: Forward scatter (FSC) versus side scatter (SSC)

B Plot: CD45 Antibody, anti-human, VioBlue®, REAfinity™ versus side scatter (SSC)

C Plot: PE versus 7-AAD Staining Solution

D Plot: CD56 Antibody, anti-human, APC, REAfinity versus CD3 Antibody, anti-human, PE

E Plot: CD14 Antibody, anti-human, VioGreen™, REAfinity versus side scatter (SSC)

F Plot: CD56 Antibody, anti-human, APC, REAfinity versus CD3 Antibody, anti-human, PE

G Plot: TCRα/β Antibody, anti-human, FITC, REAfinity versus CD3 Antibody, anti-human, PE

H Plot: CD19 Antibody, anti-human, APC-Vio® 770, REAFinity versus side scatter (SSC)

Definition of logical gates

Define and label the gates as described in the following:

Gate	Label	Definition
G1	Debris exclusion	FSC small events excluded
G2	$CD45^+$ cells	FSC small events excluded and $CD45^+$ cells
G3	Viable $CD45^+$ cells	Viable $CD45^+$ and 7-AAD^- cells
G4	$CD56^+CD3^-$ cells	Viable $CD56^+CD3^-$ cells
G5	Monocytes	Viable $CD14^+$ cells
G6	Lymphocytes	Viable SSC^{low} $CD14^-$ cells
G7	NK cells	Viable SSC^{low} $CD14^-$ $CD56^+CD3^-$ cells
G8	NKT cells	Viable SSC^{low} $CD14^-$ $CD56^+CD3^+$ cells
G9	T cells	Viable SSC^{low} $CD14^-$ $CD3^+$ cells
G10	TCRα/$β^+$ cells	Viable SSC^{low} $CD14^-$ $CD3^+$ TCRα/$β^+$ cells
G11	B cells	Viable SSC^{low} $CD14^-$ $CD19^+$ cells

Table 10: Gate definition for panel CC.

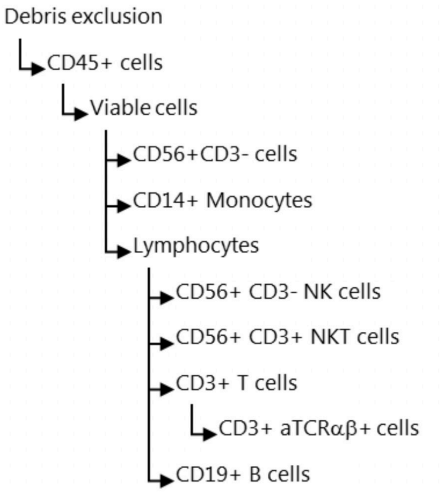


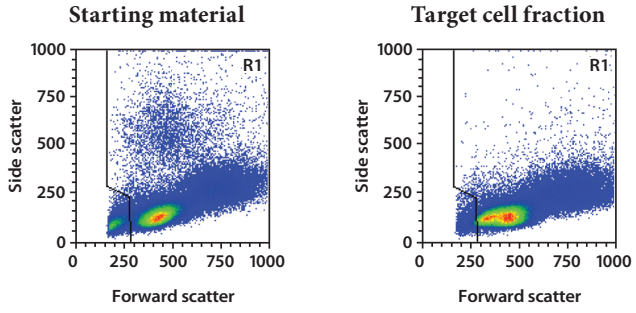
Figure 2: Hierarchical gating strategy for panel A according to table 10.

Description of the detailed gating strategy

A Plot: Forward scatter (FSC) versus side scatter (SSC) – Exclusion of FSC small events

Activated gate: no gate.

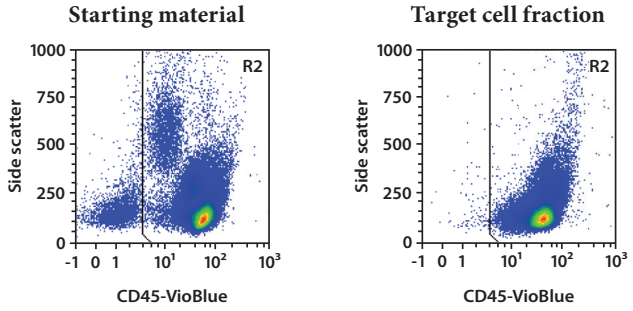
Set region "debris exclusion" (R1) including all leukocytes (WBCs) while excluding debris.



B Plot: CD45 Antibody, anti-human, VioBlue®, REAfinity™ versus side scatter (SSC) – Identification of $CD45^+$ cells (leukocytes)

Activated gate: G1=FSC small events excluded.

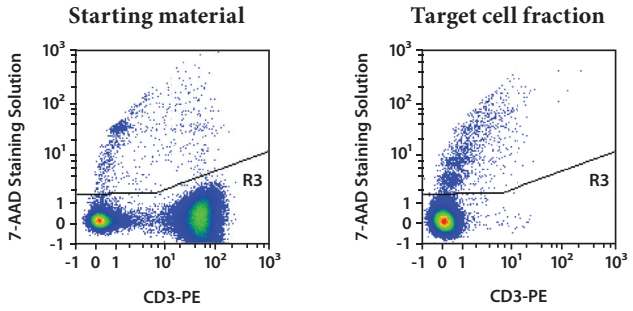
Set region '$CD45^+$ cells' (R2) to exclude $CD45^-$ events and include all $CD45^+$ cells.



C Plot: PE channel versus 7-AAD Staining Solution – Identification of viable leukocytes

Activated gate: G2=FSC small events excluded and $CD45^+$ cells.

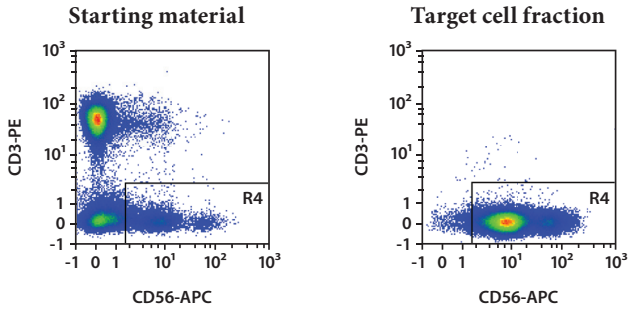
Define viable cells (R3) thereby including all 7-AAD^- viable leukocytes



D Plot: CD56 Antibody, anti-human, APC, REAfinity versus CD3 Antibody, anti-human, PE - Identification of $CD3^-$ $CD56^+$ cells

Activated gate: G3=$CD45^+$7-AAD^-.

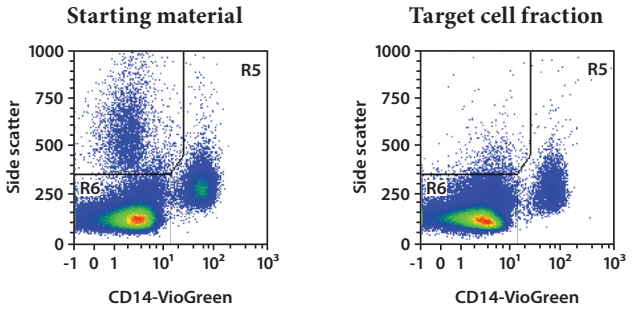
Define region "$CD56^+CD3^-$ cells" (R4) thereby including $CD3^-$ $CD56^+$ cells.



E Plot: CD14 Antibody, anti-human, VioGreen™, REAfinity™ versus side scatter (SSC) – Identification of CD14$^+$ cells and lymphocytes

Activated gate: G3=R1*R2*R3= CD45$^+$7-AAD$^-$ cells.

Define region "monocytes" (R5) thereby including CD14$^+$ cells. Define region "lymphocytes" (R6) thereby excluding CD14$^+$ and SSC^{high} cells.



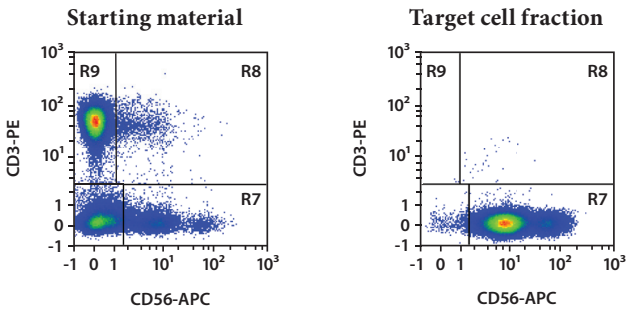
F Plot: CD56 Antibody, anti-human, APC, REAfinity versus CD3 Antibody, anti-human, PE – Identification of CD3$^-$ CD56$^+$CD14$^-$, CD3$^+$CD56$^+$CD14$^-$, and CD3$^+$CD14$^-$ cells

Activated gate: G6 = Viable SSC^{low} CD14$^-$ cells.

Define region 'NK cells' (R7) thereby including CD3$^-$CD56$^+$ cells.

Define region 'NKT cells" (R8) thereby including CD3$^+$CD56$^+$ cells.

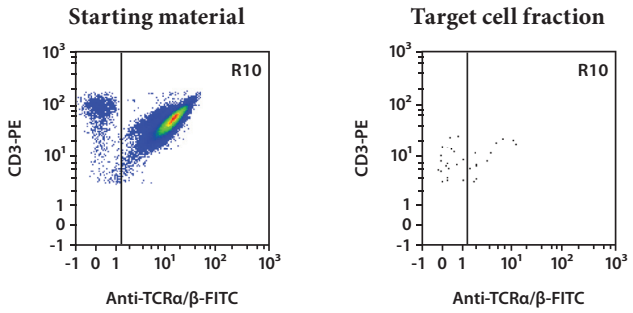
Define region 'CD3$^+$ cells' (R9) thereby including CD3$^+$ cells.



G Plot: TCRα/β Antibody, anti-human, FITC, REAfinity versus CD3 Antibody, anti-human, PE – Identification of CD3$^+$TCRα/β$^+$CD14$^-$ cells

Activated gate: G9=Viable SSC^{low}CD14$^-$CD3$^+$ cells.

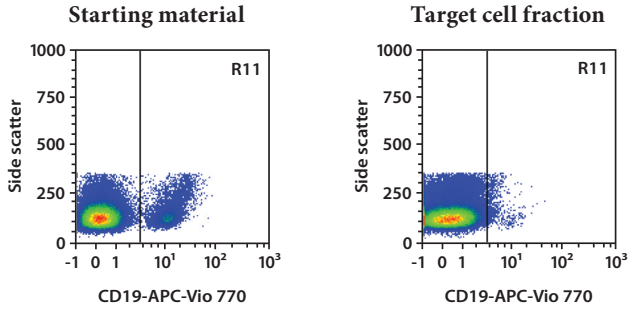
Define region "TCRα/β$^+$ cells" (R10) thereby including TCRα/β$^+$ cells.



H Plot: CD19 Antibody, anti-human, APC-Vio® 770, REAFinity versus side scatter (SSC) – Identification of CD19$^+$ cells

Activated gate: G6=Viable SSC^{low} CD14$^-$ cells.

Define region "B cells" (R11) thereby including CD19$^+$ cells.



5. Analysis of results

5.1 Calculations of cell count for CD3$^-$CD56$^+$ cells, NK, NKT, T, B, and CD14$^+$ cells and log depletion for T and B cells

For analysis of results, display a statistic of panels CC and A with following parameters:
count, count/mL, and percentage of superior gate (%#).
The following statistic (tables 11 and 12) and calculations show values determined using the starting material fraction as an example.
The grey boxes show the formula for the calculation and the blue boxes show the example calculation for the starting material fraction.

Path	%#	Count	Count/mL
	–	92028	4.60×10^5
R1	92.41	85046	4.25×10^5
R1/R2	97.36	82804	4.14×10^5
R1/R2/R3	❶ 98.15	81269	❷ 4.06×10^5

Table 11: Example statistic for the starting material fraction stained with panel CC.

Path	%#	Count	Count/mL
	–	377768	3.78×10^6
R1	90.79	342988	3.43×10^6
R1/R2	96.86	332222	3.32×10^6
R1/R2/R3	98.85	❹ 328405	3.28×10^6
R1/R2/R3/R4	10.35	❸ 33991	3.40×10^5
R1/R2/R3/R5	13.22	❺ 43421	4.34×10^5
R1/R2/R3/R6	82.22	270023	2.70×10^6
R1/R2/R3/R6/R7	10.31	❻ 27851	2.79×10^5
R1/R2/R3/R6/R8	1.84	❼ 4980	4.98×10^4
R1/R2/R3/R6/R9	76.01	❽ 205245	2.05×10^6
R1/R2/R3/R6/R9/R10	97.67	❾ 200465	2.01×10^6
R1/R2/R3/R6/R11	9.33	❿ 25203	2.52×10^5

Table 12: Example statistic for the starting material fraction stained with panel A.

5.1.1 Calculations of leukocyte cell count

The viability of WBCs can be adopted from the statistic in table 11 from R3 %# (❶).

$$\text{Viability} = \text{R3 \%\#}$$

$$\text{Viability} = 98.15\%$$

The dilution factor is calculated by multiplying the optional pre-dilution factor (e.g. 10) of the sample for the staining with the panel "CC" with the dilution factor 40 (800 µL/20 µL = 40).

$$\text{Dilution factor} = 40 \times \text{pre-dilution of sample}$$

$$\text{Dilution factor} = 40 \times 1 = 40$$

To calculate the viable WBCs [cells/mL], the count/mL R3 (❷) is multiplied with the dilution factor.

$$\text{Viable WBCs/mL} = \text{Count/mL R3} \times \text{dilution factor}$$

$$\text{Viable WBCs/mL} = 4.06 \times 10^5/\text{mL} \times 40 = 1.62 \times 10^7/\text{mL}$$

To calculate the total viable WBCs/sample, the number of viable WBCs [cells/mL] is multiplied by the sample volume [mL].

$$\text{Total viable WBCs} = \text{Viable WBCs/mL} \times \text{sample volume}$$

$$\text{Total viable WBCs} = 1.62 \times 10^7/\text{mL} \times 400\ \text{mL} = 6.48 \times 10^9$$

5.1.2 Calculation of $CD3^-CD56^+$ cell count

To calculate the frequency of $CD3^-CD56^+$ cells [%], the count of $CD3^-CD56^+$ cells (❸) is divided by the count of viable WBCs (❹) and multiplied by 100%.

$$\text{Frequency of CD3}^-\text{CD56}^+\text{ cells} = \frac{\text{Count ❸} \times 100\%}{\text{Count ❹}}$$

$$\text{Frequency of CD3}^-\text{CD56}^+\text{ cells} = \frac{33991 \times 100\%}{328405} = 10.35\%$$

To calculate the total $CD3^-CD56^+$ cells in the sample, the frequency of $CD3^-CD56^+$ cells [%] is multiplied by the total viable WBCs and divided by 100%.

$$\text{Total CD3}^-\text{CD56}^+\text{ cells} = \frac{\text{Frequency of CD3}^-\text{CD56}^+\text{ cells} \times \text{total viable WBCs}}{100\%}$$

$$\text{Total CD3}^-\text{CD56}^+\text{ cells} = \frac{10.35\% \times 6.48 \times 10^9}{100\%} = 6.71 \times 10^8$$

5.1.3 Calculation of $CD14^+$ cell count

To calculate the frequency of $CD14^+$ cells [%], the count of $CD14^+$ cells (❺) is divided by the count of viable WBCs (❹) and multiplied by 100%.

$$\text{Frequency of CD14}^+\text{ cells} = \frac{\text{Count ❺} \times 100\%}{\text{Count ❹}}$$

$$\text{Frequency of CD14}^+\text{ cells} = \frac{43421 \times 100\%}{328405} = 13.22\%$$

To calculate the total $CD14^+$ cells in the sample, the frequency of $CD14^+$ cells [%] is multiplied by the total viable WBCs and divided by 100%.

$$\text{Total CD14}^+\text{ cells} = \frac{\text{Frequency of CD14}^+\text{ cells} \times \text{total viable WBCs}}{100\%}$$

$$\text{Total CD14}^+\text{ cells} = \frac{13.22\% \times 6.48 \times 10^9}{100\%} = 8.57 \times 10^8$$

5.1.4 Calculation of NK cell count

To calculate the frequency of NK cells [%], the count of $CD3^-CD56^+CD14^-$ cells (❻) is divided by the count of viable WBCs (❹) and multiplied by 100%.

$$\text{Frequency of CD3}^-\text{CD56}^+\text{CD14}^-\text{ cells} = \frac{\text{Count ❻} \times 100\%}{\text{Count ❹}}$$

$$\text{Frequency of CD3}^-\text{CD56}^+\text{CD14}^-\text{ cells} = \frac{27851 \times 100\%}{328405} = 8.48\%$$

To calculate the total NK cells in the sample, the frequency of $CD3^-CD56^+CD14^-$ cells [%] is multiplied by the total viable WBCs and divided by 100%.

$$\text{Total CD3}^-\text{CD56}^+\text{CD14}^-\text{ cells} = \frac{\text{Frequency of CD3}^-\text{CD56}^+\text{CD14}^-\text{ cells} \times \text{total viable WBCs}}{100\%}$$

$$\text{Total CD3}^-\text{CD56}^+\text{CD14}^-\text{ cells} = \frac{8.48\% \times 6.48 \times 10^9}{100\%} = 5.50 \times 10^8$$

5.1.5 Calculation of NKT cell count

To calculate the frequency of NKT cells [%], the count of $CD3^+CD56^+CD14^-$ cells (❼) is divided by the count of viable WBCs (❹) and multiplied by 100%.

$$\text{Frequency of } CD3^+CD56^+CD14^- \text{ cells} = \frac{\text{Count ❼} \times 100\%}{\text{Count ❹}}$$

$$\text{Frequency of } CD3^+CD56^+CD14^- \text{ cells} = \frac{4980 \times 100\%}{328405} = 1.52\%$$

To calculate the total NKT cells in the sample, the frequency of $CD3^+CD56^+CD14^-$ cells [%] is multiplied by the total viable WBCs and divided by 100%.

$$\text{Total } CD3^+CD56^+CD14^- \text{ cells} = \frac{\text{Frequency of } CD3^+CD56^+CD14^- \text{ cells} \times \text{total viable WBCs}}{100\%}$$

$$\text{Total } CD3^+CD56^+CD14^- \text{ cells} = \frac{1.52\% \times 6.48 \times 10^9}{100\%} = 9.85 \times 10^7$$

5.1.6 Calculation of T cell count

To calculate the frequency of T cells [%], the count of $CD3^+CD14^-$ cells (❽) is divided by the count of viable WBCs (❹) and multiplied by 100%.

$$\text{Frequency of } CD3^+CD14^- \text{ cells} = \frac{\text{Count ❽} \times 100\%}{\text{Count ❹}}$$

$$\text{Frequency of } CD3^+CD14^- \text{ cells} = \frac{205245 \times 100\%}{328405} = 62.50\%$$

To calculate the total T cells in the sample, the frequency of $CD3^+CD14^-$ cells [%] is multiplied by the total viable WBCs and divided by 100%.

$$\text{Total } CD3^+CD14^- \text{ cells} = \frac{\text{Frequency of } CD3^+CD14^- \text{ cells} \times \text{total viable WBCs}}{100\%}$$

$$\text{Total } CD3^+CD14^- \text{ cells} = \frac{62.50\% \times 6.48 \times 10^9}{100\%} = 4.05 \times 10^9$$

5.1.7 Calculation of T cell -logP value

The –logP value describes the depletion efficiency of labeled cells (here $CD3^+$ T cells). To calculate the depletion efficiency the –log of the total T cells of the target fraction divided by the total T cells of the starting material fraction is determined.

Example for calculation: total $CD3^+$ cells in the target cell fraction = 2.93×10^5

▲ **Note:** For the $CD3^+$ T cells in the target cell fraction the arithmetic average of the triplicates is calculated.

$$-\log P\ (CD3^+ \text{ T cells}) = -\log \frac{(\text{total } CD3^+CD14^- \text{ cells target fraction})}{(\text{total } CD3^+CD14^- \text{ cells starting material fraction})}$$

$$-\log P\ (CD3^+ \text{ T cells}) = -\log \frac{2.93 \times 10^5}{4.05 \times 10^9} = 4.14$$

5.1.8 Calculation of TCRα/$β^+$ T cell count

To calculate the frequency of TCRα/$β^+$ cells [%], the count of TCRα/$β^+CD14^-$ cells (❾) is divided by the count of viable WBCs (❹) and multiplied by 100%.

$$\text{Frequency of TCR}\alpha/\beta^+CD14^- \text{ cells} = \frac{\text{Count ❾} \times 100\%}{\text{Count ❹}}$$

$$\text{Frequency of TCR}\alpha/\beta^+CD14^- \text{ cells} = \frac{200465 \times 100\%}{328405} = 61.04\%$$

To calculate the total TCRα/$β^+$ T cells in the sample, the frequency of TCRα/$β^+$ cells [%] is multiplied by the total viable WBCs and divided by 100%.

$$\text{Total TCR}\alpha/\beta^+CD14^- \text{ cells} = \frac{\text{Frequency of TCR}\alpha/\beta^+CD14^- \text{ cells} \times \text{total viable WBCs}}{100\%}$$

$$\text{Total TCR}\alpha/\beta^+CD14^- \text{ cells} = \frac{61.04\% \times 6.48 \times 10^9}{100\%} = 3.96 \times 10^8$$

5.1.9 Calculation of B cell count

To calculate the frequency of B cells [%], the count of $CD19^+CD14^-$ cells (❿) is divided by the count of viable WBCs (❹) and multiplied by 100%.

$$\text{Frequency of } CD19^+CD14^- \text{ cells} = \frac{\text{Count ❿} \times 100\%}{\text{Count ❹}}$$

$$\text{Frequency of } CD19^+CD14^- \text{ cells} = \frac{25203 \times 100\%}{328405} = 7.67\%$$

To calculate the total B cells in the sample, the frequency of $CD19^+CD14^-$ cells [%] is multiplied by the total viable WBCs and divided by 100%.

$$\text{Total } CD19^+CD14^- \text{ cells} = \frac{\text{Frequency of } CD19^+CD14^- \text{ cells} \times \text{total viable WBCs}}{100\%}$$

$$\text{Total } CD19^+CD14^- \text{ cells} = \frac{7.67\% \times 6.48 \times 10^9}{100\%} = 4.97 \times 10^8$$

5.1.10 Calcualation of B cell -logP value

The –logP value describes the depletion efficiency of unwanted cells (here $CD19^+$ B cells). To calculate the depletion efficiency the –log of the total B cells of the target fraction divided by the total B cells of the starting material fraction is determined.

Example for calculation: total $CD19^+$ B cells in the target cell fraction = 1.04×10^6

▲ **Note:** For the $CD19^+$ B cells in the target cell fraction the arithmetic average of the triplicates is calculated.

$$-\log P\,(CD19^{+}\ B\ cells) = -\log \frac{(total\ CD19^{+}CD14^{-}\ cells\ target\ fraction)}{(total\ CD19^{+}CD14^{-}\ cells\ starting\ material\ fraction)}$$

$$-\log P\,(CD19^{+}\ B\ cells) = -\log \frac{1.04 \times 10^{6}}{4.97 \times 10^{8}} = 2.68$$

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.

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.