Miltenyi Biotec

Proposal for CD45RA$^+$ T cell determination and subset analysis by flow cytometry after depletion of CD45RA$^+$ T cells

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

Several possibilities to measure naive T cells exist. Flow cytometric analysis can be performed by different gating strategies. In flow cytometric assays, a clear distinction of CD45RA$^+$ naive T cells from CD45RO$^+$ memory T cells might be imprecise when measuring CD3$^+$CD45RA$^+$ cells versus CD3$^+$CD45RO$^+$ cells alone. Further detection markers might therefore be indicated. CD95 is present on effector memory T cells and central memory T cells while being absent on naive T cells. Furthermore, CD62L and CD197 (CCR7) can be used to additionally distinguish between naive and central memory T cells.[1,2] This protocol describes the determination of human CD45RA$^+$ T cells in blood products and cell separation fractions of automated cell separation process using the CliniMACS® Plus System or the CliniMACS Prodigy®.

The described staining and analysis strategy was optimized according to the special requirements of flow cytometric detection suitable for determination of a very low percentage of remaining CD45RA$^+$ T cells in the final product after the depletion of CD45RA$^+$ T cells from the starting material.

1.2 Reagent and instrument requirements

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 technicalsupport@miltenyi.com 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.

Table 1 gives an overview of extinction and excitation wave lenght and might be helpful to transfer this protocol to your existing flow cytometer.

Fluoro-chrome	Excitation laser (nm)	Ex_{max} (nm)	Em_{max} (nm)	MACSQuant Analyzer		MACSQuant VYB	
				Channel	Filter (nm)	Channel	Filter (nm)
VioBlue	405	400	452	V1	450/50	V1	450/50
VioGreen	405	388	520	V2	525/50	V2	525/50
VioBright 515	488	488	514	B1	525/50	B1	525/50
Vio 515	488	488	514	B1	525/50	B1	525/50
VioBright FITC	488	496	522	B1	525/50	B1	525/50
FITC	488	495	520	B1	525/50	B1	525/50
PE	488 or 561	565	578	B2	585/40	Y1	586/15
PE-Vio 615	488 or 561	565	619	B3	655–730	Y2	615/20
PerCP	488	482	675	B3	655–730	N/A	N/A
PerCP-Vio 700	488	482	704	B3	655–730	N/A	N/A
PE-Vio 770	488 or 561	565	775	B4	750 LP	Y4	750 LP
APC	561 or 635	652	660	R1	655–730	Y3	661/20
APC-Vio 770	561 or 635	652	775	R2	750 LP	Y4	750 LP
N/A = not available							

Table 1: Overview of fluorochromes available from Miltenyi Biotec.

SP0071.03

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- Three-laser flow cytometer (equipped with three lasers 405 nm, 488 nm, and 635 nm to allow simultaneous analysis of VioBlue®, VioGreen™, FITC, PerCP, PE-Vio® 770, and APC) and appropriate software, e.g., MACSQuant® Analyzer 10 and MACSQuantify™ Software
- Automated hematology analyzer
- Table top centrifuge
- CD45RA-APC (clone REA1047), CD45RO-FITC (clone REA611), CD45-VioBlue (clone REA747), CD3-VioGreen (clone REA613)
- (Optional) CD62L-PE (clone REA615), CD197 (CCR7)-VioBlue (clone REA546), and CD95 (FAS)-PE-Vio770 (clone DX2)
- 7-AAD Staining Solution (# 130-111-568) or Propidium Iodide Solution (# 130-093-233) for flow cytometric exclusion of dead cells
- MACS Comp Bead Kit, anti-mouse Igκ (# 130-097-900), MACS Comp Bead Kit, anti-REA (# 130-104-693)
- Buffer, e.g., CliniMACS PBS/EDTA Buffer (# 700-25) or autoMACS® Rinsing Solution (# 130-091-222)
- Human serum albumin (HSA) or bovine serum albumin (BSA)
- Red Blood Cell Lysis Solution (10×) (# 130-094-183)
  Store at 2–8 °C in a tightly closed bottle.
- ddH_2O to dilute the Red Blood Cell Lysis Solution (10×)
- Isotype controls for REAfinity™ Antibodies (clone REA293), e.g., REA Control (S)-VioBlue, REA Control (S)-FITC, REA Control (S)-PE, REA Control (S)-PE-Vio770, REA Control (S)-APC, REA Control (S)-APC-Vio770, or REA Control (S)-VioGreen.

1.3 Preparation of solutions and staining cocktails

- 1× Red Blood Cell Lysis Solution

  To prepare 1× Red Blood Cell Lysis Solution, add 4 mL Red Blood Cell Lysis Solution (10×) to 36 mL of double-distilled water, and mix well. Store at room temperature (20–25 °C). Discard after use.
- Buffer: CliniMACS PBS/EDTA Buffer or autoMACS Rinsing Solution supplemented with HSA or BSA, final concentration 0.5%.
- Prepare staining cocktail for the cell count panel (panel "cc") and for the frequency panel of CD45RA$^+$ T cells (panel "f"):
  For each sample take the following fluorochrome-conjugated antibodies and buffer. For each reagent use the volume, which is indicated in the respective data sheet. The final volume including the fluorochrome-conjugated antibodies, buffer, and 7-AAD Staining Solution has to be 20 µL for the cell count "cc" panel and 70 µL for the frequency "f" panel.
  There are two options for preparing the "f" panel. The first option includes ingredients as indicated in section "2. Cocktail for frequency "f" panel". The second option includes all ingredients from the first option and additionally those mentioned in section "3. (Optional) Staining of naive T cells". For both options adjust the final volume to 70 µL with buffer.

1. Cocktail for cellcount "cc" panel:
   - CD45-VioBlue
   - 7-AAD Staining Solution

   Add buffer to adjust the volume to 20 µL.
2. Cocktail for frequency "f" panel:
   - CD45RO-FITC
   - 7-AAD Staining Solution
   - CD45RA-APC
   - CD3-VioGreen

   Continue with step 3 or add buffer to adjust the volume to 70 µL.
3. (Optional) Staining of naive T cells:
   Optionally, the following three fluorochrome-conjugated antibodies can be added to the frequency panel "f":
   - CD62L-PE
   - CD197 (CCR7)-VioBlue
   - CD95 (FAS)-PE-Vio770

   Add buffer to adjust the volume to 70 µL.

2. Staining protocol

An analysis of CD45RA$^+$ T 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 mL of all separated fractions) from the following samples:

Sample	Referred to as
Leukapheresis product: before labeling and washing and prior depletion	Starting material (start)
(Optional) Leukapheresis product: after labeling and washing and prior depletion	Original fraction (ori)
CD45RA$^-$ target cells after CliniMACS Plus/CliniMACS Prodigy Separation.	Target cell fraction: CD45RA$^-$ cells (mainly memory T cells) → analyze triplicates
(Optional) CD45RA$^+$ non-target cells after CliniMACS Plus/ CliniMACS Prodigy Separation.	Non–target cell fraction depletion: CD45RA$^+$ T cells
(Optional) Buffer waste fraction	Buffer waste fraction (waste)

2.1 Staining with panel "cc" to determine the cell count

▲ The sample should not be older than 24 hours.

1. Label all tubes (5 mL tubes) with "cc" and additionally as follows:
   (1) Starting material (start)
   (2) Target cell fraction (Non-CD45RA$^+$ T cells) (TCF) → **triplicates**

(Optional)

   (3) Original fraction (ori)
   (4) Non-target cell fraction (CD45RA$^+$ T cells) (NTCF)
   (5) Buffer waste fraction (waste)

2. Transfer 100 µL of each fraction into the tubes.
   ▲ **Note:** If the cell count of a sample exceeds 1×10^8/mL dilute the sample 1:10 and take 100 µL of the diluted sample for staining.
3. Add 20 µL of the prepared staining cocktail for the panel "cc" into each tube (for details refer to section 1.3). The final volume including the fluorochrome-conjugated antibodies should be 120 µL.
4. Mix well and incubate the tubes for 10 minutes in the dark in the refrigerator (2–8 °C).
5. Lyse red blood cells by adding 880 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 1000 µL (dilution factor: 10).
6. Incubate for at least 10 minutes in the dark at room temperature.
   ▲ **Note:** If the sample is not red after 10 minutes, incubate for additional 2 minutes.
7. Analyze the samples by flow cytometry. The analysis should be performed within one hour after staining.

2.2 Staining with panel "f " to determine the frequency of CD45RA$^+$ T cells

1. Label all tubes (5 mL tubes) with "f" and additionally as follows:
   (1) Starting material (start)
   (2) Target cell fraction (Non-CD45RA$^+$ T cells) (TCF) → **triplicates**

(Optional)

   (3) Original fraction (ori)
   (4) Non–target cell fraction depletion (CD45RA$^+$ T cells) (NTCF)
   (5) Buffer waste fraction (waste)
   ▲ **Note:** For correct determination of remaining CD45RA$^+$ 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, ori, and non-target cell fraction depletion).
2. Transfer the required volume for 2×10^6 cells of each fraction into the tubes and wash the cells by adding 1 mL of CliniMACS® PBS/EDTA Buffer containing 0.5% HSA.
3. Centrifuge the cells at 300×g for 5 minutes at room temperature. Aspirate the supernatants completely.
4. Resuspend the cells in 50 µL of buffer.
5. Add 70 µL of the prepared staining cocktail for the panel "f" into each tube (for details refer to section 1.3). The final volume including the fluorochrome-conjugated antibodies should be 120 µL.
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 1.3). Close the tube and mix immediately.
9. Incubate for at least 10 minutes in the dark at room temperature.
   ▲ **Note:** If the sample is not red after 10 minutes, incubate for additional 2 minutes.
10. Centrifuge the cells at 300×g (±10×g) for 5 minutes at room temperature. Aspirate supernatant completely.
11. Resuspend the cells in 0.5 mL of buffer and analyze the samples by flow cytometry.

3. Example of a flow cytometric data acquisition and analysis

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

3.1 Acquisition and analysis software

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

3.2 Instrument settings for the panel cell count and frequency

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-, PE-Vio 770, PerCP- and APC-conjugated antibodies in combination with a MACS Comp Bead Kit using the MACSQuant Analyzer, or by using commercially available fluorescence reference standards.

- Exclude debris from data acquisition by setting the threshold (trigger) on the CD45-VioBlue channel for the "cc" panel.
- Exclude debris from data acquisition by setting the threshold (trigger) on the FSC channel for the "f" panel.
- 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.

3.3 Data acquisition for the panel cell count and frequency

The samples are analyzed using a MACSQuant Analyzer according to the following table after adjustment of the regions (refer to sections 3.4.1 and 3.4.2):

	Sample	Panel cell count „cc"		Panel frequency „f"	
		Uptake volume [µL]	Measure rate [cells/s]	Uptake volume [µL]	Measure rate [cells/s]
1	Starting material	200	2000	100	2000
–	ddH_20	–	–	200	high*
3	Target cell fraction, triplicates	200	2000	450	2000
	optional				
2	Original fraction	200	2000	100	2000
4	Non–target cell fraction	200	2000	300	2000
5	Buffer waste fraction	200	2000	450	2000

* To avoid determination of false $CD45RA^+$ events, rinsing of the MACSQuant Analyzer and the measuring of ddH_2O prior to the measurement of the target cell fraction is recommended. Perform rinsing of the MACSQuant Analyzer by using the **Clean button** or choosing **Extended mode** instead of **Standard mode**.

3.4 Creation of dot plots

3.4.1 Creation of dot plots for the panel cell count

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: CD45-VioBlue versus side scatter (SSC) (Region 1)

B Plot: 7-AAD Staining Solution versus side scatter (SSC) (Region 2)

C Plot: Forward scatter (FSC) versus side scatter (SSC) (Region 3)

Definition of logical gates

Define and label the gates as described in the following:

Gate	Label	Definition	
G1	$CD45^+$ cells	R1	$CD45^+$
G2	Viable $CD45^+$ cells	R1*R2	$CD45^+$*7-AAD
G3	WBCs[#]	R1*R2*Not R3	$CD45^+$*7-AAD* leukocytes

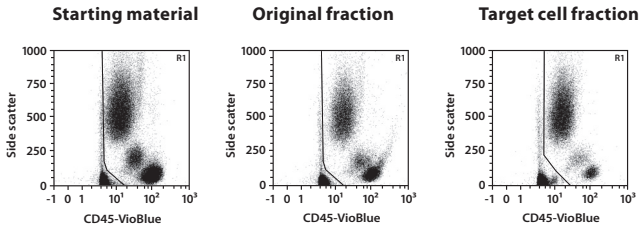
[#] WBCs = white blood cells

Description of the detailed gating strategy

A Plot: CD45-VioBlue versus side scatter (SSC) – Identification of $CD45^+$ cells (leukocytes)

Activated gate: no gate.

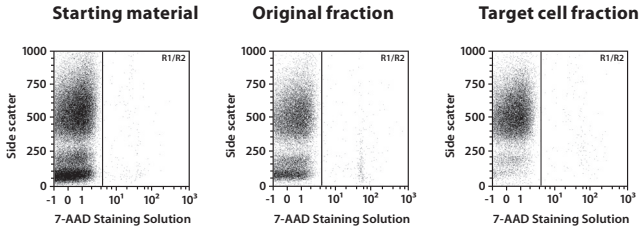
Set R1 to exclude $CD45^-$ events and include all $CD45^+$ cells



B Plot: 7-AAD Staining Solution versus side scatter (SSC) – Identification of viable $CD45^+$ cells

Activated gate: G1.

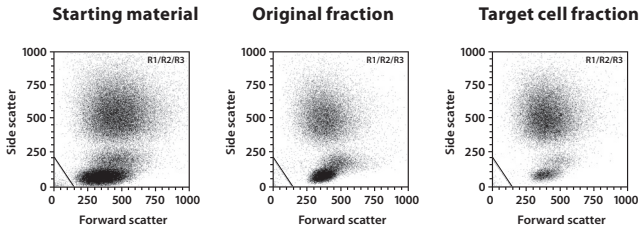
Define R2 thereby including all $7\text{-}AAD^-$ viable $CD45^+$ cells.



C Plot: Forward scatter (FSC) versus side scatter (SSC) – Exclusion of debris

Activated gate: G2=R1*R2=viable $CD45^+$ cells.

Set region R3 including all leukocytes (WBCs) while excluding debris.



3.4.2 Creation of dot plots for the panel frequency

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: 7-AAD Staining Solution versus side scatter (SSC) (R1)

B Plot: Forward scatter (FSC) versus side scatter (SSC) (R2 and R3)

C Plot: CD3-VioGreen versus side scatter (SSC) (R4)

D Plot: CD45RO-FITC versus CD45RA-APC (R5, R6, and optional R7)

(Optional)

E Plot: CD95 (FAS)-PE-Vio 770 versus CD45RO-FITC (R8)

F Plot: CD197 (CCR7)-VioBlue versus CD62L-PE (R9)

Definition of logical gates

Define and label the gates as described in the following:

Gate	Label	Definition	
G1	Viable cells	R1	7-AAD$^-$
G2	WBCs	R1*R2	7-AAD$^-$* leukocytes
G3	PBMCs‡	R1*R2*R3	7-AAD$^-$* leukocytes*PBMCs
G4	CD3$^+$ cells	R1*R2*R3*R4	7-AAD$^-$* leukocytes*PBMCs* CD3$^+$
G5	CD45RO$^+$ cells	R1*R2*R3*R4*R5	7-AAD$^-$* leukocytes*PBMCs* CD3$^+$*CD45RO$^+$
G6	CD45RA$^+$ T cells	R1*R2*R3*R4*R6	7-AAD$^-$* leukocytes*PBMCs* CD3$^+$*CD45RA$^+$
Optional			
G7	CD45RO$^-$ cells	R1*R2*R3*R4*R7	7-AAD$^-$* leukocytes*PBMCs* CD3$^+$*CD45RO$^-$
G8	CD45RO$^-$ CD95$^-$ cells	R1*R2*R3*R4*R7*R8	7-AAD$^-$* leukocytes*PBMCs* CD3$^+$*CD45RO$^-$* CD45RO$^-$CD95$^-$
G9	CD197 (CCR7)$^+$ CD62L$^+$ cells	R1*R2*R3*R4* R7*R8*R9	7-AAD$^-$* leukocytes*PBMCs* CD3$^+$*CD45RO$^-$* CD45RO$^-$CD95$^-$* CD197 (CCR7)$^+$CD62L$^+$

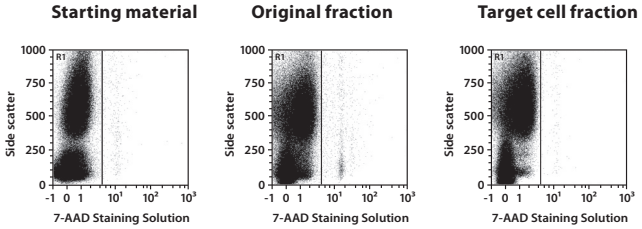
‡PBMCs = peripheral blood mononuclear cells

Description of the detailed gating strategy

A Plot: 7-AAD Staining Solution versus side scatter (SSC) – Identification of viable cells

Activated gate: no gate.

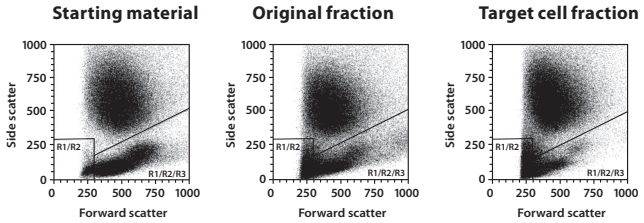
Set R1 thereby including all 7-AAD$^-$ viable cells.



B Plot: Forward scatter (FSC) versus side scatter (SSC) – Identification of peripheral blood mononuclear cells (PBMCs)

Activated gate: G1= viable cells.

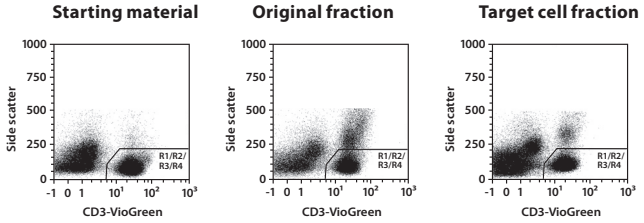
R2 is defined as an exclusion gate ("not-gate"). The events inside this region should be excluded from the further analysis.
Define R3 thereby including PBMCs.



C Plot: CD3-VioGreen versus side scatter (SSC) – Identification of CD3$^+$ cells

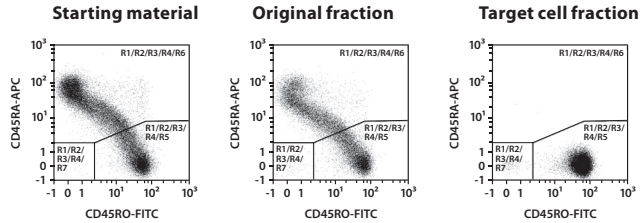
Activated gate: G3=R1*R2*R3=viable PBMCs.

Define R4 thereby including CD3$^+$SSC^{low} cells.



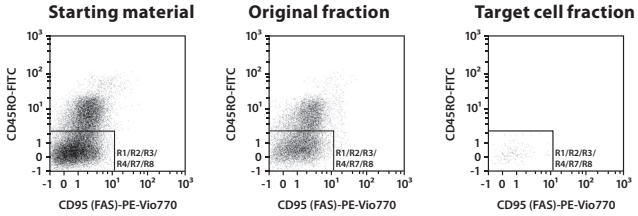
D Plot: CD45RO-FITC versus CD45RA-APC – Identification of CD45RO$^+$ and CD45RA$^+$ T cells and optional naive T cells (= CD45RO$^-$/ CD95$^-$ / CD197 (CCR-7)$^+$/ CD62L$^+$)

Activated gate: G4=R1*R2*R3*R4= CD3$^+$ cells.
Define R5 thereby including CD45RO$^+$ CD45RA$^-$ cells. It is recommended to use the target cell fraction to set the gate.
Depending to R5 CD45RO$^+$ cells, define R6 by including CD45RA$^+$ T cells.
(Optional) Define R7 by including all CD45RO$^-$ cells.



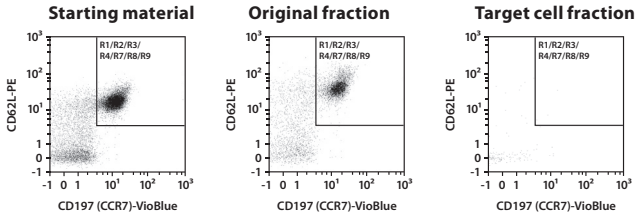
E (Optional) Plot:
CD95 (FAS)-PE-Vio 770 versus CD45RO-FITC – Identification of naive T cells (= CD45RO$^-$/ CD95$^-$ / CD197 (CCR-7)$^+$/ CD62L$^+$)

Activated gate: G7=R1*R2*R3*R4*R7= all CD45RO$^-$ cells.
Define R8 thereby including CD45RO$^-$CD95$^-$ cells.



F (Optional) Plot:
CD197 (CCR-7)-VioBlue versus CD62L-PE – Identification of naive T cells (= CD45RO$^-$/ CD95$^-$ / CD197 (CCR-7)$^+$/ CD62L$^+$)

Activated gate: G8=R1*R2*R3*R4*R7*R8= CD45RO$^-$ CD95$^-$ cells.
Define R9 thereby including CD197 (CCR7)$^+$CD62L$^+$ cells.



4. Analysis of results

4.1 Calculations of cell count and log depletion for CD45RA$^+$ T cells

For analysis of results display a statistic of panels "cc" and "f" with following parameters:
count, count/mL, and percentage of superior gate (%#).
In the following statistic (tables 2 and 3) and calculations the starting material fraction is used 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	%T	%#	Count	Count/mL
R1	27.94	27.94	101317	5.07×10^5
R1/R2	27.60	❶ 98.79	100095	5.0×10^5
R1/R2/R3	27.20	98.54	98629	❷ 4.93×10^5

Table 2: Example statistic for the starting material fraction stained with panel cell count "cc".

Path	%T	%#	Count	Count/mL
R1	99.70	99.70	292773	2.94×10^6
R1/R2	99.65	99.94	❹ 292610	2.94×10^6
R1/R2/R3	41.38	41.53	121520	1.22×10^6
R1/R2/R3/R4	20.48	49.48	60130	6.04×10^5
R1/R2/R3/R4/R5	7.51	36.66	22042	2.21×10^5
R1/R2/R3/R4/R6	12.79	62.47	❸ 37564	3.77×10^5
R1/R2/R3/R4/R7	12.78	62.39	37517	3.77×10^5
R1/R2/R3/R4/R7/R8	7.37	57.69	21642	2.17×10^5
R1/R2/R3/R4/R7/R8/R9	5.37	72.90	15776	1.58×10^5

Table 3: Example statistic for the starting material fraction stained with panel frequency "f".

The **viability of WBCs** can be adopted from the statistic in table 2 from R2 %# (❶).

Viability = R2 %#

Viability = 98.79%

The **dilution factor** is calculated by multiplying the optional pre-dilution factor (e.g. 10) of the sample for the staining with the panel cell count "cc" with the dilution factor 10 (1000 µL/100 µL = 10).

Dilution factor = 10 × pre-dilution of sample

Dilution factor = 10 × 1 = 10

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

Viable WBCs/mL = Count/mL R3 × dilution factor

Viable WBCs/mL = 4.93×10^5/mL x 10 = 4.93×10^6/mL

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

Total viable WBCs = Viable WBCs/mL × sample volume

Total viable WBCs = 4.93×10^6/mL × 188 mL = 9.27×10^8

To calculate the **frequency of CD45RA$^+$ T cells** [%], the count of CD45RA$^+$ T cells R6 (❸) is divided by the count of viable WBCs R2 (❹) (refer to table 3) and multiplied by 100%.

$$\text{Frequency of CD45RA}^+\text{ T cells} = \frac{\text{Count R6} \times 100\%}{\text{Count R2}}$$

$$\text{Frequency of CD45RA}^+\text{ T cells} = \frac{37564 \times 100\%}{292610} = 12.84\%$$

To calculate the **total CD45RA$^+$ T cells** in the sample, the frequency of CD45RA$^+$ T cells [%] is multiplied by the total viable WBCs and divided by 100%.

$$\text{Total CD45RA}^+\text{ T cells} = \frac{\text{Frequency of CD45RA}^+\text{ T cells} \times \text{total viable WBCs}}{100\%}$$

$$\text{Total CD45RA}^+\text{ T cells} = \frac{12.84\% \times 9.27\times10^8}{100\%} = 1.2\times10^8$$

The **–logP value** describes the depletion efficiency of labeled cells (here CD45RA$^+$ T cells). To calculate the depletion efficiency the –log of the total CD45RA$^+$ T cells of the target fraction divided by the total CD45RA$^+$ T cells of the starting material fraction is determined.

Example for calculation: total CD45RA$^+$ T cells in the target cell fraction = 1.95×10^3

$$-\log P = -\log \frac{(\text{total CD45RA}^+\text{ T cells target fraction})}{(\text{total CD45RA}^+\text{ T cells starting material fraction})}$$

$$-\log P = -\log \frac{1.95\times10^3}{1.2\times10^8} = 4.80$$

5. References

1. Gattinoni, L. and Restifo, N. P. (2013) Moving T memory stem cells to the clinic. Blood 121(4): 567–568.
2. Restifo, N. P. (2014) Big bang theory of stem-like T cells confirmed. Blood 124(4): 476–477.

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