



Applications for the enrichment column of the MACSQuant® Analyzer

Enumeration of circulating endothelial cells

Background

Circulating endothelial cells (cEC) are found in human peripheral blood and have been demonstrated to increase in number when vascular damage occurs in conjunction with various diseases like diabetes¹, heart disease², systemic sclerosis³, or cancer⁴. When cECs are shed from damaged vasculature into the blood, they can be detected by the expression of CD34 and CD146 and the absence of CD45 expression⁵. In healthy donors, the level of cEC expression is very low, between 1 to 20 cells/mL, creating a challenge for accurate detection by traditional flow cytometry. In the course of disease, increased numbers of cECs can be monitored in the peripheral blood. Accurate monitoring of cECs in the peripheral blood requires careful analysis of this rare cell population for the assessment of vascular injury. With the cEC Enrichment and Enumeration Kit and the MACSQuant® Analyzer, reliable assessment of the number of cECs in whole blood can be achieved by enrichment of cECs using the MACSQuant Column, a unique feature that combines the power of MACS® Technology with flow cytometry. Enrichment of cECs allows the reduction of total events that have to be analyzed, thus allowing sensitive enumeration of the total cECs present. For analysis, cECs are detected by staining with the cEC Staining Cocktail, containing CD34-FITC and CD146-APC for positive staining of cECs and CD45-PE for exclusion of lymphocytes and hematopoietic stem cells.



Figure 1: MACSQuant Column. The MACSQuant Analyzer features the enrichment of cell subsets using the MACSQuant Column prior to flow cytometric analysis and thus facilitates rare cell analysis.

Materials and methods

Reagents and solutions

- cEC Enrichment and Enumeration Kit, human (# 130-096-170)
- EDTA-anticoagulated blood (minimum of 10.2 mL)
- autoMACS® Pro Running Buffer (# 130-091-221) or prepare a phosphate buffered saline (PBS), pH 7.2, containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS Rinsing Solution (# 130-091-222); keep buffer cold (2–8 °C)
- Double-distilled water for dilutions

For one test, two samples of 5 mL of peripheral blood (cEC staining sample and isotype control sample) and one sample of 200 µL of peripheral blood (original fraction) are needed.

Materials

- MACSQuant Analyzer with MACS MiniSampler
- MACSQuant Column (# 130-094-458)
- MACSmix™ Tube Rotator
- 50 mL tubes
- 1.5 mL tubes
- Chill 5 Rack (# 130-092-951)
- Refrigerated centrifuge (2–8 °C)
- Vortex mixer
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407)

Sample preparation

The following protocol can be used for cEC enumeration out of whole blood. For best results use starting material directly after aspiration.

Before starting

Determine the total number of leukocytes per mL of blood sample. Peripheral blood should be collected in EDTA-anticoagulant. Blood samples should be stored at room temperature under agitation until analysis.

Magnetic and immunofluorescent labeling

Set up three samples as described below:

	cEC staining sample	Isotype control sample	Original fraction
Volume	5 mL whole blood	5 mL whole blood	200 µL whole blood
Purpose	Enumeration of cECs	Control of CD146 staining	Determination of CD34 ⁺ cell frequency before enrichment

cEC staining sample and isotype control sample		Original fraction
1. Take two 50 mL tubes and transfer 5 mL of whole blood into each tube (maximum of 10 ⁸ white blood cells).	1. Take one 1.5 mL tube and transfer 200 µL of whole blood into the tube.	
2. Add 45 mL of diluted (1x) Red Blood Cell Lysis Solution to each of the two tubes.	2. Add 800 µL of diluted (1x) Red Blood Cell Lysis Solution.	
3. Mix the three samples gently on an orbital shaker or MACSmix Tube Rotator (medium position; 8 rpm). Incubate all samples at room temperature for 10 minutes.		
4. Centrifuge both cell suspensions at 300xg for 10 minutes, remove both supernatants completely, and resuspend each cell pellet in 300 µL of buffer by pipetting cell suspension up and down. (Optional) Transfer the cell suspension into a 15 mL tube to simplify handling.	4. Centrifuge cell suspension at 300xg for 10 minutes, remove supernatant completely, and resuspend cell pellet in 70 µL of buffer by pipetting cell suspension up and down.	
5. Add 100 µL of FcR Blocking Reagent and 100 µL of cEC Enrichment Cocktail to each of the two samples.		
6. Mix the two samples gently and incubate both for 30 minutes in the dark in the refrigerator (2–8 °C).		
7. Add 50 µL of cEC Staining Cocktail to the cEC staining sample and 50 µL of cEC Control Cocktail to the isotype control sample.	7. Add 20 µL of FcR Blocking Reagent and 10 µL of cEC Staining Cocktail to the sample.	
8. Mix the three samples gently and incubate all samples for 10 minutes in the dark in the refrigerator (2–8 °C).		
9. Wash cells of both samples by adding 5 mL of buffer and centrifuge at 300xg for 10 minutes. Aspirate both supernatants completely.	9. Wash cells by adding 1 mL of buffer and centrifuge at 300xg for 10 minutes. Aspirate supernatant completely.	
10. Resuspend each of the three samples in 500 µL of buffer by pipetting cell suspension up and down.		

cEC enrichment and enumeration using the MACSQuant® Analyzer

- Place a pre-cooled Chill 5 Rack onto the MACS MiniSampler and select **Chill 5 tube rack** from the **Rack** drop-down menu.
- Load the instrument settings by clicking **Open, Instrument Settings** and select the correct instrument settings with compensation.
- Place the samples in the positions A1, B1, and C1 and ensure all settings are as follows:

Experiment setups	
Rack type	Chill 5 Rack, choose 3 positions
Sample ID	Position A1: cEC staining sample Position B1: control sample Position C1: original fraction
Flow rate	High
Mode	Position A1: EnrichS Position B1: EnrichS Position C1: Standard
Uptake volume	Position A1: 450 µL Position B1: 450 µL Position C1: 450 µL

Experiment setups	
Sample volume	Position A1: 450 µL Position B1: 450 µL Position C1: 500 µL
Instrument setting	PBMC compensation (needs to be generated before)
Autolabel	PI, Titer (1:100)
Annotations	Position A1: CD34-FITC, CD45-PE, CD146-APC Position B1: CD34-FITC, CD45-PE, Mouse IgG1-APC Position C1: CD34-FITC, CD45-PE, CD146-APC

- Set the autolabel function, by clicking on the **Autolabel** tab, click on **<add>** and add **Propidium iodide** from the **Universal** selection, select titer as 1:100, click **Ok**.
- PI will now be listed in the **Autolabel** tab, enable PI for all three samples.
- Start measurement.

Note: Magnetic enrichment of cECs using this kit can be performed using any of the MACS Separators, including the autoMACS Pro Separator. For full details of this process, please refer to the cEC Enrichment and Enumeration Kit data sheet (# 130-096-170).

Gating strategy

1. Open a new analysis window containing six dot plots plus the statistics display (Plot 7).

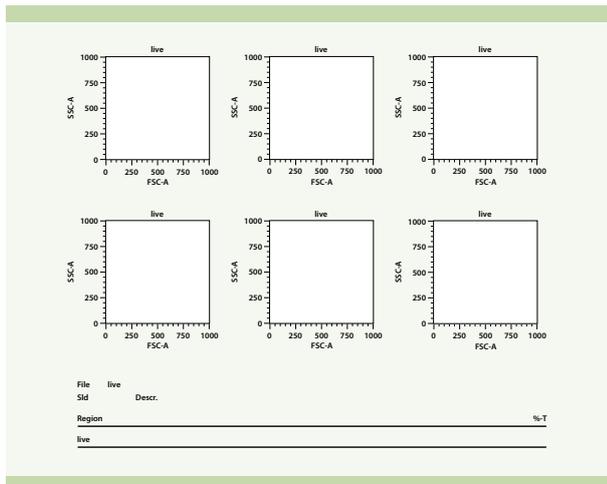


Figure 2: Open the analysis window "Plot 7" in the MACSQuantify™ Software.

2. Define the plot windows as follows:
 - a. Forward scatter (FSC) versus side scatter (SSC) – linear scale
 - b. PE versus PI (for CD45-PE versus PI) – hlog or log scale
 - c. PE versus FITC – hlog or log scale
 - d. APC versus PE – hlog or log scale

3. Gating overview

Region	Name	Gating hierarchy
P1	Leukocytes	P1
P2	Viable leukocytes	P1/P2
P3	Viable CD34 ⁺ cells	P1/P2/P3
UL4 (quadrant)	Viable CD146 ⁺ /mIgG1 cells	P1/P2/P3/UL4

Table 1: Gating strategy needed for cEc analysis.

4. With the **original fraction**, create an FSC/SSC dot plot. Draw region Population 1 (P1) to exclude debris and platelets.
5. Draw region P2 in the PE/PI dot plot gated on region P1 to exclude PI positive dead cells.
6. Draw region P3 in the PE/FITC dot plot gated on region P2 to include all CD34⁺ cells.
7. Note the displayed frequency of CD34⁺ cells in P3 (shown in the shaded region).
8. Copy all the regions into the **Isotype control** file. This can be accomplished by dragging and dropping the gates into the file in the **Samples** tab.
9. Draw a quadrant gate P4 in the APC versus PE dot plot gated on region P3 to differentiate positive and negative cells in the four quadrants. Note the displayed frequency of cells in quadrant UL4 (shown in the shaded region). This gate displays the background non-specific staining
10. Copy all the regions into the cEC staining sample file.
11. Note the displayed frequency of CD146⁺ cells in quadrant UL4 (shown in the shaded region).

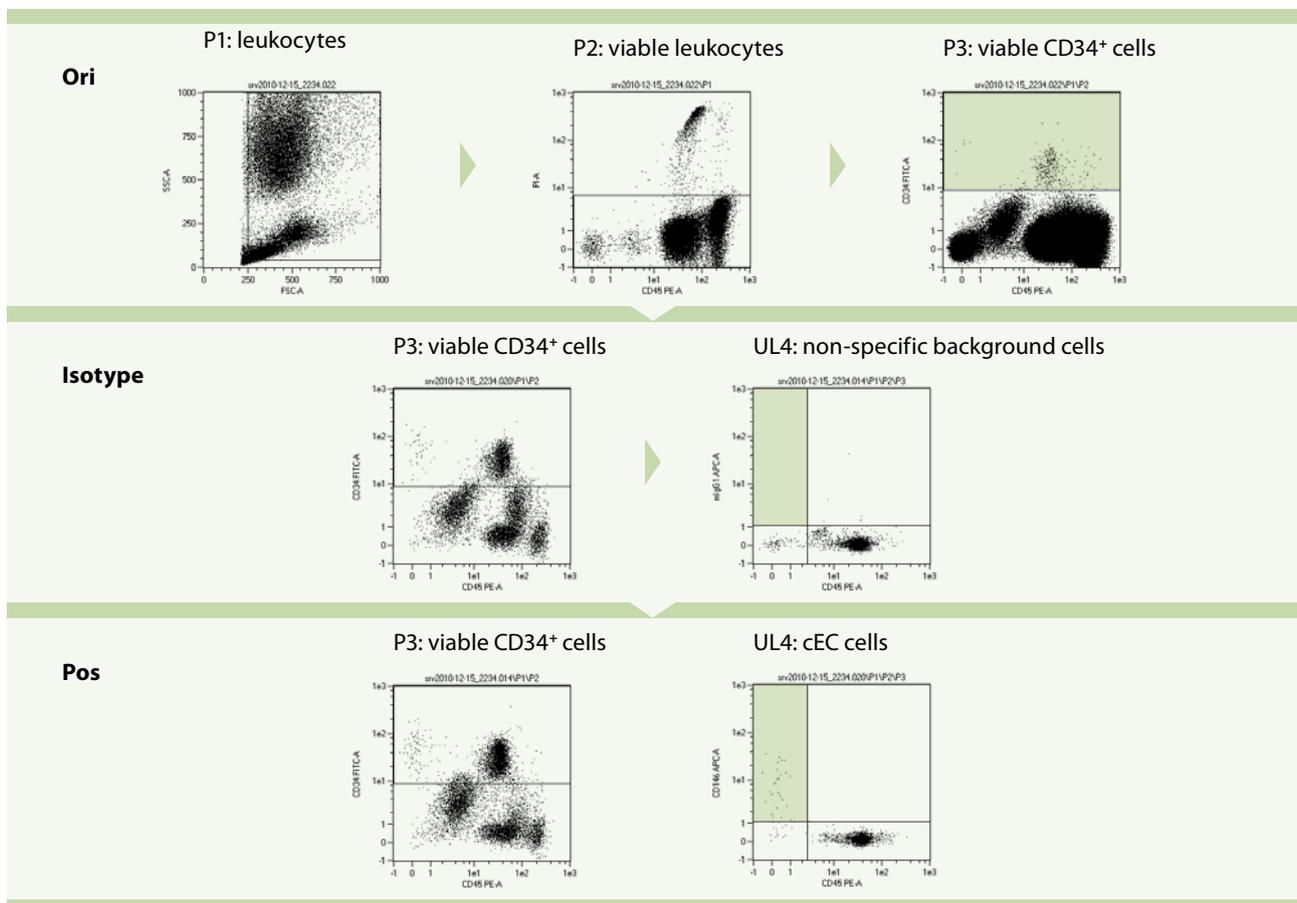


Figure 3: Gating overview. Gates were applied to original, isotype control, and positive samples.

Determination of number of CD34⁺ cells

1. Calculate the absolute number of CD34⁺ cells in 5 mL whole blood from the frequency of CD34⁺ cells in the original fraction:

$$\text{Absolute number of CD34}^+ \text{ cells in 5 mL whole blood} = (\text{absolute number of leukocytes in 5 mL sample} \times \% \text{ viable CD34}^+ \text{ cells}) / 100$$

Determination of number of cECs in 5 mL sample

1. Obtain the number of non-specifically stained events from the isotype control region UL4.
2. Subtract the percentage of gated events in the isotype control sample from the cEC sample. Calculate the absolute number of cECs in 5 mL blood from the corrected frequencies and the number of CD34⁺ cells as follows:

$$\text{Absolute number of cECs in 5 mL whole blood} = [(\% \text{ gated cEC sample} - \% \text{ gated isotype control sample}) \times \text{absolute number of CD34}^+ \text{ cells}] / 100$$

Example:

- Number of white blood cells in 5 mL whole blood: **4×10⁷**
- Frequency of CD34⁺ cells before enrichment (original fraction): **0.03%**
- Frequency of CD34⁺/mouse IgG1⁺/CD45⁻ cells (non-specific binding) (isotype control): **0%**
- Frequency of CD34⁺/CD146⁺/CD45⁻ cells (staining sample): **1.57%**

Calculation of the number of CD34⁺ cells in the starting volume:
 $(4 \times 10^7 \times 0.03\%) / 100 = 12,000$

Correction of non-specific staining:
 $1.57\% - 0\% = 1.57\%$

Calculation of the cEC number:
 $(12,000 \times 1.57\%) / 100 = 188.4$

In this example, the number of cECs in 5 mL white blood cells is 188 (= **38 cECs/mL** whole blood).

Results

The cEC Enrichment and Enumeration Kit in combination with the pre-enrichment and flow cytometric analysis on the MACSQuant Analyzer is a powerful tool to enumerate the number of cECs in 5 mL of whole blood. In the example shown, the total number of cECs in 5 mL blood is 188 or 38 cECs/mL whole blood. The cECs accounted for 1.57% of the CD34⁺ population.

Conclusions

The MACSQuant Analyzer has a novel enrichment capability that makes the enumeration of cECs fast and easy. In this example, there were 38 cECs per mL of whole blood and the entire process took less than two hours to perform from blood draw to analyzed data. After the gating strategy has been made, this can be saved for future use as an analysis template, further automating the process on the MACSQuant Analyzer.

By combining magnetic enrichment and flow cytometric analysis in one experiment, the cEC Enrichment and Enumeration Kit combined with the MACSQuant Analyzer will drastically reduce the time and difficulty of identifying and reliably monitoring this rare cell type.

References

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