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 diabetes, heart disease, systemic sclerosis, or cancer. 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 expression. 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.

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:

<table>
<thead>
<tr>
<th>cEC staining sample</th>
<th>Isotype control sample</th>
<th>Original fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>5 mL whole blood</td>
<td>200 μL whole blood</td>
</tr>
<tr>
<td>Purpose</td>
<td>Enumeration of cECs</td>
<td>Determination of CD34+ cell frequency before enrichment</td>
</tr>
</tbody>
</table>

**cEC enrichment and enumeration using the MACSQuant® Analyzer**

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

<table>
<thead>
<tr>
<th>Experiment setups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rack type</td>
</tr>
<tr>
<td>Sample ID</td>
</tr>
<tr>
<td>Flow rate</td>
</tr>
</tbody>
</table>

4. 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.
5. PI will now be listed in the Autolabel tab, enable PI for all three samples.
6. 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).

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

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

3. Gating overview

<table>
<thead>
<tr>
<th>Region</th>
<th>Name</th>
<th>Gating hierarchy</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Leukocytes</td>
<td>P1</td>
</tr>
<tr>
<td>P2</td>
<td>Viable leukocytes</td>
<td>P1/P2</td>
</tr>
<tr>
<td>P3</td>
<td>Viable CD34⁺ cells</td>
<td>P1/P2/P3</td>
</tr>
<tr>
<td>UL4 (quadrant)</td>
<td>Viable CD146⁺/mIgG1 cells</td>
<td>P1/P2/P3/UL4</td>
</tr>
</tbody>
</table>

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:

   \[
   \text{Absolute number of CD34+ cells in 5 mL whole blood} = \left( \frac{\text{absolute number of leukocytes in 5 mL sample} \times \% \text{ viable CD34+ cells}}{100} \right)
   \]

**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:

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

**Example:**

- Number of white blood cells in 5 mL whole blood: \(4 \times 10^7\)
- 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 (\(= 38 \text{ cECs/mL whole blood}\)).

**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**