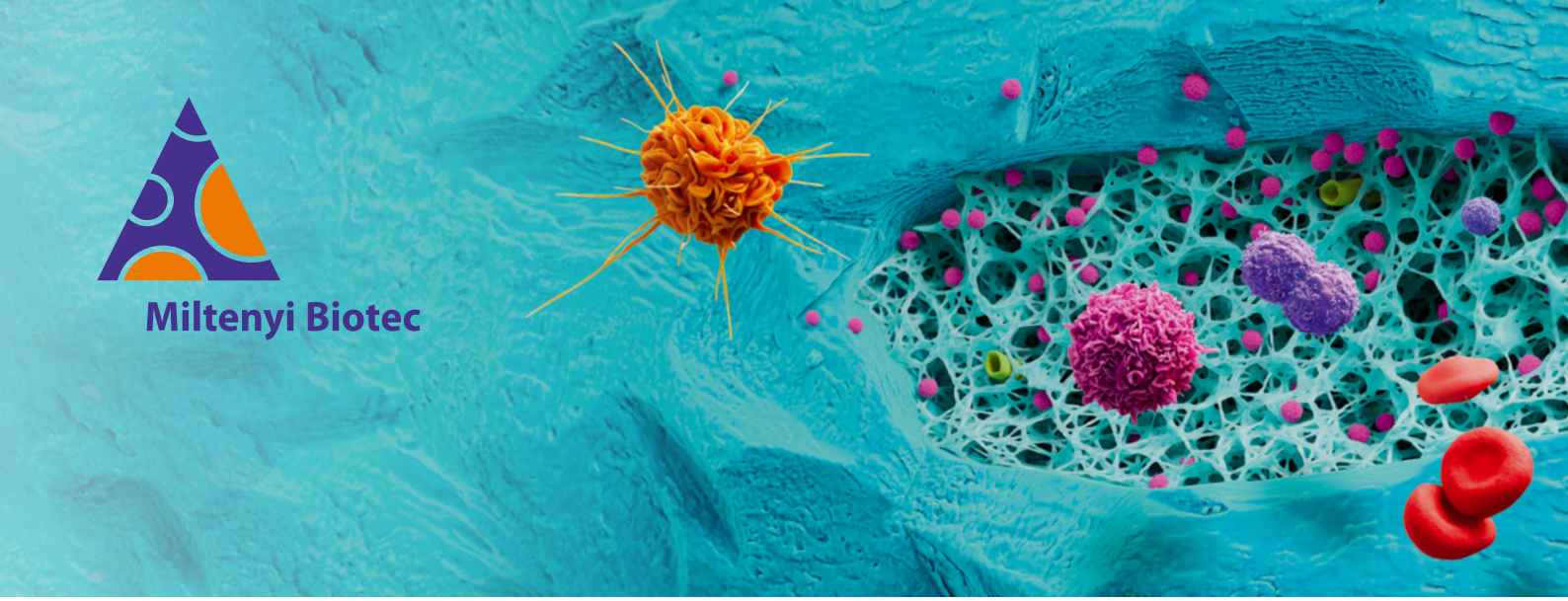
Miltenyi Biotec



StemMACS™ HSC-CFU Assay Kit, human

Flow cytometric analysis of the differentiation potential of human hematopoietic stem cells

Background

Hematopoietic stem cells (HSCs) are tissue-specific adult stem cells capable of differentiating into all blood cell types to ensure homeostasis of blood throughout life.

The current gold standard for the evaluation of the proliferation and differentiation potential of hematopoietic stem and progenitor cells (HSPCs) is the colony-forming unit (CFU) assay. In the conventional CFU assay, cells are plated in methylcellulose-based medium. After incubation for 14 days, different numbers and types of colonies (CFUs) are generated and evaluated under the microscope individually. However, the microscopic analysis in this conventional assay is highly dependent on the judgement and experience of the researcher.

The StemMACS HSC-CFU Assay Kit, human was developed to eliminate this user-dependent bias while maintaining the high-quality performance of the assay. For CFU formation, cells are diluted in methylcellulose-free StemMACS HSC-CFU Assay Medium into a round-bottom 96-well plate. Each well corresponds to the clonal progeny of a single HSC. During the incubation period, the StemMACS HSC-CFU Assay Medium promotes growth and differentiation of the cells. In order to determine the type of CFUs that are formed, each well is stained with the StemMACS HSC-CFU Assay Cocktail and analyzed by flow cytometry. By following the gating scheme described here, the type of CFU can be easily identified through the corresponding marker combination. Moreover, based on the frequency of each CFU type, the percentage of appearance can be determined versus the total number of CFUs very easily.

Taken together, the StemMACS HSC-CFU Assay Kit, human represents a perfect combination between an outstanding methylcellulose-free medium which allows easy handling, and a clear flow cytometric readout which eliminates the need for user-dependent visual scoring under a microscope.

Flow cytometric analysis with the MACSQuant® Analyzer X, in combination with a plate-feeding incubator, enables increased automation, reduced hands-on time, and high throughput sample acquisition.

In this application note, human buffy coat–derived HSPCs were analyzed using the StemMACS HSC-CFU Assay Kit, human. The kit and the procedure described here are applicable to other sources of human HSPCs, e.g., peripheral blood, cord blood, bone marrow, or mobilized leukapheresis.

Materials

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coats by density gradient centrifugation and analyzed by the StemMACS HSC-CFU Assay Kit, human (# 130-125-042).

Buffer and media

- Buffer consisting of PBS, pH 7.2, 2 mM EDTA, and 0.5% BSA (PEB) were prepared by diluting MACS® BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). The buffer was kept cold (2–8 °C)
- Dilution medium: Iscoves's Modified Dulbecco's Medium (IMDM)
- Sterile water
- Propidium Iodide Solution (# 130-093-233) for flow cytometric exclusion of dead cells without fixation
- (Optional) Red Blood Cell Lysis Solution (10×) (# 130-094-183)

Antibodies

Marker	Clone	Order no.
CD34 Antibody, anti-human, APC	AC136	130-113-176
CD45 Antibody, anti-human, FITC, REAfinity	REA747	130-110-631

Table 1: Marker panel for flow cytometry to determine the CD34 positive cell count.

Equipment

- MACSQuant® Analyzer 10
- MACSQuant Analyzer X (recommended for high throughput analysis)

Procedure

Sample preparation

15 mL of buffy coat from each of three donors were used to obtain PBMCs through density gradient centrifugation. A small sample from each resulting PBMC fraction was stained with CD34-APC and CD45-FITC in order to determine the CD34 positive cell count of the sample (fig. 1).

For each donor tested, it is recommended that three round-bottom 96-well plates are seeded with CD34⁺ cells. In this application note, twice as many plates per donor (n=3) were seeded with a concentration of 250×CD34⁺ cells per mL, to a total of 18 plates, in order to demonstrate unequivocally the robustness of the MACSQuant Analyzer X's high throughput capabilities.

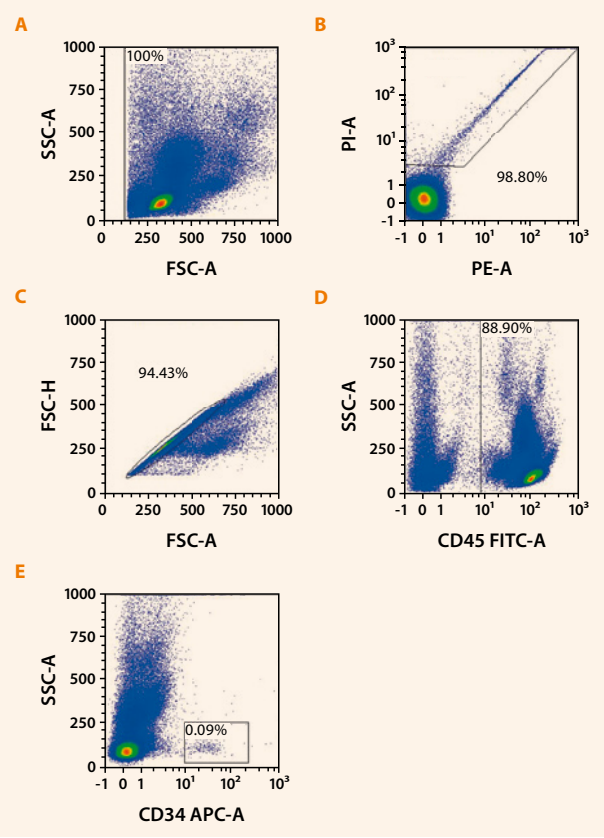


Figure 1: Gating strategy in order to determine the CD34 positive cell count. A: All cells by forward scatter (FSC-A) to side scatter (SSC-A). B: Viable cells by PE-A to propidium iodide (PI-A) channel. C: Single cells visualized by FSC-A to FSC-H. D: CD45⁺ cells by CD45 FITC-A to SSC-A (pre-gate on single cells). E: CD34⁺ cells by CD34 APC-A to SSC-A (pre-gate on CD45⁺ cells).

Experimental setup of the StemMACS™ HSC-CFU Assay Kit, human (fig. 2)

The aliquoted medium (15 mL are required for three 96-well plates) was thawed at room temperature a few hours prior to setting up the experiment.

Using a multi-channel pipette, 10 µL of cell suspension diluted in IMDM were seeded per well, followed by adding 50 µL of the StemMACS™ HSC-CFU Assay Medium (fig. 2, day 0).

The cell-seeded 96-well plates were placed in a humidity chamber containing sterile water to minimize evaporation. The humidity chamber was then placed in an incubator and incubated for 14 days at 37 °C and 5% CO_2. During this time, the cells were able to proliferate and differentiate into the different colonies.

At the end of the incubation time, the plates were stained with the StemMACS HSC-CFU Assay Cocktail (fig. 2, day 14). First, the cocktail was diluted with PEB buffer. For three 96-well plates, 45 µL of StemMACS HSC-CFU Assay Cocktail were required, diluted to a final volume of 4.5 mL. 15 µL of the diluted cocktail were added per well and incubated at 4 °C for 10 minutes. Finally, 25 µL of PEB buffer were added per well resulting in a total volume of 100 µL.

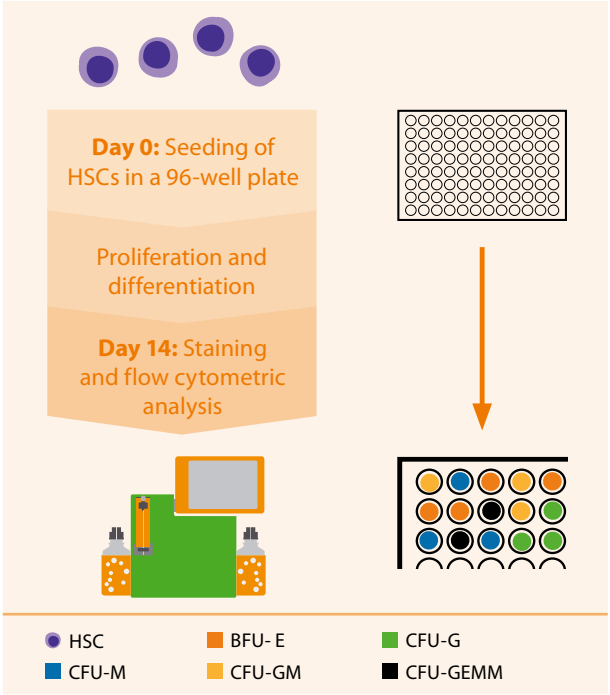


Figure 2: Workflow using the StemMACS HSC-CFU Assay Kit, human. HSCs, diluted in StemMACS HSC-CFU Assay Medium, are plated in round-bottom 96-well plates. After proliferation and differentiation for 14 days, the generated colonies are stained with the StemMACS HSC-CFU Assay Cocktail and analyzed by flow cytometry.

Flow cytometric acquisition of samples (fig. 2, day 14)
The StemMACS™ HSC-CFU Assay Cocktail contains three fluorochrome-conjugated antibodies, which allow the distinction of each colony type (table 2). As a consequence of the high dilution required to ensure that most wells are seeded initially with a single cell, 45–60 wells per 96-well plate will contain a colony and the remaining will be empty.
A small percentage of the wells might also contain 2 colonies. To balance this, at least three 96-well plates have to be seeded per donor, which further improves the statistical analysis.

Marker	% CD15$^+$ cells	% CD14$^+$ cells	% CD235a$^+$ cells
CFU-GEMM	15	15	20
CFU-GM	30	30	
CFU-M		50	
CFU-G	50		
BFU-E			50

Table 2: Detection parameters of colony types (CFU-GEMM: CFU-granulocyte erythrocyte macrophage megakaryocyte, CFU-GM: CFU-granulocyte macrophage, CFU-M: CFU-macrophage, CFU-G: CFU-granulocyte, BFU-E: burst-forming unit-erythroid). Any well which exhibits at least the shown percentages is classified as the corresponding colony type.

For this experiment, we employed a convenient way to analyze a large number of plates with increased speed and truly hands-free operation by using the MACSQuant® Analyzer X connected to an automated incubator (Cytomat™ II, Thermo Fisher). Built with speed in mind, the MACSQuant Analyzer X allows the recording of large amounts of data in a short period of time, with each sample being saved as an individual file. The setup with the automated incubator, which can continuously provide the MACSQuant Analyzer X with new plates, enables high throughput acquisition while simultaneously keeping all plates in a cooled and temperature-controlled environment, thus preserving the cells throughout the process. The automated flow-cytometric acquisition allowed the measurement of 18 plates with an average time of about 55 minutes per plate using the following parameters.

MACSQuant Analyzer X parameters

- Uptake volume: 50 µL
- Sample volume: 100 µL
- Flow rate: high
- Mix sample: Select rows A and E, select "Shake gentle" from the drop-down menu
- Mode: Select "Fast" from the drop-down menu

Setting gates for the analysis
To set the correct gating for all samples, a well with CD235a-positive events (corresponding to a BFU-E colony) was selected and the following steps performed.

- Use a forward scatter (FSC-A) vs. side scatter (SSC-A) plot to set a gate that excludes debris and includes all cells (fig. 3A).
- Open the events of gate A on a new plot and display FSC-A versus FSC-H. Gate the single cells (fig. 3B).
- Display the single cells in two new plots (fig. 3C and D). On the first plot (fig. 3C), adjust the y-axis to CD15-APC channel and the x-axis to CD235a-PE channel. On the second plot (fig. 3D) adjust the y-axis to CD15-APC channel and the x-axis to CD14-VioBlue® channel. Set a quadrant to separate the populations as shown in figure 3C and D.

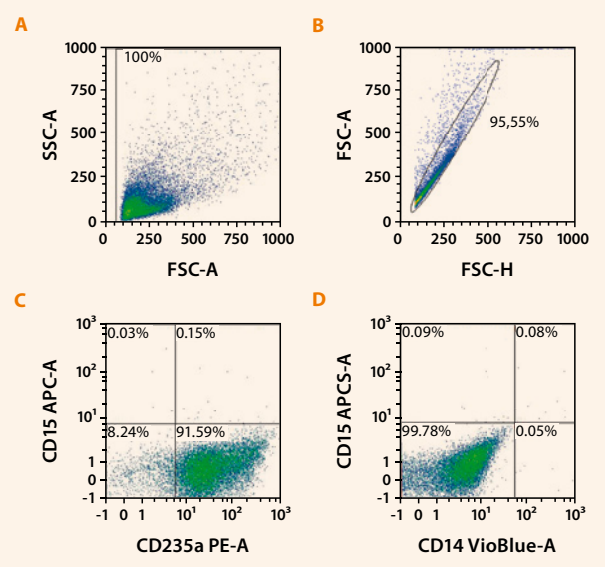


Figure 3: Setting gates for the analysis.

Data analysis
Once the gates are set for each colony (BFU-E, CFU-G, CFU-M, CFU-GM, CFU-GEMM), the same gates can be applied for all samples (fig. 4). The most important information on the plots are the percentages of CD14$^+$, CD15$^+$, and CD235a$^+$ cells, which can be displayed on each quadrant and exported to an excel spreadsheet. According to the percentages of CD14$^+$, CD15$^+$, and CD235a$^+$, the colony type is determined (table 2).
The results from all plates were analyzed and summarized in figure 5.

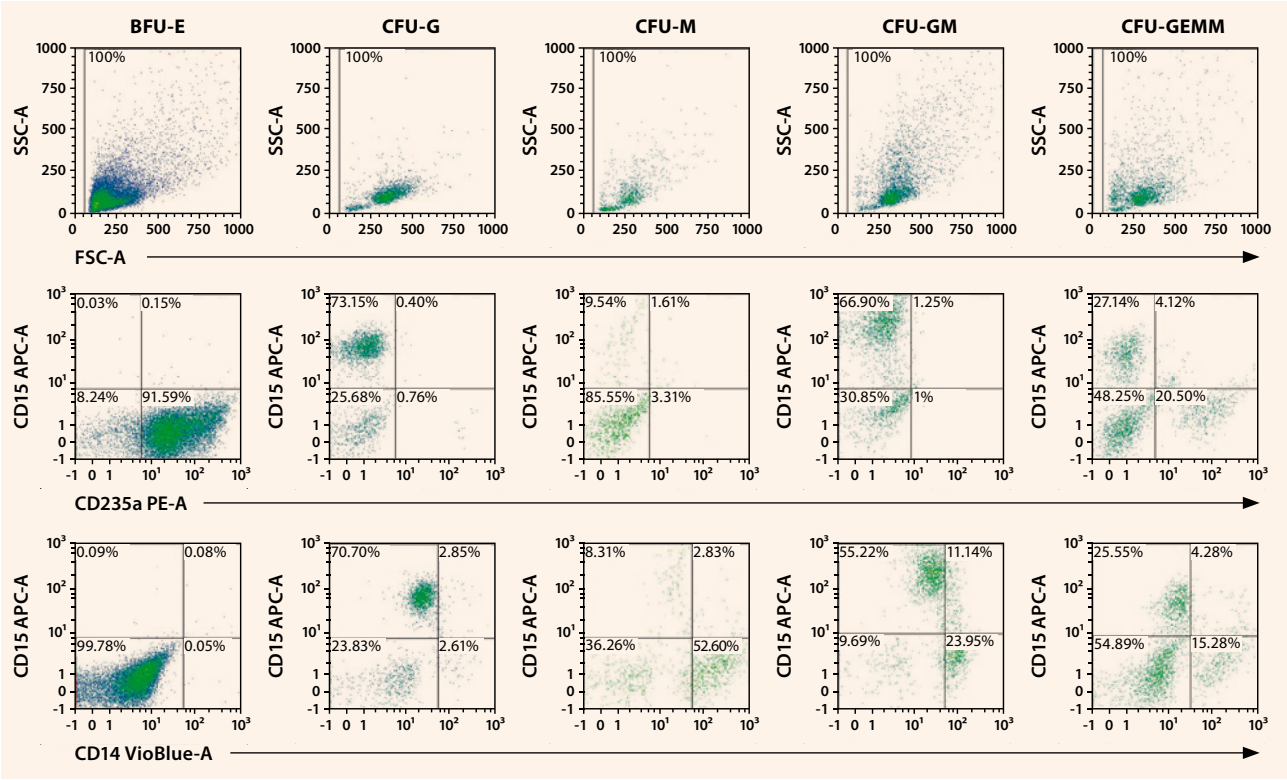


Figure 4: Plots of the different colony types. The percentage of CD14⁺, CD15⁺, and CD235a⁺ cells can be displayed on each quadrant and exported to an excel spreadsheet.

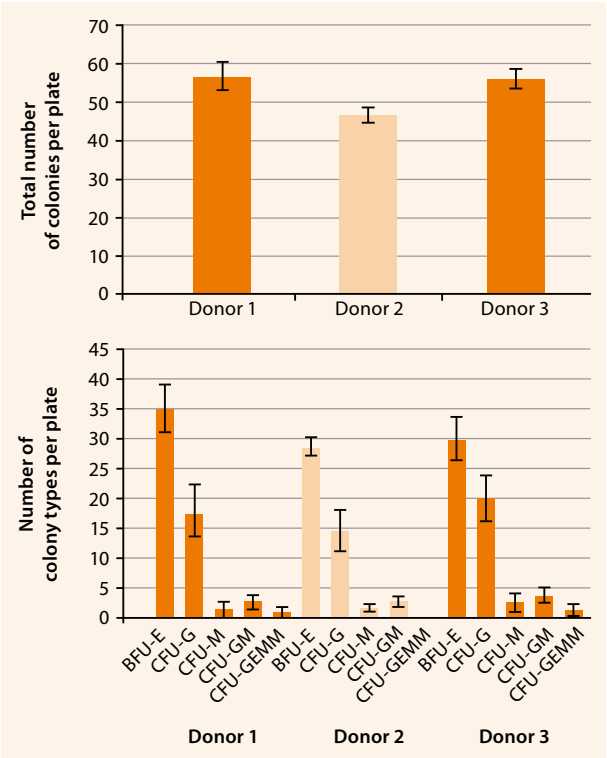


Figure 5: Total number of colonies per plate and number of colony types per plate for three donors.

Conclusion

- The StemMACS™ HSC-CFU Assay Kit, human combines an easy-to-handle, methylcellulose-free medium and a highly standardized analysis.
- Compared to the conventional assay, which is based on a visual readout using a microscope, the StemMACS HSC-CFU Assay Kit, human allows a flow cytometric readout, providing clear and user-independent results.
- Flow cytometric analysis is amenable to automation when using the MACSQuant® Analyzer and thus allows for automated, high throughput measurement and analysis.

Miltenyi Biotec B.V. & Co. KG | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macsde@miltenyi.com | **www.miltenyibiotec.com**
Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

130-127-522