Miltenyi Biotec

Frequently asked questions

StemMACS™ HSC-CFU Assay Kit, human

What are the differences between the hematopoietic stem cell (HSC) colony-forming unit (CFU) assay based on the StemMACS HSC-CFU Assay Kit, human and a conventional HSC-CFU assay based on semi-solid culture media?
The StemMACS HSC-CFU Assay Kit, human supports growth and differentiation of hematopoietic myeloid progenitor colonies just as the conventional HSC-CFU assay. However, there are some differences: The conventional HSC-CFU assay uses methylcellulose-based semi-solid culture medium in 35 mm dishes or wells, whereas the StemMACS HSC-CFU Assay Kit, human is based on methylcellulose-free culture medium to be used in 96-well round bottom plates. Methylcellulose-free medium is not only easier to handle, it also allows for a different analysis method. With the conventional assay, colonies are analyzed by visual scoring with a light microscope. With the StemMACS HSC-CFU Assay Kit, human, colonies are analyzed by flow cytometry, resulting in user-independent data and clear results. Table 1 summarizes the assay specifications and differences.

	User-independent HSC-CFU assay with StemMACS HSC-CFU Assay Kit, human	Conventional HSC-CFU assay with StemMACS HSC-CFU Media, human
Culture medium	Methylcellulose-free formulation	Based on methylcellulose
Culture ware	96-well round bottom plates	6-well plates or 35 mm dishes
Medium volume needed	5 mL per 96-well plate	1.1 mL per well or dish
Number of wells, dishes, or plates seeded	Three 96-well plates per sample	Two wells of a 6-well plate or two 35 mm dishes per sample
Cell number for seeding	250 $CD34^+$ cells per 96-well plate (donor or material dependencies possible, see also "How many colonies are expected when using the recommended seeding concentration of 250 $CD34^+$ cells per 96-well round bottom plate?" and Table 2) → 750 cells in total across three 96-well plates	250 $CD34^+$ cells seeded per well or dish (donor or material dependencies possible) → 500 cells in total across two dishes
Seeding tool	Multi-channel pipette	16 gauge blunt-end needle
Incubation parameters	12–14 days at 37 °C, 5% CO_2 placed in a separate humidity chamber	12–14 days at 37 °C, 5% CO_2
Culture robustness	No impact from movement.	Movement of incubator by closing or opening the incubator door can lead to unidentifiable colonies.
Analysis	Flow cytometric readout	Visual scoring by microscopy
Product capacity	Up to 30 samples	Up to 30 samples

Table 1: Direct comparison of the StemMACS HSC-CFU Assay Kit, human with the conventional HSC-CFU assay.

Which cellular starting materials can be used with the StemMACS HSC-CFU Assay Kit, human?
The StemMACS HSC-CFU Assay Kit, human can be used with human CD34+ cells from a variety of sources, such as cord blood, buffy coat, peripheral blood mononuclear cells (PBMCs), leukapheresis, and bone marrow. The assay can be started with separated cells as wells as with unseparated cells, such as cord blood–derived mononuclear cells (CB-MNCs) or PBMCs. Cells can be separated (CD34 MicroBead Kit Ultrapure, human) manually or automatically by using the autoMACS® Pro Separator. Moreover, the CliniMACS® Plus or the CliniMACS Prodigy® can be used to isolate CD34+ cells from a leukapheresis sample. Cells that were sorted by HSC marker expression with instruments like the MACSQuant® Tyto® can also be used with the StemMACS HSC-CFU Assay Kit, human. Fresh and frozen cells can be used as starting material. For frozen HSCs, it is recommended to culture those cells for at least six hours in HSC expansion media supplemented with cytokines, such as the StemMACS HSC Expansion System (composed of the StemMACS HSC Expansion Media XF, human supplemented with the StemMACS HSC Expansion Cocktail) to ensure cell recovery after thawing.

Cells from cord blood, buffy coat, PBMCs, leukapheresis, and bone marrow can be used as:

- separated hematopoietic stem and progenitor cells (HSPCs), such as CD34+ cells and CD133+ cells
- thawed HSPCs
- transduced or electroporated HSPCs
- sorted HSPCs
- MNCs
- NH_4Cl^- treated (lysis of erythrocytes)

Does the medium of the StemMACS HSC-CFU Assay Kit, human have the same composition as the methylcellulose-based medium used for the conventional HSC-CFU assay?
Yes, the medium of the StemMACS HSC-CFU Assay Kit, human can be used in the same way as that for the conventional HSC-CFU assay, just without methylcellulose. Its composition is identical to our StemMACS HSC-CFU complete with Epo, human without methylcellulose.

Can I use 6- or 12-well plates when using the StemMACS HSC-CFU Assay Kit, human?
No, you have to use round-bottomed 96-well plates for the StemMACS HSC-CFU Assay Kit, human in order to allow for colony formation in this methylcellulose-free medium. Use three 96-well round bottom plates per sample.

When is the endpoint of the cultivation?
Just as with the conventional assay, cells cultivated in the StemMACS HSC-CFU Assay Medium require 12–14 days at 37 °C and 5% CO_2. During this time, cells form colonies and proliferate, reaching numbers that can be acquired by a flow cytometer. Colonies can be analyzed as early as 12 days after beginning of culture and as late as 14 days. Longer incubation periods lead to bigger colonies, which introduces the risk of cell death due to lack of nutrients and/or buildup of metabolic byproducts.

What results do I get when performing the flow cytometric HSC-CFU assay compared to the conventional HSC-CFU assay and how comparable are they?
Our flow cytometric HSC-CFU assay will give you the same results as the conventional assay based on visual scoring (fig. 1).

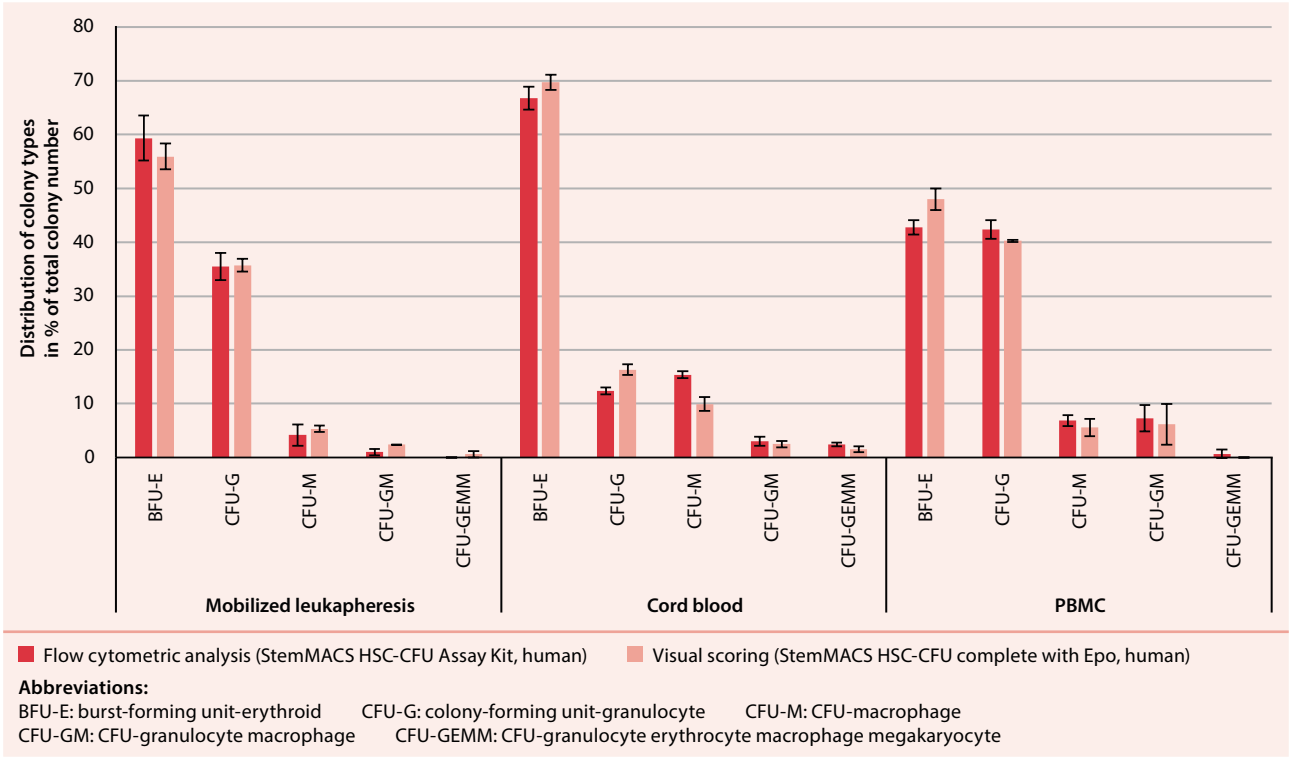

Figure 1: Flow cytometric analysis of an HSC-CFU assay achieves similar results compared to visual scoring of an HSC-CFU assay based on methylcellulose-based StemMACS HSC-CFU complete with Epo, human.

Can plates be stored until acquisition after colony staining? If yes, for how long?
After colony staining, plates should be analyzed with the MACSQuant® Analyzer (or similar flow cytometer) in a timely manner. This, however, can be challenging when many plates have to be analyzed on the same day. Therefore, it is possible to store stained plates for up to 18 hours at 2–8 °C before acquisition. We recommend using the MACSQuant X for reliable high-throughput flow cytometric analysis.

Does the storage time at 2–8 °C until measurement have an impact on the results?
No. We have assessed whether a prolonged storage in an cooled incubator impacts sample quality. We found that different plates with cells derived from the same donor showed similar results, regardless of cooled storage time before measurement (data not shown).

How many colonies are expected when using the recommended seeding concentration of 250 CD34⁺ cells per 96-well round bottom plate?
The recommended cell concentration used for this assay results in a high probability of seeding one colony-forming cell (CFC) per well, which gives rise to one specific colony type. However, the seeding concentration depends on (i) the frequency of CD34⁺ cells in your sample and (ii) their CFU potential.

i It is important to determine the frequency of CD34⁺ cells in your sample, for example by staining, before starting the CFU assay.

ii The CFU potential is the number of CFUs to be expected in relation to the number of CD34⁺ cells. If seeding of 250 CD34⁺ cells results in 75 colonies, the CFU potential of the samples is 30% (75 colonies out of 250 CD34⁺ cells). In our experience, typical values range from 15 to 30%.

Note: (i) and (ii) depend on the material, the donor, and the quality of the starting material (fresh or frozen).

The cell concentration is calculated based on the mathematical Poisson distribution. The recommended seeding concentration of 250 CD34⁺ cells per plate (which corresponds to 2.5 cells per well) with a CFU potential of 30% results in around 51 total colonies per plate (tab. 2). In the remaining 45 wells no colony is formed, thus, they are defined as empty.

For the seeding concentration of 250 CD34⁺ cells per plate, we recommend working with at least 45 empty wells per plate, which is achieved for a sample with a maximal CFU potential of 30%. When using CD34⁺ cells with a higher CFU potential, e.g. 50%, it may be important to adapt the cell concentration, e.g. to 1.5 cells per well.

Is every well of a 96-well plate occupied by one colony?
The number of wells with more than one colony type can be calculated according to the mathematical Poisson distribution. In a scenario with 45 empty wells and 51 wells with colonies, we expect 13 wells to contain two colony types; with 66 empty wells and 30 wells with colonies, five wells with two colonies are expected. Due to the specific staining patterns, it is possible to analyze most cases with two colonies (see next question "Can I still analyze wells with more than one colony?").

Number of seeded CD34⁺ cells per well	CFU potential	Number of empty wells	Number of wells with colonies
2.5	30%	45	51
2.5	15%	66	30
1.5	50%	45	51

Table 2: Expected number of wells with colonies based on the number of seeded CD34⁺ cells and their CFU potential.

Can I still analyze wells with more than one colony?
If two colonies are present in the same well, it is possible to distinguish them based on specific staining patterns in most cases. For example, a BFU-E colony expresses CD235a (fig. 2A) while a CFU-G colony expresses CD15 (fig. 2B). If a BFU-E and a CFU-G colony were in the same well, they could still be distinguished based on their different expression patterns (fig. 2C).

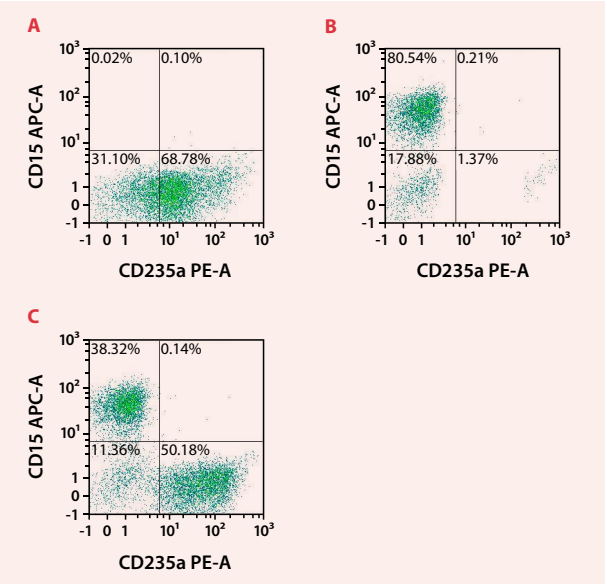

Figure 2: HSC colonies display specific marker expressions which makes them distinguishable with flow cytometric analysis. A single BFU-E (A) and a single CFU-G (B) were analyzed separately by flow cytometry. Due to their different expression of CD15 and CD235, these colonies can be identified, even if they occupy the same well (C).

The same principle can also be applied when a BFU-E (CD235a⁺) and a CFU-M (CD14⁺) are in the same well. The typical colony type–specific marker expression patterns are shown in figure 3.

What does the flow cytometric analysis look like for different colony types?
The StemMACS HSC-CFU Assay Kit, human uses colony type-specific marker expression patterns for colony identification. Figure 3 shows typical dot plots for all colony types and their expression patterns.

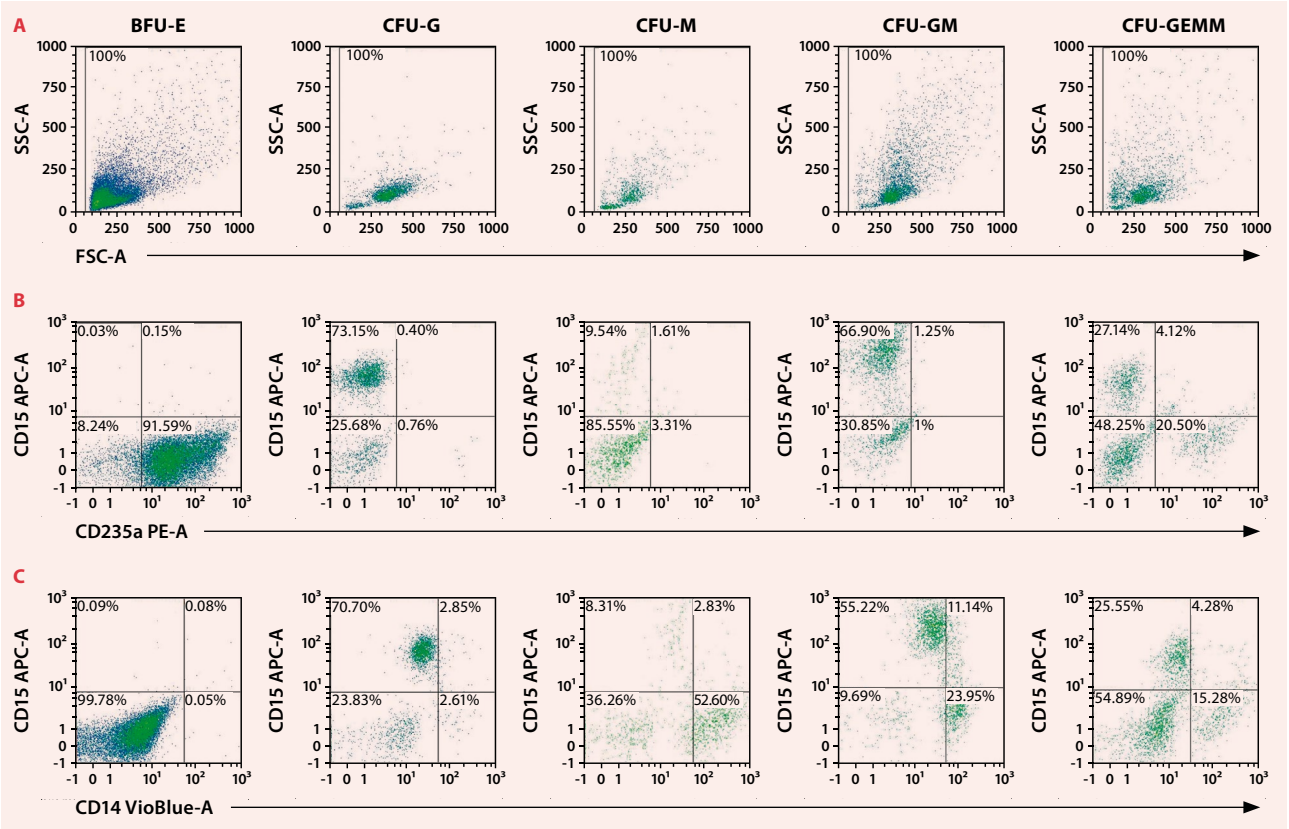

Figure 3: Flow cytometric analysis of colonies with the StemMACS HSC-CFU Assay Kit, human. Colonies were analyzed for expression of CD14, CD235a, and CD15. The percentages of CD14$^+$, CD235a$^+$, CD15$^+$ cells are displayed in each quadrant (B and C).

What do I need to consider when choosing flow cytometry settings?

First, the forward scatter (FSC) and side scatter (SSC) gains should be adjusted by using either PBMCs or a cultivated cell aliquot. For the cell aliquot, we recommend cultivating 500–1,000 CD34$^+$ cells in 500 µL StemMACS HSC-CFU Assay Kit Medium in a suitable culture vessel (e.g., a 48-well plate) for 12–14 days, and to use the resulting colony mixture to adjust the flow cytometry settings. Second, it is important to make sure that the trigger is set in a way to include BFU-E colonies (fig. 3A BFU-E). Third, the height has to be activated to gate single cells. This workflow has to be performed once for the first experiment, and the resulting setting should be suitable for following experiments as well, as long as the compensation and calibration settings are up to date.

How many events are required to get reliable results?

The colony size can vary depending on the source material (such as cord blood or peripheral blood), the donor, and the colony type (e.g., BFU-E colonies usually consist of more cells than CFU-M colonies). This means that the event count of each measured sample will be different. Furthermore, unseparated samples with a low number of CD34$^+$ cells (e.g. PBMCs) include cells that will not survive in the medium formulated specifically for HSCs, resulting in dead cells and cell debris. These factors can affect the flow cytometric readout by increasing the number of events per well, even in wells that would otherwise be considered empty. Therefore, we recommend acquiring at least 250 events per well, of which at least 35 events need to be positive for CD14, CD15, or CD235a, and at the same time fulfill the specific colony detection parameters (refer to datasheet 3.4 Data analysis).

Relevant products

Product	Order no.
StemMACS HSC-CFU Assay Kit, human	130-125-042
CD34 MicroBead Kit UltraPure, human	130-100-453
StemMACS HSC Expansion Media XF, human	130-100-463
StemMACS HSC Expansion Cocktail, human	130-100-843
CD34 Antibody, anti-human, APC (clone AC136)	130-113-176
CD45 Antibody, anti-human, FITC, REAfinity (clone REA747)	130-110-631
StemMACS HSC-CFU complete with Epo, human	130-091-280

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