Miltenyi Biotec

StemMACS™ HSC-CFU Assay Kit
human

Order no. 130-125-042

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1. Description

This product is for research use only.

Components

450 mL StemMACS HSC-CFU Assay Medium, human

1350 µL StemMACS HSC-CFU Assay Cocktail, human:

Cocktail of fluorochrome-conjugated antibodies: CD235a Antibody, anti-human, PE, REAfinity® (clone: REA175, isotype: recombinant human IgG1),
CD15 Antibody, anti-human, APC (clone: VIMC6, isotype: mouse IgM),
CD14 Antibody, anti-human, VioBlue® (clone: TÜK4, isotype: mouse IgG2aκ).

Capacity For up to 30 tests.

Product format Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide.

Storage Store StemMACS HSC-CFU Assay Medium, human protected from light at –20 °C. Store StemMACS HSC Assay Cocktail, human protected from light at +2 to +8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background information

The StemMACS HSC-CFU Assay Kit, human offers a highly standardized method for analyzing hematopoietic stem and progenitor cells (HSPCs). The kit combines differentiation in cell culture with a standardized, flow cytometry-based readout and eliminates the need for user-dependent, visual scoring under a microscope. For HSC-CFU formation, cells are diluted in methycellulose-free StemMACS HSC-CFU Assay Medium into a 96-well plate. Each well corresponds to the clonal progeny of a single HSPC. During the following incubation period, the StemMACS HSC-CFU Assay Medium will promote growth and differentiation of the deposited cells in suspension. In order to analyze the type of HSC-CFU per well, each well is stained with the StemMACS HSC-CFU Assay Cocktail and analyzed by flow cytometry. Following the gating scheme described in this protocol, the type of colony can be easily identified through the corresponding marker combination. Based on the determined colony type, the percentage of each colony type versus the total number of colonies can be determined. This setup provides a standardized, user-independent analysis of HSC-CFU assays and allows for automation in combination with the MACSQuant® Analyzer.

1.2 Product formulation

StemMACS HSC-CFU Assay Medium is a ready-to-use medium formulation that supports the growth of human BFU-E, CFU-E, CFU-G, CFU-M, CFU-GM, and CFU-GEMM colonies.

Components	Concentration in medium
Fetal bovine serum (FBS)	30%
Bovine serum albumin (BSA)	1%
L-glutamine	2 mM
2-mercaptoethanol	0.1 mM
Stem cell factor (SCF)	50 ng/mL
GM-CSF	20 ng/mL
G-CSF	20 ng/mL
IL-3	20 ng/mL
IL-6	20 ng/mL
Erythropoietin (Epo)	3 U/mL

Table 1: Composition of StemMACS HSC-CFU Assay Medium.

1.3 Reagent and instrument requirements

- Flow cytometer with the ability to discriminate APC, PE, and VioBlue, e.g., MACSQuant Analyzer 10
- Accessories for processing 96-well plates at the flow cytometer, e.g., MACS® Chill 96 Rack (# 130-094-459), when using the MACSQuant Analyzer 10
- Sterile 15 mL polypropylene tube
- Sterile disposable pipette tips
- Sterile pipettes
- 96-well round bottom plates
- Humidity chamber
- Dilution medium: Iscoves´s Modified Dulbecco´s Medium (IMDM)

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- PBS/EDTA buffer with 0.5% BSA (PEB)
- Sterile water
- Multi-channel pipettor
- Reagent reservoirs
- CD34 Antibody, anti-human, APC (clone: AC136)
- CD45 Antibody, anti-human, FITC (clone: REA747)
- (Optional) Red Blood Cell Lysis Solution (10×) (# 130-094-183)

2. Protocol

▲ Kit components should not be substituted or mixed with those from other kits or lots.

2.1 Preparation of StemMACS HSC-CFU Assay Medium

▲ To avoid repeated freeze-thaw cycles, StemMACS HSC-CFU Assay Medium should be dispensed into appropriate aliquots.

1. Thaw the medium overnight at +4 °C.
2. Shake the bottle vigorously.
3. Aliquot into sterile tubes (15 mL/tube) using a sterile pipette.
4. Freeze aliquots at –20 °C. Thaw at room temperature before use or overnight at +4 °C.

2.2 Preparation of cell samples

Hematopoietic colony-forming assays can be performed using mononuclear cells from bone marrow, cord blood, or peripheral blood. Likewise, enriched hematopoietic stem and progenitor cells, e.g., enriched lineage marker-negative (Lin^-), $CD133^+$, or $CD34^+$ cells, or ES and iPS cell-derived progenitors can be used.
For details about the preparation of mononuclear cells refer to the protocol section at www.miltenyibiotec.com/protocols.
For pre-enrichment of Lin^-, $CD133^+$, or $CD34^+$ cells, refer to the data sheet of the respective separation product.

2.3 Set-up of the HSC-CFU assay

The cells are diluted to the recommended concentration of 250 HSPCs per plate. This results with a high probability in one colony-forming cell per well and gives rise to one specific type of colony that can be stained and acquired by flow cytometry. For more information refer to FAQs at **www.miltenyibiotec.com/130-125-042** under the resources tab.

▲ **Note:** In general, the seeding concentration depends on (i) the frequency of $CD34^+$ cells and (ii) the colony-forming unit (CFU) potential of the $CD34^+$ cells in the material to be plated.

2.3.1 Count of $CD34^+$ cells

1. Thaw the required number of aliquoted StemMACS HSC-CFU Assay Medium at room temperature or overnight at +4 °C. Each 15 mL aliquot corresponds to 1 test/assay and is sufficient for processing three 96-well plates.

   (Optional) For samples with high erythrocyte content, it is highly recommended to perform red blood cell lysis before determining the $CD34^+$ cell count. Follow the instructions of the data sheet of the Red Blood Cell Lysis Solution (10×). Resuspend the cell pellet in sterile PEB.
2. Using sterile conditions, take a small sample and determine the cell number.
3. Remove an aliquot of up to 10^6 cells using sterile technique.
4. Centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
5. Resuspend cell pellet in appropriate amount of non-sterile PEB.
6. Add CD34 antibody, anti-human, APC and CD45 antibody, anti-human, FITC according to manufacturer's recommendation.
7. Mix well and incubate for 10 minutes in the dark in the refrigerator (+2 to +8 °C).
8. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
9. Resuspend the cell pellet in a suitable amount of non-sterile PEB for flow cytometric analysis.
10. Determine the percentage of $CD34^+CD45^+$ cells by flow cytometry.
11. Adjust the cell concentration to 250 $CD34^+CD45^+$ cells per 1 mL medium using plain IMDM.

    ▲ **Note:** For each sample, three 96-well round-bottom plates are required. For pipetting with a multichannel pipette, it is recommended to prepare 10^3 $CD34^+$ cells in 4 mL IMDM.

    ▲ **Note:** It might be necessary to adapt the cell concentration for materials with high CFU potential. Refer to FAQs for more information.
12. Continue with chapter 2.3.2 Assay preparation.

2.3.2 Assay preparation

1. Vortex the tube to ensure even distribution of cells.
2. Transfer cell suspension into a reagent reservoir.
3. Pipette 10 µL (250 cells/mL) into each well of three 96-well round bottom plates.

   ▲ **Note:** The cells are diluted to a concentration that results with a high probability in one colony-forming cell per well.
4. Transfer 15 mL of StemMACS HSC-CFU Assay Medium into a new reagent reservoir.
5. Add 50 µL of StemMACS HSC-CFU Assay Medium into each well of the 96-well plates.
6. Place plates into a humidity chamber or sterile enclosure filled with sterile water to minimize evaporation of cell culture medium during cultivation.
7. Incubate the plates placed in a humidity chamber for 12–14 days at +37 °C and 5% CO_2.

   ▲ **Note:** Make sure to have PBCMs for the setup of the flow cytometric analysis at day 12–14. If this is not feasible, cultivate 0.5–1×10^3 $CD34^+$ cells in 500 µL StemMACS HSC-CFU Assay Medium for 12–14 days and use the resulting mixture of colonies for the flow cytometric settings.

2.4 Immunofluorescent staining for flow cytometric analysis

1. Prepare the staining cocktail by diluting 45 µL of StemMACS HSC-CFU Assay Cocktail with PBS/EDTA/0.5% BSA buffer (PEB) to a final volume of 4.5 mL.
2. Add 15 µL of staining cocktail into each well of the three 96-well round bottom plates.

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3. Incubate for 10 minutes at +2 to +8 °C.
4. Add 25 µL of PEB to each well for a total volume of 100 µL.
5. Proceed with chapter 3. Flow cytometric acquisition and data analysis.

▲ **Note:** Make sure that the flow cytometer has been set up for processing of 96-well plates. A gentle mixing mode is recommended if supported by the flow cytometer.

3. Flow cytometric acquisition and data analysis

3.1 Flow cytometer setup

Using MACSQuant Analyzer 10

Define parameters for the HSC-CFU Assay Kit using the MACSQuant Analyzer 10.

Parameters are:

- Select all wells of the 96-well plate to be measured.
- Mix sample: mix gentle
- Mode: fast
- Uptake volume: 50 µL
- Sample volume: 100 µL
- Flow rate: high

Using MACSQuant Analyzer X

Define parameters for the HSC-CFU Assay Kit using the MACSQuant Analyzer X.

Parameters are:

- Select all wells of the 96-well plate to be measured.
- Mode: fast
- Uptake volume: 50 µL
- Sample volume: 100 µL
- Flow rate: high

For the first (row A) and fifth row (row E) of the plate select the following mixing type:

- Mix sample: Shake gentle.

Using another flow cytometer

Proceed according to manufacturer's recommendations for acquisition from 96-well plates.

3.2 Flow cytometric acquisition

1. Perform the following general steps:

   - Adjust Forward scatter (FSC) and Side scatter (SSC) gains by using PBMCs or the cultivated cell aliquot (refer to chapter 2.3.2, note below step 7).

   - Make sure that the trigger is set in a way to include erythrocytes/BFU-E colonies.

   - Activate Height for gating of single cells.
2. Select a well with positive signal of CD235a (Glycophorin A), e.g,. a BFU-E colony.
3. Draw a gate to include all events (figure 1, plot A).
4. Next, exclude all doublets by gating on single cells in a FSC-A versus FSC-H plot. (figure 1, plot B).
5. Display all single cells in two new plots: For the first one (figure 1, plot C) adjust a rectangle gate to include the CD235a (Glycophorin A)-PE channel events and another rectangle gate to include events that will be CD15-APC–positive. For the second one (figure 1, plot D) add a rectangle gate in the CD14-VioBlue–positive area.
6. Name the regions of interest: Add a name to the CD235a (Glycophorin A)-PE–positive and CD15-APC–positive regions of figure 1, plot C respectively, and the CD14-VioBlue–positive region of figure 1, plot D.
7. Start flow cytometric acquisition.

▲ **Note:** Make sure that the general gating parameters still apply, and that no regions have been shifted after re-compensation of the flow cytometer.

3.3 Example of immunofluorescent staining with the StemMACS HSC-CFU Assay Cocktail

$CD34^+$ cells from mobilized peripheral blood were isolated using magnetic separation and cultivated in StemMACS HSC-CFU Assay Medium for 14 days. The resulting colonies were stained with the StemMACS HSC-CFU Assay Cocktail as described above and analyzed on a MACSQuant Analyzer 10. Figure 1 to 5 show the plots of each colony type: BFU-E (figure 1), CFU-G (figure 2), CFU-M (figure 3), CFU-GM (figure 4), and CFU-GEMM (figure 5).

Figure 1: BFU-E

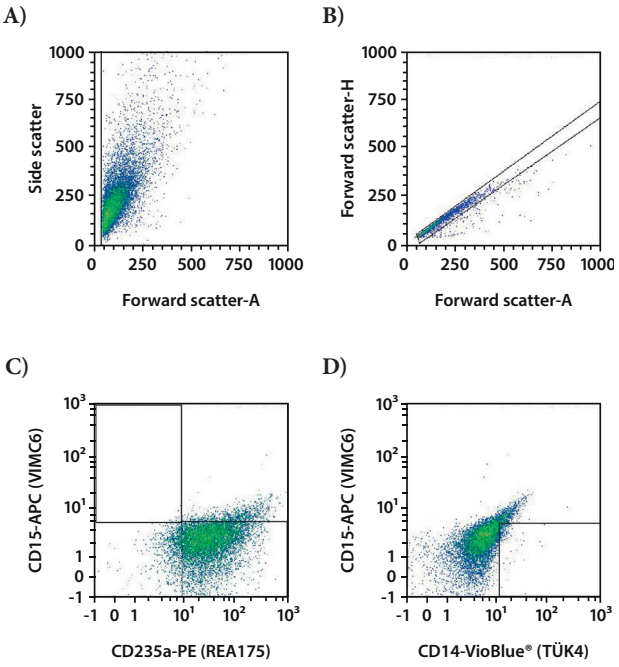


Figure 2: CFU-G

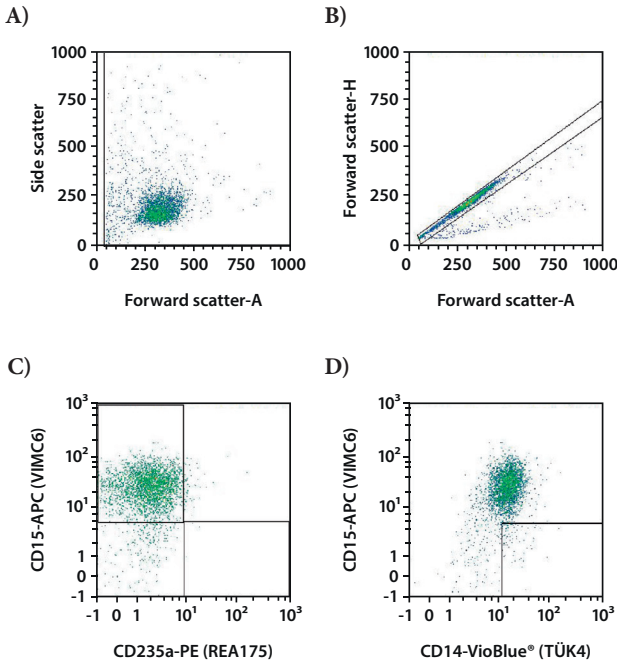


Figure 3: CFU-M

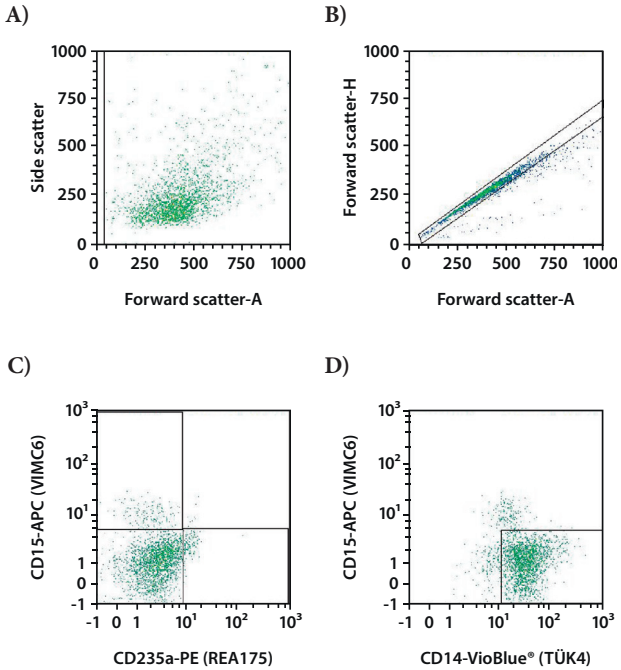


Figure 4: CFU-GM

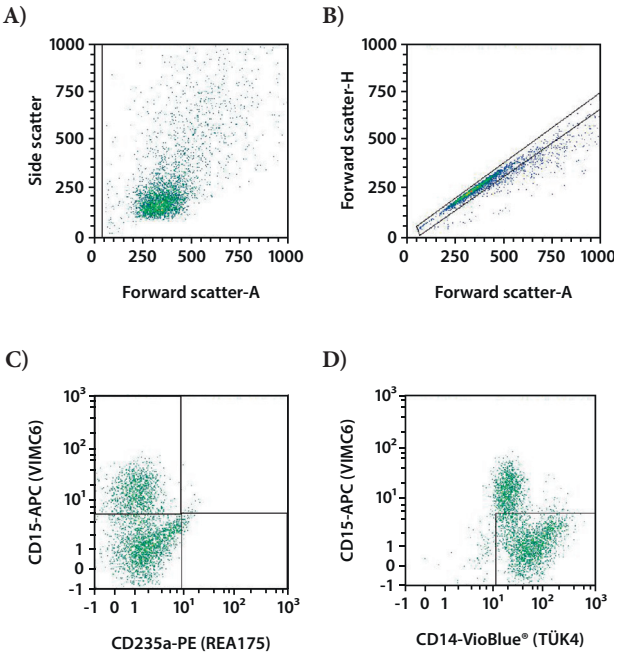
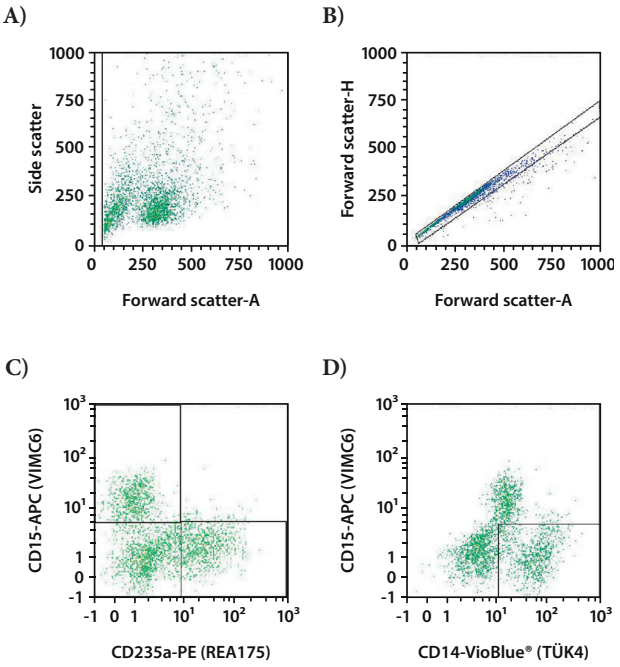


Figure 5: CFU-GEMM



3.4 Data analysis

Apply the analysis template (refer to chapter 3.2, steps 2 to 6) to all remaining wells. Information about the percentage of $CD14^+$, $CD15^+$, and $CD235a\ (Glycophorin)^+$ cells in each gate is generated and can be exported for further analysis into a Microsoft® Excel® worksheet by using the MACSQuant Analyzer.

Based on the specific staining for each well the colonies can be identified using the detection parameters listed in table below. For example, any well which exhibits over 15% $CD15^+$, 15% $CD14^+$, and 20% $CD235a\ (Glycophorin\ A)^+$ events, would be a CFU-GEMM colony.

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The minimal number of events required to have a reliable readout is 250 events per well of which at least 35 events need to be positive for one or more of the stained markers and also correspond to the colony detection parameters (table 2). Refer to FAQs for more details.

Refer to **www.miltenyibiotec.com/130-125-042** to get more information on the available analysis tools when using the MACSQuant Analyzer.

▲ **Note:** Most of the wells containing colonies originate from a single founder cell. To ensure this, it is recommended to work with at least 45 empty wells per 96-well plate. Refer to FAQs for more details.

	% $CD15^+$ cells	% $CD14^+$ cells	% CD235a (Glycophorin A) cells
CFU-GEMM	>15	>15	>20
CFU-GM	>20	>10	
CFU-M		>19	
BFU-E			>20
CFU-G	>19		

Table 2: Colony detection parameters.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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