Miltenyi Biotec

EPC Analysis Cocktail Kit, anti-human

Order no. 130-093-477

Contents

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

1. Description

This product is for research use only.

Components

1 mL EPC Analysis Cocktail, anti-human containing:

CD34 Antibody, anti-human, FITC (clone: AC136, isotype: mouse IgG2a)

CD133/2 Antibody, anti-human, PE (clone: 293C3, isotype: mouse IgG2b)

CD14 Antibody, anti-human, PerCP (clone: Tük4, isotype: mouse IgG2a)

CD309 (VEGFR-2) Antibody, anti-human, APC (clone: ES-20E6, isotype: mouse IgG1)

1 mL EPC Control Cocktail CD309, anti-human containing:

CD34 Antibody, anti-human, FITC (clone: AC136, isotype: mouse IgG2a)

CD133/2 Antibody, anti-human, PE (clone: 293C3, isotype: mouse IgG2b)

Isotype Control Antibody, mouse IgG1, APC (clone: IS5-21F5)

CD14 Antibody, anti-human, PerCP (clone: Tük4, isotype: mouse IgG2a)

200 µL EPC Control Cocktail CD133, anti-human containing:

CD34 Antibody, anti-human, FITC (clone: AC136, isotype: mouse IgG2a)

Isotype Control Antibody, mouse IgG2b, PE (clone: IS6-11E)

CD14 Antibody, anti-human, PerCP (clone: Tük4, isotype: mouse IgG2a)

4 mL EPC Enrichment Cocktail, anti-human

4.4 mL FcR Blocking Reagent, human

2×50 mL Red Blood Cell Lysis Solution (25×)

0.5 mL Propidium Iodide Solution

Capacity

For 20 tests. One test corresponds to 20.5 mL whole blood, cord blood, or bone marrow or to 2×10^8 white blood cells for leukapheresis product.

Product format

Antibodies and FcR Blocking Reagent, human are supplied in buffer containing stabilizer and 0.05% sodium azide.

Storage

Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background information

The EPC Analysis Cocktail Kit, anti-human is designed for the enumeration of circulating endothelial progenitor cells (cEPCs) from peripheral blood, cord blood, leukapheresis products, or EPCs from bone marrow based on the expression of CD34 and CD309 (VEGFR-2/KDR) as well as of CD133 following the enrichment of EPCs.

1.2 Applications

- Identification and enumeration of $CD34^+CD309$ (VEGFR-2/KDR)$^+$ and $CD133^+$ endothelial progenitor cells from human peripheral blood, leukapheresis, cord blood, and bone marrow.

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140-002-193.06

1.3 Reagent and instrument requirements

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 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).

  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- Flow cytometer equipped with a red (640 nm) and a blue (488 nm) laser, e.g., MACSQuant Analyzer 10 (# 130-096-343) or MACSQuant Analyzer 16 (# 130-109-803).
- Roation device for tubes, e.g., MACSmix™ Tube Rotator (# 130-090-753).
- Double distilled water (ddH_2O).
- (Optional) Membrane filter (100 µm).
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407) to remove cell clumps.
- (Optional) MACS Chill 5 Rack, 1 rack (# 130-092-951).
- (Optional) MACS Comp Bead Kit, anti-mouse Igκ (# 130-097-900) or anti-human Igκ (# 130-104-187) for optimal compensation of the fluorescence spillover from fluorochrome-conjugated antibodies.

2. Protocol

2.1 Sample preparation

▲ For best results use starting material directly after aspiration.

▲ All blood samples have to be supplemented with an anticoagulant, for example, EDTA for whole blood and Heparin for bone marrow.

▲ Blood samples should be stored at room temperature under agitation until analysis.

▲ (Optional) When using bone marrow aspirate pass the material through a 100 µm nylon membrane filter.

1. Dilute 25× Red Blood Cell Lysis Solution 1:25 with double-distilled water (ddH_2O), for example, dilute 4 mL of 10× Red Blood Cell Lysis Solution with 96 mL of ddH_2O.

   ▲ **Note:** Do not dilute with deionized water. Store prepared 1× Red Blood Cell Lysis Solution at room temperature. Discard unused solution at the end of the day.

2. Determine cell number. For the enumeration of EPCs from one patient, the following three samples of whole blood, cord blood, bone marrow, or leukapheresis product are necessary.

Sample	EPC sample	Control sample CD309	Control sample CD133
Volume	10 mL*	10 mL*	200 µL
Purpose	Enumeration of EPCs	Control of CD309 staining	Control of CD133 staining

* 10 mL sample or maximum of 10^8 white blood cells

2.2 Magnetic labeling and staining of EPC sample and control sample CD309

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters (30 µm), # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

1. Take two 50 mL tubes and transfer 10 mL of cell sample into each tube (maximum of 10^8 white blood cells).
2. Add 40 mL of 1× Red Blood Cell Lysis Solution to each of the two tubes.
3. Mix the samples gently on an orbital shaker or MACSmix Tube Rotator (medium position; 8 rpm).
4. Incubate all samples at room temperature for 10 minutes.
5. Centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. Resuspend cell pellet in 300 µL of buffer by pipetting up and down.
7. Add 100 µL of FcR Blocking Reagent, human and 100 µL of EPC Enrichment Cocktail, anti-human to each sample.
8. Mix gently and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).
9. Add 50 µL of EPC Analysis Cocktail, anti-human to the EPC sample and 50 µL of EPC Control Cocktail CD309, anti-human to the control sample CD309.
10. Mix gently and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
11. Wash cells by adding 5 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
12. Resuspend cell pellet in 1 mL of buffer by pipetting up and down.
13. For cell separation using MS columns, the MACSQuant Cell Enrichment Unit, or the EPC Enrichment and Enumeration Express Mode proceed to chapter 2.4.

2.3 Magnetic separation and preparation of EPC sample and control sample CD309 for flow cytometric analysis

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters (30 µm), # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

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▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

Magnetic separation with MS Columns

1. Place one columns per sample in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
2. Prepare column by rinsing with 500 µL of buffer.
3. Apply one of the cell suspensions onto each column. Collect flow-through containing unlabeled cells.
4. Wash column three times with 500 µL of buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 3.

   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette 1 mL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into each column.
7. Proceed to flow cytometric analysis. Add 10 µL of Propidium Iodide Solution immediately before flow cytometric analysis.

   ▲ **Note:** Analyze samples by flow cytometry directly after isolation.

2.4 Staining of control sample CD133

1. Take one 1.5 mL tube and transfer 200 µL of cell sample into the tube.
2. Add 800 µL of 1× Red Blood Cell Lysis Solution.
3. Mix the sample gently on an orbital shaker or MACSmix Tube Rotator (medium position; 8 rpm).
4. Incubate at room temperature for 10 minutes.
5. Centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. Resuspend cell pellet in 70 µL of buffer by pipetting up and down.
7. Add 20 µL of FcR Blocking Reagent, human and 10 µL of EPC Control Cocktail CD133, anti-human.
8. Mix gently and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
9. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
10. Resuspend cell pellet in 1 mL of buffer by pipetting up and down.
11. Proceed to flow cytometric analysis. Add 5 µL of Propidium Iodide Solution immediately before flow cytometric analysis.

2.5 Automatic separation and analysis with the MACSQuant Analyzer

For automated flow cytometric analysis using the MACSQuant Analyzer flow cytometers, the Express Mode **EPC_Enrichment_Enumeration_Kit_h** can be used. Express Modes are unique add-on features for the MACSQuantify™ Software. They are standardized data analysis tools that are optimized to automate flow cytometric measurements and analyses via predefined experiment settings, acquisition, and automated gating. Derived from mathematical algorithms, they reduce human error and therefore increase experimental reproducibility.
For details refer to the MACSQuant user manual, the MACSQuantify Software guide, or visit www.macsquant.com. For more information on the usage of Express Modes refer to the application note "How to use a MACSQuant® Instrument Express Mode in Custom Login" in the Resources section at www.miltenyibiotec.com.
If analyses are performed using the Express Mode, these have to be done as a group analysis.

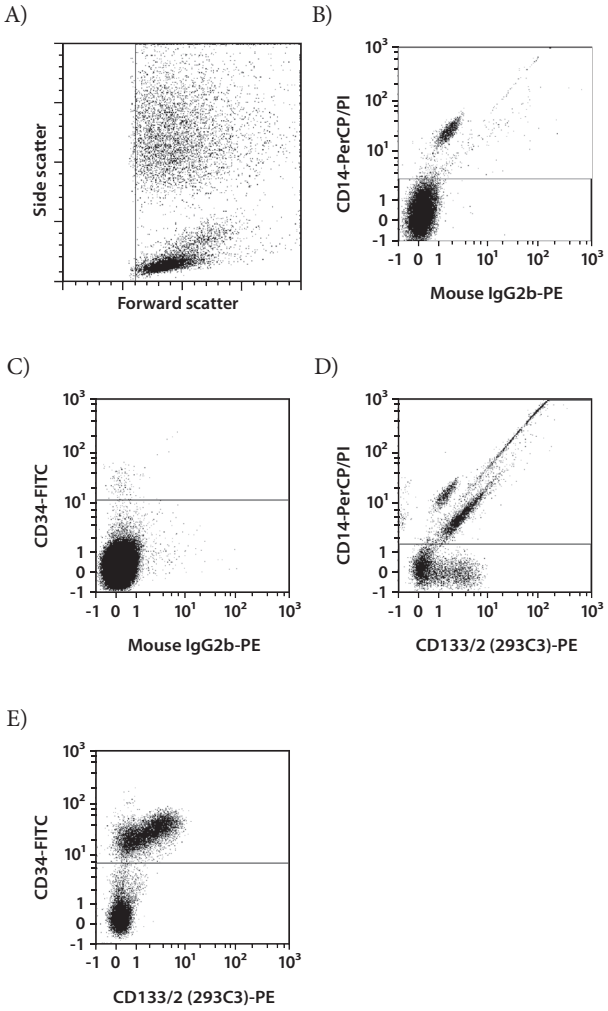
Define the experiment as follows using the Custom mode for analysis:

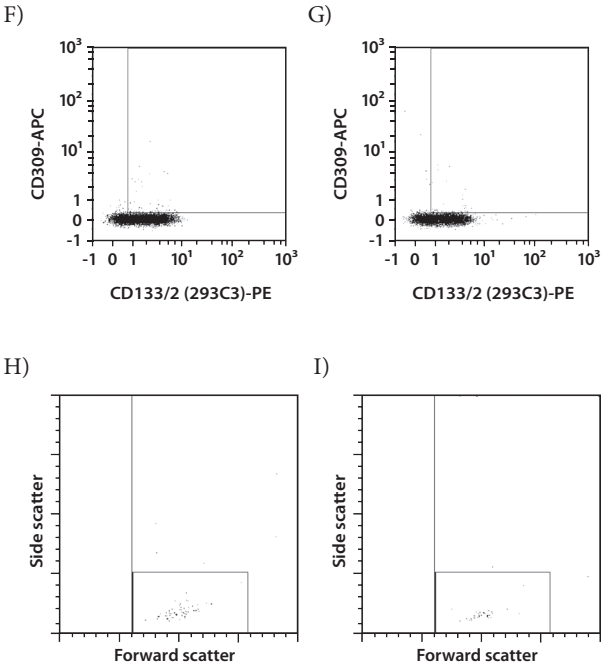
Experiment setups	
Rack type	MACS Chill 5 Rack, choose three positions
Sample ID	1. position: control CD133 2. position: control CD309 3. position: EPC enumeration
Flow rate	1. position: medium 2. position: high 3. position: high
Mode	1. position: standard 2. position EnrichS 3. position: EnrichS
Uptake volume	1. position: 400 µL 2. position 450 µL 3. position: 450 µL
Sample volume	1. position: 1 mL 2. position 900 µL 3. position: 900 µL
Instrument setting	PBMC compensation (needs to be generated before)
Autolabel	PI, Titer (1:99)
Annotations	1. position: CD34-FITC, Mouse IgG2b-PE, CD14-PerCP 2. position: CD34-FITC, CD133-2-PE, Mouse IgG1-APC, CD14-PerCP 3. position: CD34-FITC, CD133-2-PE, CD309-APC, CD14-PerCP

3. Example of immunofluorescent staining with the EPC Analysis Cocktail Kit, anti-human

Whole blood was stained with the EPC Analysis Cocktail Kit, anti-human. Cells were analyzed by flow cytometry using the MACSQuant Analyzer 10.

As a preliminary step for elimination of debris and platelets a gate in forward scatter (FSC) versus side scatter (SSC) was set (A). To identify $CD34^+$ cells in the control sample CD133, a gate around viable, non-monocyte cells was set (B) and CD34-FITC and Mouse IgG2b-PE were displayed (C). To identify $CD34^+$ cells within the EPC sample and the control sample CD309, the same gating strategy was applied and adjusted. A gate around viable, non-monocyte cells was set (D) and CD34-FITC and CD133/2-PE were displayed (E). To define $CD34^+CD133^+CD309^+$ cells, CD309-APC and CD133/2-PE were displayed within the EPC sample (F) and the control sample CD309 (G). A gate around $FSC^{low}SSC^{low}$ cells was set to define EPCs within the EPC sample (H) and the control sample CD309 (I).





3.1 Determination of the EPC frequency

EPC sample

Region	Gated [%]	Count
$CD34^+$ cells	47.45	9164
$CD34^+CD133^+CD309^+$ cells	0.96	88
$FSC^{low}SSC^{low}$ cells	92.05	81

Control sample CD309

Region	Gated [%]	Count
$CD34^+$ cells	47.45	8674
$CD34^+CD133^+CD309^+$ cells	0.56	51
$FSC^{low}SSC^{low}$ cells	96.08	49

Calculate the frequency of EPCs with low forward and side scatter among $CD34^+$ cells as shown below.

$$\frac{100\%}{CD34^+ \text{ cell number}/FSC^{low}SSC^{low} \text{ cell number}}$$

$$\text{Frequency of } FSC^{low}SSC^{low} \text{ EPCs} = \frac{100\%}{9164/81} = 0.88\%$$

$$\text{Frequency of } FSC^{low}SSC^{low} \text{ control sample CD309} = \frac{100\%}{8674/49} = 0.56\%$$

3.2 Determination of the EPC number

	Description	Example
a	Number of white blood cells in 10 mL whole blood	7.3×10^7
b	Frequency of $CD34^+$ cells before enrichment (control sample CD133)	0.06%
c	Frequency of $FSC^{low}SSC^{low}$ EPCs	0.88%
d	Frequency of $FSC^{low}SSC^{low}$ from control sample CD309	0.56%

Calculation of the number of $CD34^+$ cells in the starting volume

	Description	Calculation	Example
e	Number of $CD34^+$ cells in 10 mL whole blood	(a × b) / 100	$(7.3\times10^7 \times 0.06\%)/100$ = 43,800

Correction of non-specific staining

	Description	Calculation	Example
f	Frequency of $FSC^{low}SSC^{low}$ EPCs after enrichment	c – d	0.88% – 0.56% = 0.32%

Calculation of the EPC number

	Description	Calculation	Example
g	Number of EPCs in the starting volume	(e × f) / 100	(43,800 × 0.32%) / 100 = 140

In the given example, the number of EPCs in 10 mL whole blood is 140 (= 14 EPCs/mL whole blood).

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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