Standardized enumeration of human endothelial progenitor cells (EPCs) based on a flow cytometric assay

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Introduction

In the last decade, a number of experimental data and clinical observations have suggested that bone marrow represents a reservoir of endothelial progenitor cells (EPCs) that participate in regeneration and repair of many vascular tissues. These EPCs are activated and mobilized to the blood stream by environmental stimuli for physiological tissue regeneration. EPCs are defined by coexpression of the cell surface markers CD34, CD133 and CD309 (VEGFR-2, KDR). CD133 expression seems to be restricted to the most primitive EPCs and is lost during maturation, rendering its analysis important for the identification of maturation state of EPCs[1–3].

Many attempts have been made to quantify EPCs in human whole blood or bone marrow by flow cytometry. Quantifying EPCs from human blood sources is challenging due to the rare nature: Numbers of EPCs range from 10 to 2500 EPCs/mL[1,4–7]. Different protocols for isolation and quantification complicate the comparison and interpretation of results.

In this study, a fast and reliable flow-cytometric assay is introduced to enumerate rare EPCs from human whole blood samples defined by CD34, CD133, and CD309 (VEGFR-1, KDR).

Results

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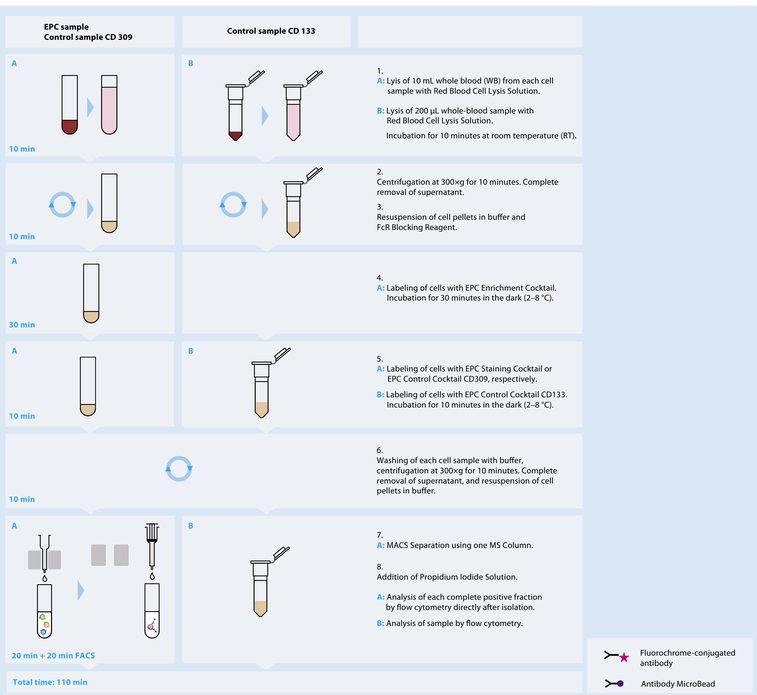

Figure 1: Overview of experimental steps with optimized magnetized and immunofluorescent labeling
To isolate and quantify EPCs, the EPC Enrichment and Enumeration Kit (Miltenyi Biotec GmbH) was used. The kit includes the EPC Enrichment Cocktail, EPC Staining Cocktail, EPC Control Cocktail CD309, EPC Control Cocktail CD133, FcR Blocking Reagent, Red Blood Cell Lysis Solution, and Propidium Iodide Solution (PI). An optimized labeling and enrichment procedure was performed as described above. For one test, two samples of 10 mL each (EPC sample and control sample CD309) and one sample of 200 µL (control sample CD133) were used. Erythrocytes in all samples were lysed and EPCs were stained and enriched from the EPC and control sample CD309. Enrichment of EPCs was performed by incubating the EPC Enrichment Cocktail and FcR Blocking Reagent followed by the EPC sample, with EPC Staining Cocktail (CD34-FITC, CD133/2 (293C3)-PE, CD309 (VEGFR-2/KDR)-APC, CD14-PE-Cy5) and the control sample CD309 with EPC Control Cocktail CD309 (CD34-FITC, CD133/2 (293C3)-PE, Mouse IgG1-APC, CD14-PE-Cy5). After incubation, cells were separated with MACS® Technology. Control sample CD133 was incubated with FcR Blocking Reagent and the EPC Control Cocktail CD133 (CD34-FITC, Mouse IgG2b-PE, CD14-PE-Cy5). Finally, the complete enriched positive fraction of EPC and control sample CD309 and partly the control sample CD133 were analyzed by four-color flow cytometry for enumeration of EPCs.

1 Flow-cytometric detection of CD34/CD133/CD309-positive EPCs

1A

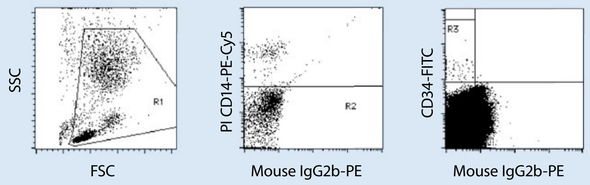

Figure 1A: Control sample CD133: identification of CD34⁺ cells and specificity of CD133
To investigate CD34 frequency in control sample CD133, cells were stained with EPC Control Cocktail CD133 (CD34-FITC, Mouse IgG2b-PE, CD14-PE-Cy5) and analysed by flow cytometry. Gating of leukocytes was performed according to light scatter signal (R1). Dead cells were excluded according to PI immunofluorescence and exclusion of monocytes and autofluorescent cells by CD14-PE-Cy5 staining (R2). The final dot plot shows all cells gated for R1 and R2. CD34 positve cells are defined in R3. The quadrant defines CD133 specificity by staining of mouse IgG2b-PE isotype control.

1B

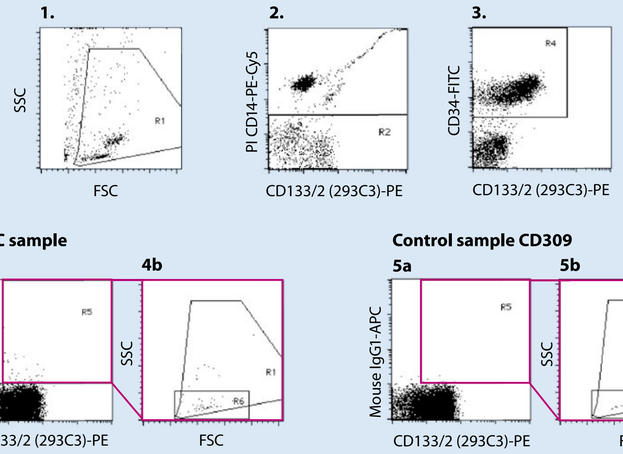

Figure 1B: EPC sample and control sample CD309: identification of EPCs
To investigate EPC frequency in the EPC sample, cells were stained with EPC Staining Cocktail (CD34-FITC, CD133/2 (293C3)-PE, CD309 (VEGFR-2/KDR)-APC, CD14-PE-Cy5). To assess CD309 specificity, control sample CD309 was stained with EPC Control Cocktail CD309 (CD34-FITC, CD133/2 (293C3)-PE, Mouse IgG1-APC, CD14-PE-Cy5) and gated in the same way as the EPC sample.
1: Exclusion of debris is achieved by gating of leukocytes according to light scatter signal (R1).
2: Exclusion of dead cells is done according to PI immunofluorescense and exclusion of monocytes and autofluorescent cells by CD14-PE-Cy5 staining (R2).
3: All CD34 positive cells are defined in R4 gated on R1 and R2.
4a+b: Shown is the EPC sample gated on R1, R2, and R4. Region 5 defines all CD34/CD133/CD309-positive cells. Progenitor cells show low forward side scatter properties. Therefore, events in R5 are backgated and cells with low forward side scatter are defined in R6 (4b).
5a: The control sample CD309 is also gated on R1, R2, and R4. Region 5 defines all CD34/CD133 positive cells labeled with CD309 isotype control Mouse IgG1-APC.
5b: Comparable to the EPC sample events in R5 are back gated for low forward-side scatter properties (R6).

2 Intra-experimental variation in six parallel experiments (n=6)

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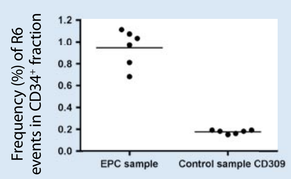

Figure 2: Frequencies of events detected in R6 among CD34⁺ cells in the EPC sample and control sample CD309 were evaluated in six parallel experiments to investigate intra-experimental variation. Median frequencies of R6 events detected in the EPC sample were 1.00% (0.68%–1.11%, SD: 0.16%, CV: 17%) and 0.19% (0.15%–0.18%, SD: 0.02%, CV: 9,4%) in the control sample CD309.

3 Increased sensitivity of EPC enumeration using dead cell exclusion, negative marker and backgating

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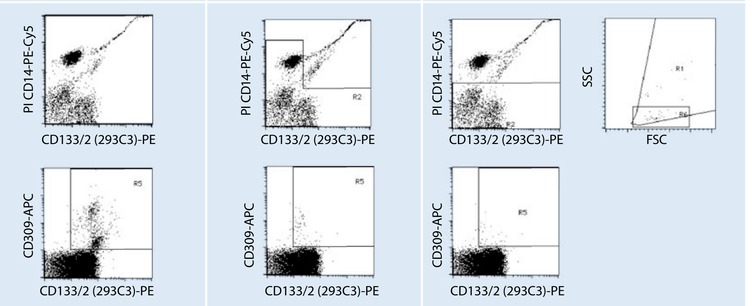

	A	B	C	D
EPC sample	8.60	0.85	0.35	-
Control sample CD309	7.65	0.16	0.08	0.08
EPC control	0.95	0.69	0.27	0.18
Absolute number of EPCs/mL	48	35	14	9

Figure 3: Comparison of dead-cell exclusion strategies including negative marker and backgating of target cells
The scenarios above show the importance of dead-cell exclusion with propidium iodide, inclusion of the negative marker CD14 and backgating of target cells.
A: No restriction for R5 leads to a high background noise (8.6% EPCs in CD34⁺ cells) that can be lowered by subtraction of corresponding isotype controls (0.95% EPCs among CD34⁺ cells).
B: If non-viable cells are excluded, the background lowers to 0.69% EPCs among CD34⁺ cells.
C: Staining of unwanted cells or autofluorescence is excluded with a negative marker (0.27% EPCs among CD34⁺ cells).
D: A further reduction of unwanted events is achieved by back gating criteria regarding to light scatter properties.

4 Determination of EPC frequency among CD34⁺ cells for healthy males (n=11) and females (n=9)

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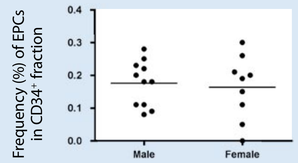

Figure 4: Median frequency (F) of EPCs among CD34⁺ cells and absolute EPC numbers (no.) per mL of whole blood in healthy eleven males and nine women aged 23–44 years were as follows:

Male	F = 0.18% (0.08–0.28%)	no. = 4.5 (1.4–58.2)
Female	F = 0.19% (0.00–0.30%)	no. = 2.9 (0.0–4.8)

Conclusion

The assay is fast (2 hours), reproducible, and copes with the requirements of rare-cell analysis using flow cytometry. Reliable standardized results can be achieved using FcR Blocking Reagent, optimized positive and negative markers as well as an optimized gating strategy combining dead-cell exclusion, back gating of target cells, and exclusion of unwanted and autofluorescent cells.

References
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