MACS
Miltenyi Biotec

REF: 170-070-702

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IVD CE

CD133/2-PE

For *in vitro* diagnostic use.
Clone: 293C3 (isotype mouse IgG2b).
1 mL antigen-specific staining reagent containing monoclonal mouse anti-human CD133/2 antibody conjugated to R-Phycoerythrin (PE).

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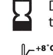
1. Product description

Clone: 293C3 (isotype mouse IgG2b).
Each vial contains 1 mL solution of monoclonal CD133/2 antibodies conjugated to R-Phycoerythrin (PE) in PBS/EDTA buffer and 0.05% sodium azide and a stabilizer.

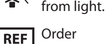
2. Glossary of symbols

For 100 tests or up to 10^9 cells.

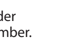
 Do not use after the use-by date.

Store protected from light.

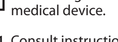
Store at 2–8 °C. Do not freeze.

 REF Order number.

Manufacturer.

 IVD *In vitro* diagnostic medical device.

LOT Batch code.

 Consult instructions for use.

P/N Part number.

After opening, the reagent is allowed to be used until the use-by date.

3. Abbreviations

APC	Allophycocyanin
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HPC	Hematopoietic progenitor cell
ISHAGE	International Society of Hematotherapy and Graft Engineering
PE	R-Phycoerythrin
RBC	Red blood cell
SSC	Side scatter
WBC	White blood cell
7-AAD	7-aminoactinomycin D

4. Background information

CD133 is a 5-transmembrane cell surface antigen with a molecular weight of 117 kDa. It is expressed on a subset of CD34 ^{bright} stem and progenitor cells in human fetal liver, bone marrow, cord blood, and peripheral blood but is not found on mature blood cells[1]. In contrast to the CD34 antigen, CD133 is not expressed by late progenitors such as pre–B cells, CFU-E, and CFU-G[2,3]. CD133 has also been found to be expressed on circulating endothelial progenitor cells and fetal neural stem cells as well as on other tissue-specific stem cells such as renal, prostate, and corneal stem cells[4,5,6,7].

The CD133/2-PE (clone 293C3) antibody recognizes epitope 2 of the CD133 antigen.

5. Intended use

CD133/2-PE is intended for the *in vitro* diagnostic identification of CD133-positive cells by flow cytometry. The cell source can be whole blood, mobilized whole blood, leukapheresis harvest, mobilized leukapheresis harvest, bone marrow, or the target cell fraction of a CD133 enrichment with the CliniMACS® CD133 System.

6. Sample preparation

CD133/2-PE can be used for the identification of CD133-positive cells by flow cytometry with a variety of cell sources, including whole blood, mobilized whole blood, leukapheresis harvest, mobilized leukapheresis harvest, bone marrow, and the target cell fraction of a CD133 enrichment with the CliniMACS® CD133 System. Each cell source can have different storage conditions and limitations that should be considered prior to collection and analysis.

The samples must be stained within 24 hours. Whole blood samples must be stained within 12 hours.

The viability of the cells should be at least 90%.

For the cell staining procedure a minimum of 300 µL sample is required. The WBC count should not exceed 10^8 cells/mL.
Important: The WBC count of the target cell fraction of a CD133 enrichment with the CliniMACS® CD133 System should not be below 10^6 cells/mL.

7. Warnings and precautions

For *in vitro* diagnostic use.

For all handling, consideration of good laboratory practice (GLP) regulations is recommended.

The use of CD133/2-PE must be performed by trained and qualified persons only.

Analysis results obtained by the use of CD133/2-PE shall never be the sole basis for diagnosis and/or therapy of patients with hematological malignancies.

The interpretation of the results are under the full responsibility of the user.

To verify the analysis results every determination should be repeated.

The reagent should not be used if signs of leakage are observed.

All biological specimens and all materials that come into contact with blood and blood products must be treated as infectious material. Regulations for the treatment of infectious material must be observed.

CD133/2-PE contains sodium azide (NaN_3), a chemical highly toxic in pure form. However, at product concentrations, it is not classified as hazardous. Sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing. Safety guidelines and the material safety data sheet must be observed.

For material required but not provided the manufacturer´s recommendations and safety regulations must be followed.

8. Reagents and materials required but not provided

1. **Buffer (PBS/EDTA):**
   E.g. MACS® Rinsing Solution, Miltenyi Biotec, # 130-091-222, or equivalent.
2. **7-aminoactinomycin D (7-AAD):**
   E.g. 7-AAD Viability Staining Solution, eBioscience, Order. no. 00-6993-50, or equivalent. For flow cytometric exclusion of dead cells without cell fixation.
   Warning: 7-AAD staining solution (50 µg/mL) is recommended. In the concentration of 50 µg/mL the 7-AAD is no longer classified as hazardous. The manufacturer´s recommendations and safety regulations must be followed.
3. **CD45-FITC:**
   E.g. CD45-FITC, Miltenyi Biotec, # 130-080-202, or equivalent.
4. **CD34-APC:**
   E.g. CD34-APC, Miltenyi Biotec, # 130-090-954, or equivalent.
5. **Mouse IgG2b-PE isotype control antibody:**
   E.g. Mouse IgG2b-PE, Miltenyi Biotec, # 130-092-215, or equivalent.
6. **RBC lysing solution:**
   E.g. Red Blood Cell Lysis Solution (10×), Miltenyi Biotec, # 130-094-183, or equivalent.
7. **Disposable capped polystyrene tubes, 12×75 mm**
8. **Vortex mixer**
9. **Low speed centrifuge:**
   Minimum speed 300×g, with 12×75 mm tube carriers.
10. **Micropipettes with disposable tips:**
    Variable micropipettes with volume ranges of 0.5–10 µL, 10–100 µL, and 100–1000 µL.
    Important: Use calibrated micropipettes only.
11. **Device for cell counting:**
    E.g. automated cell counter, Neubauer chamber, or equivalent.
    Important: Use a calibrated and maintained automated hematology analyzer only. The counting of cells with the Neubauer chamber should be performed by experienced users only.
12. **Flow cytometer:**
    CD133/2-PE is to be used with a 4-color flow cytometer or a 3-color flow cytometer.

    4-color flow cytometer
    The flow cytometer must be able to detect 4-color fluorescence (488 nm argon laser, 635 nm red diode laser) and must be equipped with appropriate computer hardware, and -software. Refer to instrument user manual for instructions.

    3-color flow cytometer
    The flow cytometer must be able to detect 3-color fluorescence (488 nm argon laser) and must be equipped with appropriate computer hardware, and -software. Refer to instrument user manual for instructions.

    It is recommended to run a commercially available whole blood control to optimize instrument settings and as a quality control of the system.

9. Staining procedure for 4-color and 3-color flow cytometric analysis

The staining procedures for the 4-color and the 3-color analysis are the same. Exception 3-color analysis: no CD34-APC is added to the staining reaction (see table 1: Cell staining). Bone marrow samples must only be stained for 4-color flow cytometric analysis.

The flow cytometric analysis for the 4-color staining and for the 3-color staining analysis is described in the chapter "Flow cytometric analysis".

A WBC count must be obtained from the sample before staining. The WBC count should not exceed 10^8 cells/mL, otherwise the sample should be diluted accordingly with an appropriate buffer.

Every sample must be run in duplicate.

1. For each sample, label three 12×75 mm tubes with A, B, and C (isotype control antibody).
2. For each sample tube use a fresh micropipette tip.
   - Carefully pipette 100 µL of well-mixed cell suspension into the bottom of each labeled tube.
     Important: In case the staining volume exceeds 100 µL, the volume has to be reduced to 100 µL.
   - If a target cell fraction of a CD133 enrichment with the CliniMACS® CD133 System is analyzed use the cellular starting product for the IgG2b isotype control.
3. Add 1 mL of RBC lysing solution to 100 µL of sample. Immediately vortex thoroughly for 3 seconds and incubate for 10 minutes at room temperature.
   Important: Avoid prolonged exposure of the cells to lytic reagents, which may cause white blood cell destruction.
4. Immediately after incubation, centrifuge tubes at 300×g for 5 minutes at room temperature.
5. Aspirate the supernatant and avoid disturbing the pellet. Resuspend the pellet thoroughly in the residual fluid.
6. Stain the cells according to table 1.

Table 1: Cell staining

Staining procedure	Tube A / Tube B	Tube C (isotype control IgG2b)
4-color analysis	CD45-FITC*	CD45-FITC*
	CD34-APC*	CD34-APC*
	10 µL CD133/2-PE	Mouse IgG2b-PE*
	Add buffer to 110 µL	Add buffer to 110 µL

(continued)

Table 1: Cell staining (continued)

Staining procedure	Tube A / Tube B	Tube C (isotype control IgG2b)
3-color analysis	CD45-FITC*	CD45-FITC*
	10 µL CD133/2-PE	Mouse IgG2b-PE*
	Add buffer to 110 µL	Add buffer to 110 µL

* Refer to the manufacturer´s recommendations for dilutions and required quantities.

7. Vortex thoroughly for 3 seconds and incubate for 10 minutes at 2–8 °C in the dark.
8. Immediately after incubation, add 1 mL of buffer to the reaction, vortex for 3 seconds, and centrifuge the tubes at 300×g for 5 minutes at room temperature.
9. Aspirate the supernatant and avoid disturbing the pellet. Resuspend the pellet thoroughly in the residual fluid.
10. Add 1 mL of buffer and 5 µL of 7-AAD solution in the concentration of 50 µg/mL. Incubate for 5 minutes at 2–8 °C and proceed immediately to flow cytometric analysis.
    Important: Minimize exposure of samples to light.

10. Flow cytometric analysis

10.1 4-color fluorescence analysis

10.1.1 Gating strategy according to the ISHAGE guidelines

For data acquisition create the following dot plots (the regions are set according to the figures shown below):

I. Dot plot: FSC vs. SSC
II. Dot plot: CD45-FITC vs. SSC (region 1)
III. Dot plot: 7-AAD vs. SSC (region 2)
IV. Dot plot: CD34-APC vs. SSC (region 3)
V. Dot plot: CD133/2-PE (or IgG2b-PE) vs. CD34-APC (region 4)
VI. Dot plot: CD45-FITC vs. SSC (region 5)
VII. Dot plot: FSC vs. SSC (region 6)

Define and label the gates as described in table 2.

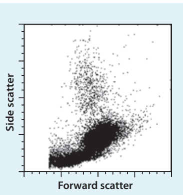
Table 2: Definition of gates

Gate	Label	Definition
G1	WBCs	R1
G2	viable WBCs	R1*R2
G3		R1*R2*R3
G4		R1*R2*R3*R4
G5		R1*R2*R3*R4*R5
G6	CD133-positive HPCs	R1*R2*R3*R4*R5*R6

10.1.2 Description of the detailed gating strategy

The description of the detailed gating strategy is shown using a mobilized leukapheresis harvest.
The regions are set while the IgG2b isotype control is acquired in setup mode.

I. Dot plot: FSC vs. SSC
Activated gate: no gate.
In this dot plot the proper setting of the threshold is checked. No regions are defined.
Important: Make sure not to cut off small lymphocytes by the threshold.



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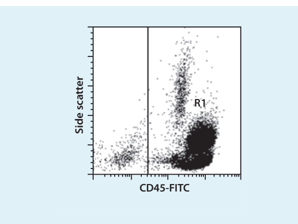
For information only

II. Dot plot: CD45-FITC vs. SSC - Discrimination of all WBCs

Activated gate: no gate.

Set R1 thereby excluding all CD45-negative events from the analysis while all CD45-positive cells are included.

Important: CD133-positive progenitor cells express CD45 with lower staining intensity than lymphocytes. Be careful when setting region R1 and do not exclude these events from the analysis.

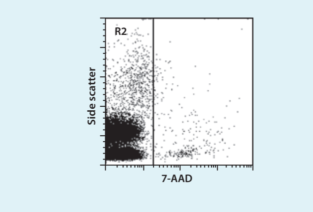


III. Dot plot: 7-AAD vs. SSC - Discrimination of dead cells

Activated gate: G1=R1=WBCs.

Define R2 thereby including all viable WBCs.

Generate a gate statistic of the dot plot III.

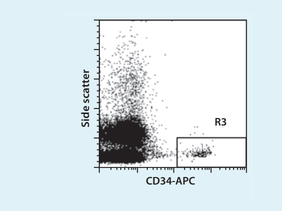


IV. Dot plot: CD34-APC vs. SSC – Gating of all CD34-positive events

Activated gate: G2=R1*R2=viable WBCs.

Define R3 thereby including all CD34-positive cells with a low SSC.

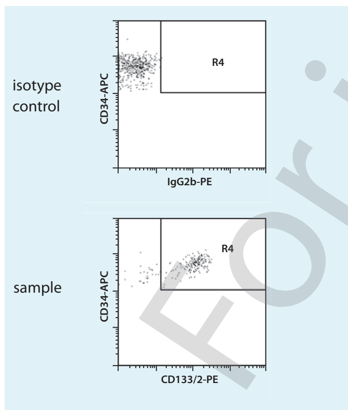
Generate a gate statistic of the dot plot IV.



V. Dot plot: CD133/2-PE (or IgG2b-PE) vs. CD34-APC – Gating of all CD133-positive events

Activated gate: G3=R1*R2*R3.

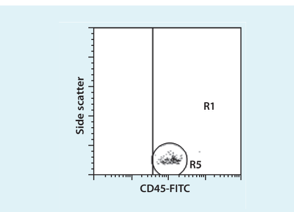
Define R4 with the isotype control to include all CD133-positive events. It is important to set region R4 as close as possible to the CD133-negative events to include all CD133-positive and CD133-dim/positive cells.



VI. Dot plot: CD45-FITC vs. SSC – Cluster control CD133-positive events

Activated gate: G4=R1*R2*R3*R4.

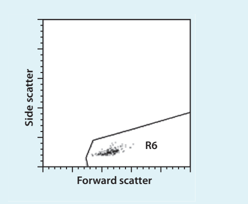
The CD133-positive cells form a cluster with low SSC and dim CD45 fluorescence and are gated in region R5. Non-specifically stained events are excluded from this region.



VII. Dot plot: FSC vs. SSC – Identification of CD133-positive HPCs

Activated gate: G5=R1*R2*R3*R4*R5.

Define a region R6 that identifies a cluster of events meeting all the fluorescence and light scatter criteria of CD133-positive progenitor cells. Cells clustered in region R6 exhibit slightly higher forward scatter than that of small lymphocytes and uniformly low side scatter. Any events falling outside region R6 are not included in the % viable CD133-positive cells determination.



10.2 3-color fluorescence analysis

10.2.1 Gating strategy according to the ISHAGE guidelines

For data acquisition create the following dot plots (the regions are set according to the figures shown below):

I. Dot Plot: FSC vs. SSC
II. Dot Plot: CD45-FITC vs. SSC (region 1)
III. Dot Plot: 7-AAD vs. SSC (region 2)
IV. Dot Plot: CD133/2-PE (or IgG2b-PE) vs. CD45-FITC (region 3)
V. Dot Plot: CD45-FITC vs. SSC (region 4)
VI. Dot Plot: FCS vs. SSC (region 5)

Define and label the gates as described in table 3.

Table 3: Definition of gates

Gate	Label	Definition
G1	WBCs	R1
G2	viable WBCs	R1*R2
G3		R1*R2*R3
G4		R1*R2*R3*R4
G5	CD133-positive HPCs	R1*R2*R3*R4*R5

10.2.2 Description of the detailed gating strategy

The description of the detailed gating strategy is shown using a mobilized leukapheresis harvest.

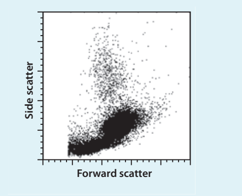
The regions are set while the IgG2b isotype control is acquired in setup mode.

I. Dot plot: FSC vs. SSC

Activated gate: no gate.

In this dot plot the proper setting of the threshold is checked. No regions are defined.

Important: Make sure not to cut off small lymphocytes by the threshold.

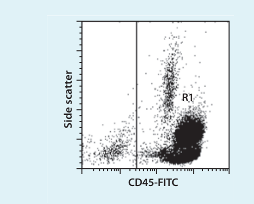


II. Dot plot: CD45-FITC vs. SSC - Discrimination of all WBCs

Activated gate: no gate.

Set R1 thereby excluding all CD45-negative events from the analysis while all CD45-positive cells are included.

Important: CD133-positive progenitor cells express CD45 with lower staining intensity than lymphocytes. Be careful when setting region R1 and do not exclude these events from the region.

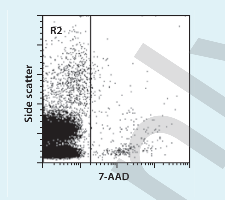


III. Dot plot: 7-AAD vs. SSC - Discrimination of dead cells

Activated gate: G1=R1=WBCs.

Define R2 thereby including all viable WBCs.

Generate a gate statistic of the dot plot III.

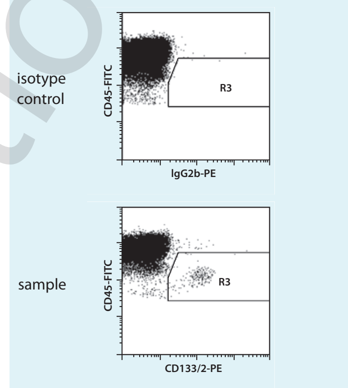


IV. Dot plot: CD133/2-PE (or IgG2b-PE) vs. CD45-FITC – Gating of all CD133-positive events

Activated gate: G2=R1*R2=viable WBCs.

Define R3 with the isotype control to include all CD133-positive events. It is important to set region R3 as close as possible to the CD133-negative events to include all CD133-positive and CD133-dim/positive cells.

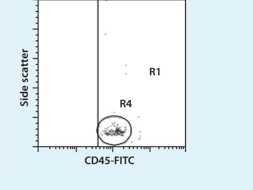
Generate a gate statistic of the dot plot IV.



V. Dot plot: CD45-FITC vs. SSC – Cluster control CD133-positive events

Activated gate: G3=R1*R2*R3.

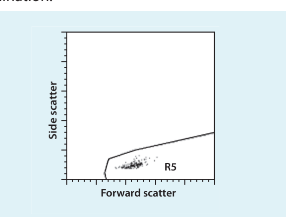
The CD133-positive cells form a cluster with low SSC and dim CD45 fluorescence and are gated in region R4. Non-specifically stained events are excluded from this region.



VI. Dot plot: FSC vs. SSC – Identification of CD133-positive HPCs

Activated gate: G4=R1*R2*R3*R4.

Define a region R5 that identifies a cluster of events meeting all the fluorescence and light scatter criteria of CD133-positive progenitor cells. Cells clustered in region R5 exhibit slightly higher forward scatter than that of small lymphocytes and uniformly low side scatter. Any events falling outside region R5 are not included in the % viable CD133-positive cells determination.



11. Data acquisition and analysis

After adjustment of the regions the samples are analyzed in the following order:

1.) Isotype control IgG2b (Tube C)
2.) Sample (Tube A and Tube B)

Set the collection criteria of „acquisition mode" to „event count" of gate G6 (G5) and collect 100 CD133-positive HPCs per tube. Use this gate G6 (G5) only as collecting criteria, but not as storage gate (data file will contain all events).

Important: Not less than 100.000 CD45-positive events per tube and a minimum of 100 CD133-positive events should be counted. When analyzing a target cell fraction of a CliniMACS Separation at least 10.000 CD45-positive events should be counted.

Analysis of results

For analysis of the results take the percentage value of living cells from the statistics generated from dot plot III and the percentage value of CD133-positive HPCs from the statistics generated from dot plot IV.

In table 4 the percentage of CD133-positive cells among viable WBCs is given in % Gated in G2 (99,16%).

Table 4: Gate statistic dot plot III (4-color analysis)

Gate	Events	% Gated	% Total
G1 = R1 = WBC	40871	100.00	98.75
G2 = R1+R2 = viable	40527	99.16	97.92
G3 = R1+R2+R3 = viable CD34+	221	0.54	0.53
G4 = R1+R2+R3+R4 = viable CD133+	190	0.46	0.46
G5 = R1+R2+R3+R4+R5 = cluster control CD133	188	0.46	0.45
G6 = R1+R2+R3+R4+R5+R6 = CD133+ HPCs	187	0.46	0.45

In table 5 the percentage of CD133-positive HPCs is given in % Gated in G6 (0.46%).

Table 5: Gate statistic dot plot IV (4-color analysis)

Gate	Events	% Gated	% Total
G1 = R1 = WBC	40527	100.00	97.92
G2 = R1+R2 = WBC	40527	100.00	97.92
G3 = R1+R2+R3 = viable CD34+	221	0.55	0.53
G4 = R1+R2+R3+R4 = viable CD133+	190	0.47	0.46
G5 = R1+R2+R3+R4+R5 = cluster control CD133	188	0.46	0.45
G6 = R1+R2+R3+R4+R5+R6 = CD133+ HPCs	187	0.46	0.45

12. Performance

All cell sources defined in the intended use of CD133/2-PE have been tested during performance evaluation.

CD133/2-PE showed a very good performance and had a reproducibility of (CV)<0.1 for all cell sources tested.

In the case of overnight storage, a decrease in performance has been observed. Whole blood should not be stored longer than 12 hours.

13. References

1. Yin, A. H. *et al.* (1997) Blood 90: 5002–5012.
2. Freund, D. *et al.* (2006) Cell Prolif. 39: 325–332.
3. Matsumoto, K. *et al.* (2000) Stem Cells 24: 2456–2465.
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6. Uchida, N. *et al.* (2000) Proc. Natl. Acad. Sci. USA 97: 14720–14725.
7. Tondreau, T. *et al.* (2005) Stem cells 23: 14105–11012.

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