



Miltenyi Biotec

# **MSC Enumeration Kit**

## human

For 25 tests

Order no. 130-106-646

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## 1. Description

<b>Components</b>	<p><b>250 µL MSC Staining Cocktail, human:</b> cocktail of fluorochrome-conjugated monoclonal antibodies: CD45-FITC (clone: 5B1, isotype: mouse IgG2a), CD235a (Glycophorin A)-VioBlue® (clone: REA175, isotype: recombinant human IgG1), CD271 (LNGFR)-PE (clone: Me20.4-1H.4, isotype: mouse IgG1).</p> <p><b>250 µL Anti-MSCA-1 (W8B2)-APC, human:</b> anti-MSCA-1 (W8B2) monoclonal antibody (clone: W8B2, isotype: mouse IgG1) conjugated to APC.</p> <p><b>250 µL MSC Isotype Cocktail, human:</b> cocktail of Mouse IgG1-APC (clone: IS5-21F5.12.2) and fluorochrome-conjugated monoclonal antibodies: CD45-FITC (clone: 5B1, isotype: mouse IgG2a), CD235a (Glycophorin A)-VioBlue (clone: REA175, isotype: recombinant human IgG1), CD271 (LNGFR)-PE (clone: Me20.4-1H.4, isotype: mouse IgG1).</p> <p><b>5 mL Red Blood Cell Lysis Solution (10×)</b></p> <p><b>1 mL FcR Blocking Reagent, human</b></p> <p><b>500 µL 7-AAD Solution (52.5 µg/mL)</b></p>
<b>Capacity</b>	For 25 tests with 200 µL human cell sample per test.
<b>Product format</b>	MSC Staining Cocktail, Anti-MSCA-1 (W8B2)-APC, MSC Isotype Cocktail, and FcR Blocking

Reagent are supplied in buffer containing stabilizer and 0.05% sodium azide.

### Storage

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

### 1.1 Principle of the assay

The MSC Enumeration Kit has been designed for the quantification of mesenchymal stromal cells (MSCs) in human bone marrow samples, based on the expression of CD271 (LNGFR) and Anti-MSCA-1 (W8B2). The kit allows the identification of CD45<sup>+</sup> leukocytes and CD271 (LNGFR)<sup>+</sup>/Anti-MSCA-1 (W8B2)<sup>+</sup> MSCs, which are also CD45<sup>dim</sup>. CD235a (Glycophorin A) is used to exclude remaining red blood cells, which were not completely lysed by the Red Blood Cell Lysing Solution. For one test, two 100 µL samples of bone marrow are needed.

The MSCs are detected by staining one 100 µL sample with the MSC Staining Cocktail as well as with Anti-MSCA-1 (W8B2)-APC. Subsequently, erythrocytes are lysed using the Red Blood Cell Lysis Solution.

Additionally, a 100 µL control sample is stained with the MSC Isotype Cocktail. This is required for the proper setting of relevant gates during flow cytometry analysis.

The FcR Blocking Reagent increases the specificity of immunofluorescent staining.

Dead cells are excluded from the analysis by adding of DNA stain 7-aminoactinomycin D (7-AAD). 7-AAD diffuses through the cell membrane of dead cells and intercalates with their DNA.

Finally, cells are analyzed with flow cytometers able to detect 4-color fluorescence (488 nm argon laser, 635 nm red diode laser, optional: 405 nm violet laser). The use of the MACSQuant® Flow Cytometers enable an automated and accurate cell count. If using flow cytometers other than the MACSQuant Flow Cytometer, it is necessary to determine the absolute cell count by analysis of commercially available counting particles such as present in BD Trucount™ Tubes.

## 1.2 Background information

The large scale isolation and expansion of MSCs from human bone marrow is time consuming, cost effective, and highly dependent on the quality of the starting material. Therefore it is desirable to qualify the bone marrow source with regard to MSC content. The common way to quantify MSCs within human blood sources is the colony forming unit-fibroblast (CFU-F) assay, which is time consuming, dependent on serum lots, and plating densities, as well as considerably subjective in defining and scoring colonies. It was shown that Anti-MSCA-1 (W8B2) only detects CD271 (LNGFR)<sup>bright</sup> cells and only these cells give rise to CFU-F<sup>1</sup>. A close linear relationship was observed between the number of CFU-F colonies counted manually after 14 days of culture and the number of CD271 (LNGFR)<sup>bright</sup> cells per milliliter of aspirate<sup>2</sup>.

## 1.3 Application

- Enumeration of MSCs in human bone marrow samples

### 1.4 Description of kit components

#### MSC Staining Cocktail

*Components of the MSC Staining Cocktail, human*

Antibody	Fluorochrome	Specificity
CD45	FITC	Leukocytes
CD235a (Glycophorin A)	VioBlue	Erythrocytes
CD271 (LNGFR)	PE	MSCs, lymphocyte progenitors

Fluorescein (FITC), Phycoerythrin (PE)

#### Anti-MSCA-1 (W8B2)-APC

Antibody	Fluorochrome	Specificity
Anti-MSCA-1 (W8B2)	APC	MSCs

Allophycocyanin (APC)

#### MSC Isotype Cocktail

*Components of the MSC Isotype Cocktail, human*

Antibody	Fluorochrome	Specificity
CD45	FITC	Leukocytes
CD235a (Glycophorin A)	VioBlue	Erythrocytes
CD271 (LNGFR)	PE	MSCs, lymphocyte progenitors
Mouse IgG1	APC	Isotype control

## Red Blood Cell Lysis Solution

Upon incubation of the cells in 1× Red Blood Cell Lysis Solution erythrocytes are selectively lysed. Dilute Red Blood Cell Lysis Solution (10×) 1:10 with double-distilled water (ddH<sub>2</sub>O).

### 1.5 Reagents and instruments requirements

- Double-distilled water (ddH<sub>2</sub>O)
- 5 mL tubes
- Micropipettes with tips
- Centrifuge (2–8 °C)
- Vortex mixer
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753)
- Three-laser flow cytometer and software (405 nm, 488 nm, and 638 nm laser), e.g., MACSQuant Analyzer 10 (# 130-096-343) with MACSQuantify™ Software.  
  
▲ **Note:** Alternatively, a dual-laser flow cytometer and software (488 nm and 638 nm laser) can be used. Exclusion of remaining red blood cells using CD235a is then not possible.
- (Optional) BD Trucount Absolute Counting Tubes (# 340334)
- (Optional) MACS® SmartStrainers (100 μm) (# 130-098-463)
- (Optional) MACS Comp Bead Kit, anti-mouse Igκ (# 130-097-900)
- (Optional) MACS Comp Bead Kit, anti-REA (# 130-104-693)

## 2. Protocols

The following protocol has been optimized for mesenchymal stromal cell (MSC) enumeration in a human bone marrow sample. For best results use starting material directly after aspiration.

▲ Bone marrow samples have to be supplemented with an anticoagulant, for example, heparin.

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

▲ When using bone marrow aspirate pass the material through a MACS SmartStrainer (100  $\mu\text{m}$ ).

▲ Dilute **Red Blood Cell Lysis Solution (10 $\times$ )** 1:10 with double-distilled water (ddH<sub>2</sub>O), for example, dilute 200  $\mu\text{L}$  of Red Blood Cell Lysis Solution (10 $\times$ ) with 1800  $\mu\text{L}$  of ddH<sub>2</sub>O. Do not use deionized water!

Store the prepared 1 $\times$  Red Blood Cell Lysis Solution at room temperature. Discard unused solution at the end of the day.

### 2.1 Immunofluorescent labeling

▲ Volumes given below are for one test with two 100  $\mu\text{L}$  samples of bone marrow.

1. Label two tubes with **A** (control sample) and **B** (MSC sample).

▲ **Note:** When using other flow cytometer than the MACSQuant Flow Cytometers use two BD Trucount Absolute Counting Tubes.

2. Carefully pipette 100  $\mu\text{L}$  of well-mixed human bone marrow to the bottom of each tube.  
**▲ Note:** For each sample tube use a fresh micropipette tip.
3. Add 20  $\mu\text{L}$  FcR Blocking Solution into each tube.
4. Add 10  $\mu\text{L}$  of MSC Isotype Cocktail into tube **A** and 10  $\mu\text{L}$  of MSC Staining Cocktail as well as 10  $\mu\text{L}$  of Anti-MSCA-1 (W8B2)-APC into tube **B**.
5. Immediately vortex thoroughly and incubate for 10 minutes at 2–8  $^{\circ}\text{C}$  in the dark.
6. Add 860  $\mu\text{L}$  of 1 $\times$  Red Blood Cell Lysis Solution into tube **A** and 850  $\mu\text{L}$  in tube **B**.
7. Immediately vortex and incubate for 10 minutes at room temperature in the dark using the MACSmix Tube Rotator.
8. Add 10  $\mu\text{L}$  of the 7-AAD Solution into each tube.
9. Directly proceed with flow cytometric analysis.  
**▲ Note:** Analysis should be performed within 20 minutes after staining.

### **2.2 Flow cytometric acquisition and data analysis using the MACSQuant® Analyzer 10**

#### **2.2.1 Flow cytometric data acquisition**

Before starting to acquire the samples make sure that the MACSQuant® Analyzer 10 flow cytometer has been carefully cleaned in order to allow the detection of rare MSCs.

#### **Instrument setting and data acquisition**

Choose an instrument setting for standard four-color cell analysis of human blood leukocytes. A proper instrument setting can be reached by adjusting parameters with separate FITC-, PE-, VioBlue-, and APC-conjugated antibodies or by using the MACS Comp Bead Kit, anti-mouse Igκ or the MACS Comp Bead Kit, anti-REA.

▲ Exclude debris from data acquisition by an appropriate value for the threshold on forward scatter (FSC).

#### **Creation of dot plots**

For data acquisition and analysis create the following dot plots (region settings are shown in the examples below):

A Plot: Forward scatter (FSC) versus side scatter (SSC)

B Plot: 7-AAD Solution versus SSC

C Plot: FSC versus CD235a (Glycophorin A)-VioBlue

D Plot: CD45-FITC versus CD271 (LNGFR)-PE

E Plot: Mouse IgG1-APC versus CD271 (LNGFR)-PE and Anti-MSCA-1 (W8B2)-APC versus CD271 (LNGFR)-PE

### Define the experiment as follows:

Experiment setup	
Rack type	Chill 5 Tube Rack, choose 2 positions
Sample ID	1. position: Control sample 2. position: MSC sample
Flow rate	medium
Mode	standard
Uptake volume	450 $\mu$ L
Sample volume	1 mL
Instrument setting	PBMC compensation (needs to be performed before data analysis)*
Annotations	1. position: CD45-FITC, CD235a (Glycophorin A)-VioBlue, CD271 (LNGFR)-PE, Mouse IgG1-APC, 7-AAD Solution 2. position: CD45-FITC, CD235a (Glycophorin A)-VioBlue, CD271 (LNGFR)-PE, Anti-MSCA-1 (W8B2)-APC, 7-AAD Solution

\* Please refer to the user guide of the MACSQuantify Software for detailed instructions.

### 2.2.2 Flow cytometric analysis

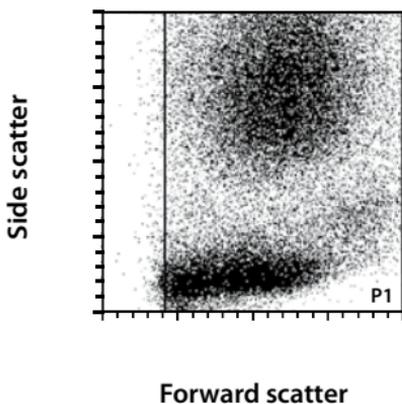
▲ The gating strategy below describes the flow cytometric analysis of a bone marrow sample using the MACSQuantify Software.

▲ Perform the analysis of the MSC sample stained with the MSC Staining Cocktail in the same way as the analysis of the control sample stained with the MSC Control Cocktail.

#### MSC sample

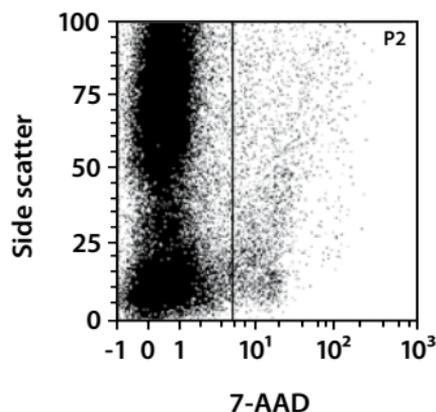
A Plot: FSC versus SSC (no gate)

Draw region P1 to exclude debris and platelets.



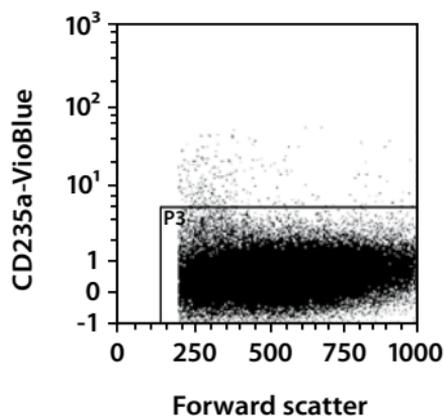
B Plot: 7-AAD Solution versus SSC (gated on P1)

Draw region P2 to exclude dead cells.

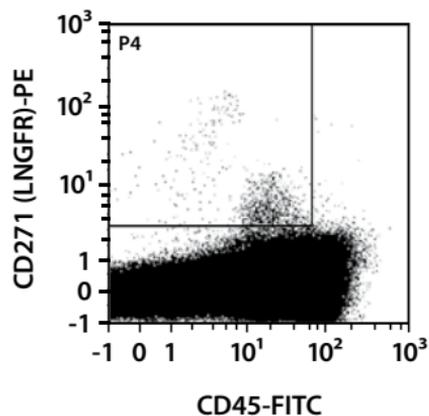


C Plot: FSC versus CD235a (Glycophorin A)-VioBlue (gated on P1/P2)

Draw region P3 to exclude CD235a<sup>+</sup> erythrocytes.



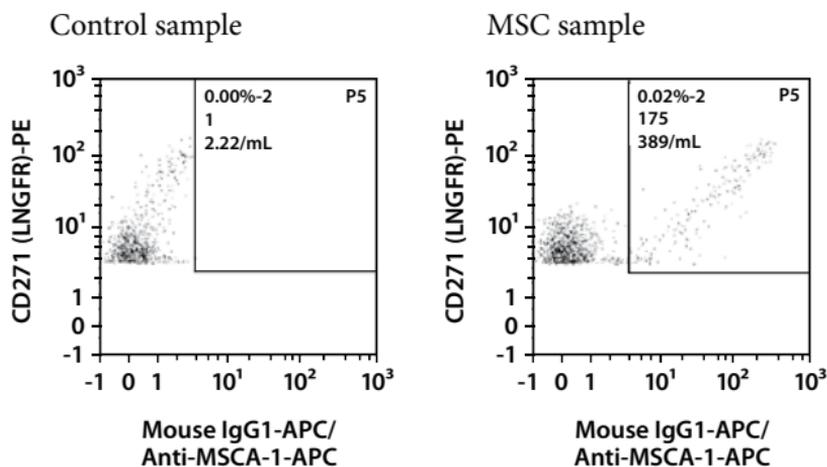
D Plot: CD45-FITC versus CD271 (LNGFR)-PE (gated on P1/P2/P3)  
Define region P4 thereby including all CD271 (LNGFR)<sup>+</sup> cells.



E Plot: Mouse IgG1-APC/Anti-MSCA-1 (W8B2)-APC versus CD271 (LNGFR)-PE (gated on P1/P2/P3/P4)

Copy all gates into the control sample.

Define region P5 with the control sample to include all Anti-MSCA-1 (W8B2)-APC<sup>+</sup> events within the MSC sample. It is important to set gate P5 as close as possible to the Anti-MSCA-1 (W8B2)<sup>-</sup> events to include all Anti-MSCA-1 (W8B2)<sup>+</sup> and Anti-MSCA-1 (W8B2)<sup>dim+</sup> cells.



### 2.2.3 Determination of MSC number

For the calculation the gate statistic of plot E of the MSC sample has been used. Following region functions are needed:

	Parameter	Description	Example
a	Count/mL	Number of events per mL staining volume	389/mL
b	%-2	Percent grand parent, for information only	0.02%
c	Staining volume	Complete volume after labeling including cocktails, antibody, Red Blood Cell Lysis Solution, FcR Blocking Reagent, and 7-AAD Solution (1000 $\mu$ L in standard applications)	1000 $\mu$ L
d	Sample volume	Bone marrow volume used for one staining (100 $\mu$ L in standard applications)	100 $\mu$ L

Calculation of dilution factor

	Parameter	Calculation	Example
e	Dilution factor	$c / d$	$1000 \mu\text{L} / 100 \mu\text{L} = 10$

Calculation of MSC number

	Parameter	Calculation	Example
f	Number of MSCs/mL bone marrow	$a \times e$	$389/\text{mL} \times 10 = 3890/\text{mL}$

In the given example, the number of MSCs is 3890/mL human bone marrow aspirate.

## 2.3 Flow cytometric acquisition and data analysis using flow cytometer other than MACSQuant® Analyzer 10

### 2.3.1 Flow cytometric data acquisition

Before starting to acquire the samples make sure that the flow cytometer has been carefully cleaned in order to allow the detection of rare MSCs.

#### Instrument setting and data acquisition

Choose a flow cytometer instrument setting for standard four-color cell analysis of human blood leukocytes. A proper instrument setting can be reached by adjusting parameters with separate FITC-, PE-, and APC-conjugated antibodies.

- ▲ Exclude debris from data acquisition by an appropriate value for the threshold on forward scatter (FSC).
- ▲ A minimum of 100 CD271 (LNGFR)<sup>+</sup> Anti-MSCA-1 (W8B2)<sup>+</sup> events should be counted. Additionally a minimum of 1000 counting particle or a maximum of 10 minutes acquisition time should be analyzed.

#### Creation of dot plots

For data acquisition and analysis create the following dot plots (region settings are shown in the examples below):

A Plot: Forward scatter (FSC) versus side scatter (SSC)

B Plot: 7-AAD Solution versus SSC

C Plot: CD45-FITC versus CD271 (LNGFR)-PE

D Plot: Mouse IgG1-APC versus CD271 (LNGFR)-PE and Anti-MSCA-1 (W8B2)-APC versus CD271 (LNGFR)-PE

E Plot: CD45-FITC versus CD271 (LNGFR)-PE

F Plot: Time versus CD271 (LNGFR)-PE

### Definition of logical gates

Define and label the gates described in the following table:

Gate	Label	Definition
G1	WBCs	R1
G2	Viable WBCs	R1*R2
G3	CD271 (LNGFR) <sup>+</sup>	R1*R2*R3
G4	CD271 (LNGFR) <sup>+</sup> aMSCA-1 <sup>+</sup>	R1*R2*R3*R4
G5		R5
G6	Counting beads	R5*R6

### 2.3.2 Flow cytometric analysis

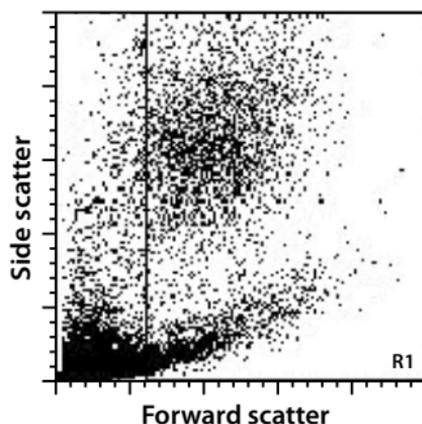
▲ The general gating strategy below describes the flow cytometric analysis of a bone marrow sample when using another flow cytometer than the MACSQuant® Analyzer 10.

▲ Perform the analysis of the MSC sample stained with the MSC Staining Cocktail in the same way as the analysis of the control sample stained with the MSC Control Cocktail.

#### MSC sample

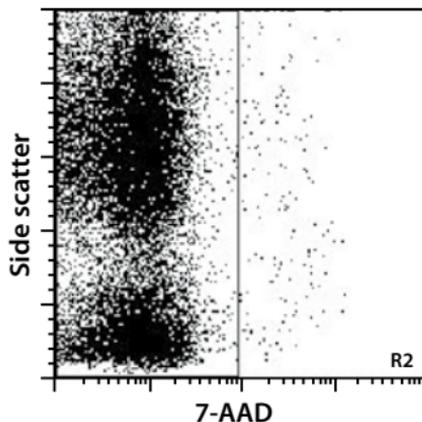
A Plot: FSC versus SSC (no gate)

Draw region R1 to exclude debris and platelets.



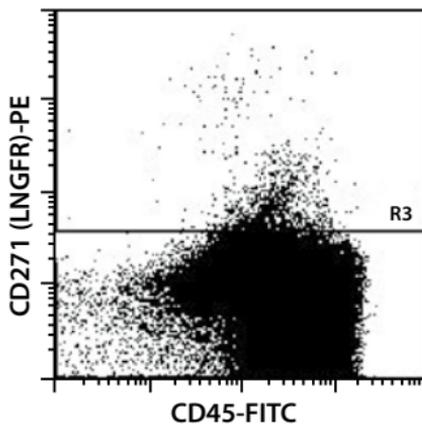
B Plot: 7-AAD Solution versus SSC (gated on R1)

Draw region R2 to exclude dead cells.



C Plot: CD45-FITC versus CD271 (LNGFR)-PE (gated on R1/R2)

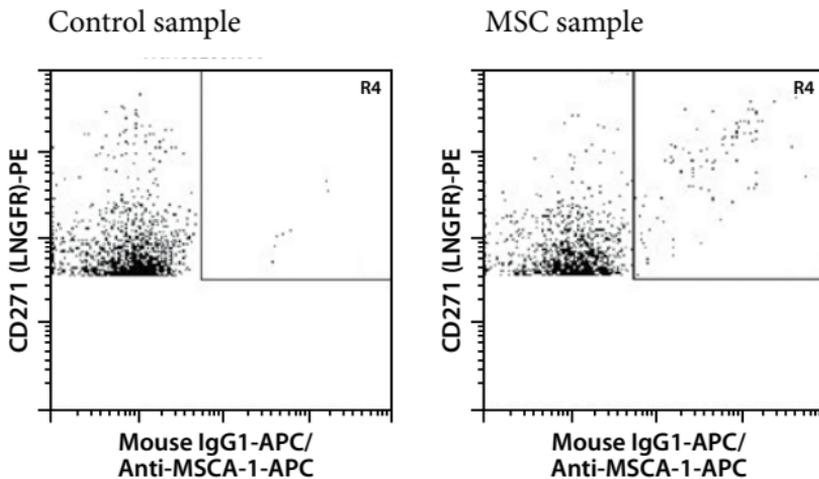
Draw region R3 thereby including all CD271 (LNGFR)<sup>+</sup> cells. Generate a gate statistic for later calculation.



D Plot: Mouse IgG1-APC/Anti-MSCA-1 (W8B2)-APC versus CD271 (LNGFR)-PE (gated on R1/R2/R3)

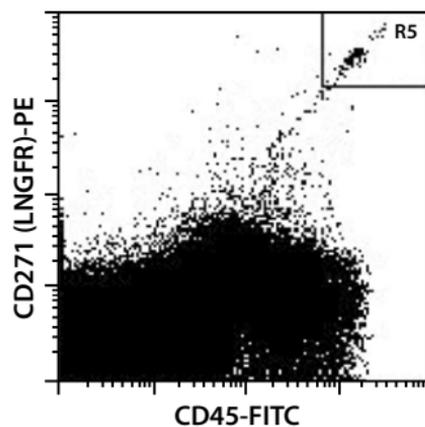
Copy all gates into the control sample file.

Define region R4 with the control sample to include all Anti-MSCA-1 (W8B2)-APC<sup>+</sup> events within the MSC sample. It is important to set gate R4 as close as possible to the Anti-MSCA-1 (W8B2)<sup>-</sup> events to include all Anti-MSCA-1 (W8B2)<sup>+</sup> and Anti-MSCA-1 (W8B2)<sup>dim+</sup> cells.



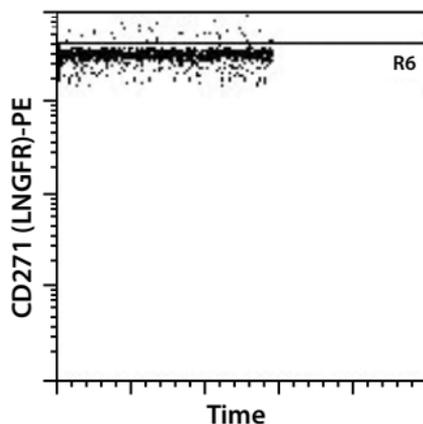
E Plot: CD45-FITC versus CD271 (LNGFR)-PE (no gate)

Define region R5 by including all FITC and PE<sup>bright</sup> counting beads.



F Plot: Time versus CD271 (LNGFR)-PE (gated on R5)

Define region R6 by including all monodisperses and bidisperses-counting beads. Generate a gate statistic for later calculation.



### 2.3.3 Analysis of the results using counting particles

For the calculations the gate statistics of plot C and F are used. A summary of these statistics are shown in the table below.

Gate	Events	Gated [%]
G1 (WBCs)	140141	100.00
G2 (Viable WBCs)	140141	100.00
G3 (CD271 (LNGFR) <sup>+</sup> )	7731	5.52
G4 (CD271 (LNGFR) <sup>+</sup> aMSCA-1 <sup>+</sup> )	114	0.08
G5	16530	100.00
G6 (Counting beads)	16040	97.04

### Calculation of dilution factor

The dilution factor is calculated by dividing the staining volume (c, complete volume after labeling) by the sample volume (d, bone marrow volume used for one staining). Refer to section 2.2.3 for details.

Parameter	Calculation	Example
Dilution factor	$c / d$	$1000 \mu\text{L} / 100 \mu\text{L} = 10$

### Calculation of MSCs number

Parameter	Calculation
Number of MSCs/ mL bone marrow	$\frac{G4 \times \text{total counting beads per tube}^*}{G6} \times \text{dilution factor}$
	Example: $\frac{114 \times 50950}{16040} \times 10 = 3621/\text{mL}$

\* As indicated in the manufacturer's instruction.

In the given example, the number of MSCs is 3621/mL human bone marrow aspirate.

### 3. Appendix

MSCA-1 (W8B2) is identical to the tissue non-specific alkaline phosphatase (TNAP). It was demonstrated that only MSCA-1<sup>+</sup> but not MSCA-1<sup>-</sup> bone marrow cells express the enzymatically active form of the TNAP. In addition, it was shown in different bone marrow sources that only MSCA-1<sup>+</sup> cells gave rise to MSCs<sup>3</sup> as defined by the ISCT consortium<sup>4</sup>. Nevertheless there are further MSC specific antibodies which can be used in combination with CD271 (LNGFR) for MSC detection and enumeration. The following table lists some of these markers with suggested titers that can be used within this assay instead of Anti-MSCA-1 (W8B2)-APC.

Specificity	Titer	Synonym	Order number
CD73-APC	1:40	ecto-5'-nucleotidase	# 130-095-183
CD90-APC	1:40	Thy-1	# 130-095-402
CD140b-APC	1:10	PDGF-receptor	# 130-105-280

### 4. References

1. Bühring, H. J. (2007) Novel markers for the prospective isolation of human MSC. *Ann. N. Y. Acad. Sci.* 6: 262–271.
2. Cuthbert, R. (2012) Single-platform quality control assay to quantify multipotential stromal cells in bone marrow aspirates prior to bulk manufacture or direct therapeutic use. *Cytotherapy* 14( 4):431–440.
3. Sobiesiak, M. *et al.* (2010) The mesenchymal stem cell antigen MSCA-1 is identical to tissue non-specific alkaline phosphatase. *Stem Cells Dev.* 19(5): 669–677.
4. Dominici, M. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8 (4): 315–317.

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## 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.

## Warranty

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**[www.miltenyibiotec.com](http://www.miltenyibiotec.com)**

For technical questions, please contact your local subsidiary or distributor.

Technical Support Team, Germany:

Phone: +49 2204 8306-830

[macstec@miltenyibiotec.de](mailto:macstec@miltenyibiotec.de)



**Miltenyi Biotec**

**Miltenyi Biotec GmbH**

Friedrich-Ebert-Straße 68

51429 Bergisch Gladbach

Germany

Phone +49 2204 8306-0

Fax +49 2204 85197

[macs@miltenyibiotec.de](mailto:macs@miltenyibiotec.de)

**Miltenyi Biotec Inc.**

2303 Lindbergh Street

Auburn, CA 95602-9562, USA

Phone 800 FOR MACS

Phone +1 530 888 8871

Fax +1 877 591 1060

[macs@miltenyibiotec.com](http://macs@miltenyibiotec.com)