

## Contents

1. Description
  - 1.1 Background information
  - 1.2 Principle of the CD105<sup>+</sup> Sca-1<sup>+</sup> LTR-HSC isolation procedure
  - 1.3 Reagent and instrument requirements
2. Protocol
  - 2.1 Preparation of bone marrow cells
  - 2.2 Magnetic labeling of CD105<sup>+</sup> cells
  - 2.3 Magnetic separation of CD105<sup>+</sup> cells
  - 2.4 Removal of MultiSort MicroBeads using the MultiSort Release Reagent and magnetic labeling of Sca-1<sup>+</sup> cells
  - 2.5 Magnetic separation of Sca-1<sup>+</sup> cells
3. Example of a separation using the CD105 MultiSort Kit (PE), mouse, and the Anti-Sca-1 MicroBead Kit (FITC), mouse
4. References

## 1. Description

### 1.1 Background information

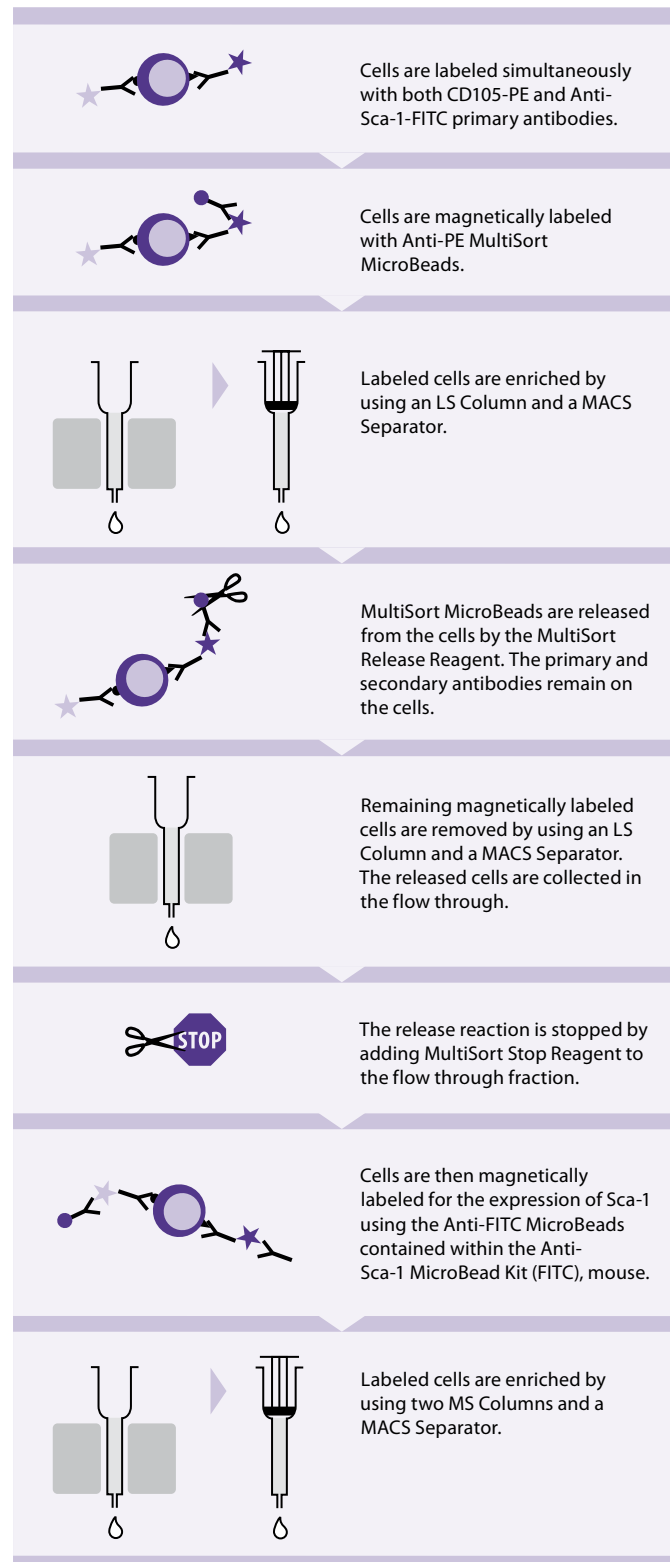
CD105, also known as endoglin, is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells.<sup>1</sup> In mouse bone marrow (BM), CD105 was also found to be expressed on a population of Sca-1<sup>+</sup> hematopoietic stem cells (HSCs), which were further characterized to exclusively possess a long-term hematologic repopulating (LTR) ability in mice, and are therefore termed LTR-HSCs.<sup>2-3</sup>

### 1.2 Principle of the CD105<sup>+</sup> Sca-1<sup>+</sup> LTR-HSC isolation procedure

This special protocol describes the isolation of CD105<sup>+</sup> Sca-1<sup>+</sup> LTR-HSCs (long-term repopulating hematopoietic stem cells) from mouse bone marrow using a two-step magnetic purification procedure. After initial selection for CD105 expression, purified cells are magnetically labeled for a second time to select for Sca-1 expression. After the second step, the resulting cell population will be CD105<sup>+</sup> Sca-1<sup>+</sup> LTR-HSCs.

Sequential magnetic purification of the target cells is facilitated by using the CD105 MultiSort Kit (PE), mouse (# 130-092-924) in the first dimension, which includes a MicroBead removal step before cells are labeled for a second time with the Anti-Sca-1 MicroBead Kit (FITC), mouse (# 130-092-529).

The whole procedure is schematically illustrated on the right side of this page and is designed for the enrichment of CD105<sup>+</sup> Sca-1<sup>+</sup> LTR-HSCs from a starting population of 2×10<sup>8</sup> bone marrow cells.





## 2.2 Magnetic labeling of CD105<sup>+</sup> cells

### 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). Degas buffer before use, as air bubbles could block the column.

▲ **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 mouse serum albumin, mouse serum, or fetal bovine serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.

- CD105 MultiSort Kit (PE), mouse (# 130-092-924) and the Anti-Sca-1 MicroBead Kit (FITC), mouse (# 130-092-529) for the sequential selection of CD105<sup>+</sup> Sca-1<sup>+</sup> LTR-HSCs.
- MACS Columns and MACS Separators: CD105<sup>+</sup> should be enriched (positive selection) by using LS Columns; Sca-1<sup>+</sup> cells should be enriched using MS Columns.

Column	Max. number of labeled cells	Max. number of total cells	Separator
MS	10 <sup>7</sup>	2×10 <sup>8</sup>	MiniMACS, OctoMACS, VarioMACS, SuperMACS
LS	10 <sup>8</sup>	2×10 <sup>9</sup>	MidiMACS, QuadroMACS, VarioMACS, SuperMACS

▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ Separators. For details see the respective MACS Separator data sheet.

- (Optional) Fluorochrome-conjugated CD117 antibody, e.g., CD117-PE (# 130-091-730) or CD117-APC (# 130-091-729), or the Lineage Cell Detection Cocktail-Biotin, mouse (# 130-092-613) for flow cytometric analysis.
- Pre-Separation Filters (# 130-041-407) to remove cell clumps.
- (Optional) Propidium iodide (PI) or 7-AAD for flow cytometric exclusion of dead cells.

## 2. Protocol

### 2.1 Preparation of bone marrow cells

▲ All steps should be performed on ice.

1. Collect mouse bone marrow cells from femurs and tibiae by flushing the shaft with buffer using a 26-gauge needle.
2. Disaggregate cells by gently pipetting up and down several times.
3. Pass cells through 30 μm nylon mesh (Pre-Separation Filter, # 130-041-407) to remove cell clumps which may block the column. Wet filter with buffer before use.
4. Wash cells by adding 50 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
5. Resuspend cells in 10 mL of buffer. Proceed to magnetic labeling of CD105<sup>+</sup> cells (2.2).

▲ 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.

▲ Volumes for magnetic labeling given below are for up to 2×10<sup>8</sup> total cells. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 4×10<sup>8</sup> total cells, use twice the volume of all indicated reagent volumes and total volumes).

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

▲ The optimal relative centrifugal force (RCF) and centrifugation time may differ and are dependent on the cell sample.

▲ Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 1600 μL of buffer per 2×10<sup>8</sup> total cells. Add 400 μL of CD105-PE and 200 μL of Anti-Sca-1-FITC.
4. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
5. Wash cells by adding 50 mL of buffer per 2×10<sup>8</sup> cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. (Optional) Repeat washing step.
7. Resuspend cell pellet in 1600 μL of buffer per 2×10<sup>8</sup> total cells and add 400 μL of Anti-PE MultiSort MicroBeads.
8. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
9. Wash cells by adding 50 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
10. Resuspend up to 2×10<sup>8</sup> cells in 1 mL of buffer.
  - ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
11. Proceed to magnetic separation of CD105<sup>+</sup> cells (2.3).



### 2.3 Magnetic separation of CD105<sup>+</sup> cells

▲ To achieve highest purities, perform two consecutive column runs.

1. Place an LS Column in the magnetic field of a MidiMACS™ Separator. For details see the LS Column data sheet.
2. Prepare LS Column by rinsing with 3 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 3×3 mL of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
5. Remove LS Column from the MidiMACS Separator and place it on a suitable collection tube.
 

▲ **Note:** To perform a second column run, you may elute the cells directly from the first onto the second, equilibrated column instead of a collection tube.
6. Pipette 5 mL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
7. To increase the purity of the enriched CD105<sup>+</sup> cells, the eluted fraction can be enriched over a second LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.
8. Determine cell number.
9. Proceed to removal of MultiSort MicroBeads (2.4).



### 2.4 Removal of MultiSort MicroBeads using MultiSort Release Reagent and magnetic labeling of Sca-1<sup>+</sup> cells

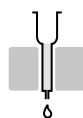
▲ Volumes for magnetic labeling given below are for up to 10<sup>7</sup> total cells. When working with fewer than 10<sup>7</sup> cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10<sup>7</sup> total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.

1. Remove a sample for analysis by flow cytometry and proceed with the remaining magnetically labeled fraction.
2. Add 20 μL of the MultiSort Release Reagent per 1 mL of cell suspension.
3. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
4. (Optional) To remove any residual magnetically labeled cells, repeat the magnetic separation procedure as described in section 2.3. Separate cells over a new LS Column. Collect magnetic (unreleased) and non-magnetic (released) cell fractions to determine the efficiency of the release reaction (see section 2.5).

▲ **Note:** This step is extremely important if the target cells of the second parameter separation are present in a low concentration after selection for CD105 expression (<10% target cells in the positive fraction after the first separation).

5. Wash cells from the released fraction carefully by adding 1–2 mL of buffer per 10<sup>7</sup> cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. Resuspend cells at a final concentration of 10<sup>7</sup> total cells per 50 μL of buffer.
7. Add 30 μL of MultiSort Stop Reagent and mix well.
8. Add 20 μL of Anti-FITC MicroBeads to magnetically label CD105<sup>+</sup> Sca-1<sup>+</sup> LTR-HSCs.
9. Mix well and incubate for 15 minutes in the dark in the refrigerator (2–8 °C).
10. Proceed to magnetic separation of Sca-1<sup>+</sup> cells (2.5).



### 2.5 Magnetic separation of Sca-1<sup>+</sup> cells

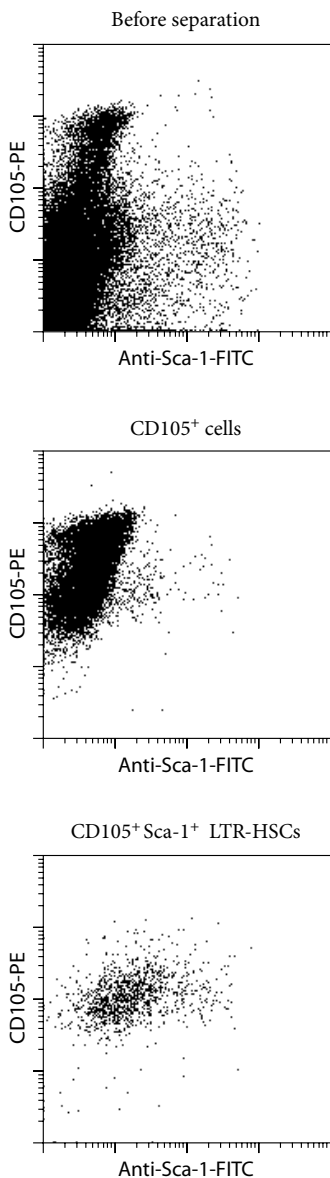
▲ To achieve highest purities, perform two consecutive column runs.

1. Place an MS Column in the magnetic field of a MiniMACS™ Separator. For details see the MS Column data sheet.
2. Prepare MS Column by rinsing with 500 μL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 3×0.5 mL of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
5. Remove MS Column from the separator and place it on a suitable collection tube.
 

▲ **Note:** To perform a second column run, you may elute the cells directly from the first onto the second, equilibrated column instead of a collection tube.
6. Pipette 1 mL of buffer onto the MS Column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the MS Column.
7. To increase the purity of the enriched CD105<sup>+</sup> Sca-1<sup>+</sup> cells, the eluted fraction can be enriched over a second MS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

### 3. Example of a separation using the CD105 MultiSort Kit (PE), mouse, and the Anti-Sca-1 MicroBead Kit (FITC), mouse

Separation of CD105<sup>+</sup> Sca-1<sup>+</sup> LTR-HSCs from mouse bone marrow using the CD105 MultiSort Kit (PE), mouse, and two LS Columns for the first positive selection followed by the Anti-Sca-1 MicroBead Kit (FITC), mouse, and two MS Columns in the second positive selection step.



### 4. References

1. Duff, S. E. *et al.* (2003) CD105 is important for angiogenesis: evidence and potential applications. *FASEB J.* 17: 984–992.
2. Chen, C. Z. *et al.* (2002) Identification of endoglin as a functional marker that defines long-term repopulating hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* 99: 15468–15473.
3. Chen, C. Z. *et al.* (2003) The endoglin<sup>positive</sup> Sca-1<sup>positive</sup> rhodamine<sup>low</sup> phenotype defines a hear-homogeneous population of long-term repopulating stem cells. *Immunity* 19: 525–533.

### 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.

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