Miltenyi Biotec

Isolation of CD105$^+$ Sca-1$^+$ LTR-HSCs from mouse bone marrow

Contents

1. Description

This product is for research use only.

1.1 Background information

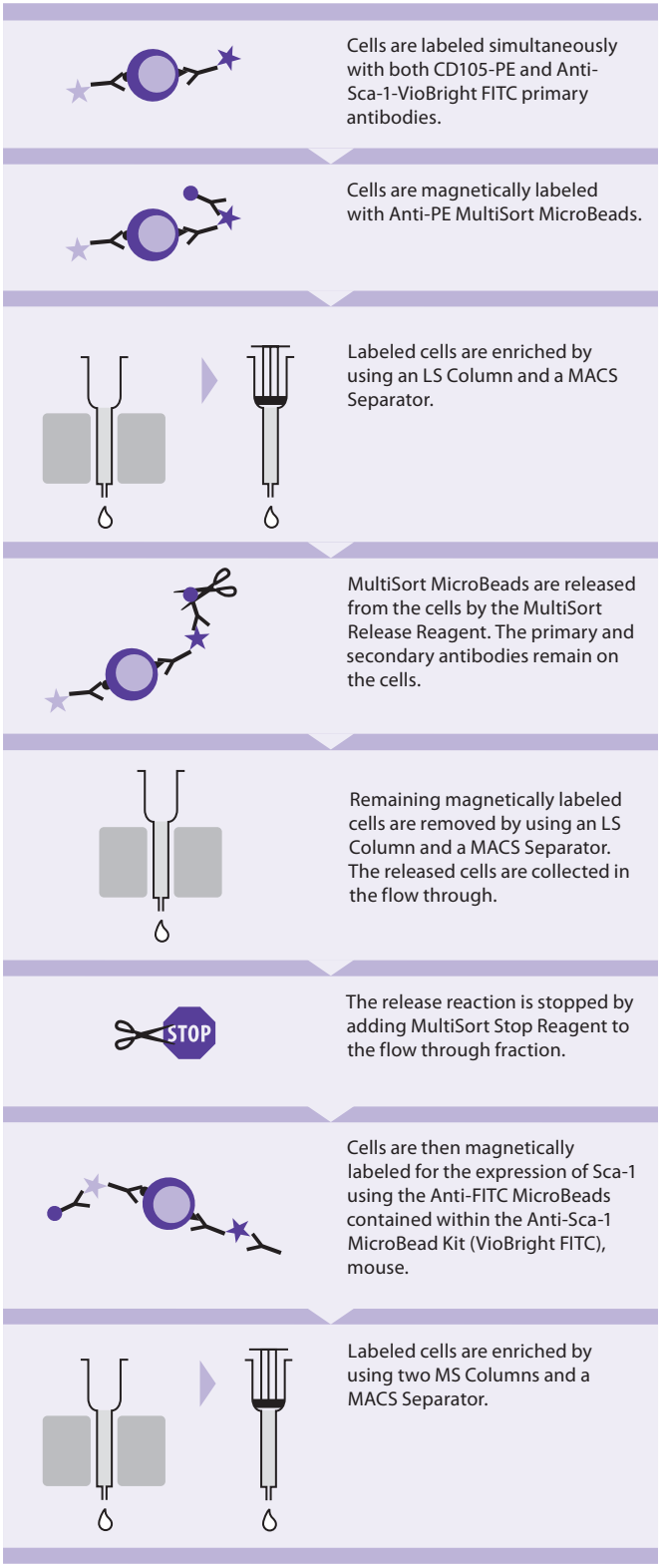
CD105, also known as endoglin, is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells.[1] In mouse bone marrow (BM), CD105 was also found to be expressed on a population of Sca-1$^+$ hematopoietic stem cells (HSCs), which were further characterized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

1.2 Principle of the CD105$^+$ Sca-1$^+$ LTR-HSC isolation procedure

This special protocol describes the isolation of CD105$^+$ Sca-1$^+$ long-term repopulating hematopoietic stem cells (LTR-HSCs) 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$^+$ Sca-1$^+$ 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 (VioBright™ FITC), mouse (# 130-123-124).

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



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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 (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- CD105 MultiSort Kit (PE), mouse (# 130-092-924) and the Anti-Sca-1 MicroBead Kit (VioBright™ FITC), mouse (# 130-123-124) for the sequential selection of $CD105^+$ $Sca\text{-}1^+$ LTR-HSCs.

- MACS Columns and MACS Separators: $CD105^+$ should be enriched by using LS Columns; $Sca\text{-}1^+$ cells should be enriched using MS Columns.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II

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

- (Optional) Fluorochrome-conjugated CD117 antibody, e.g., CD117-PE, or the Lineage Cell Detection Cocktail-Biotin, mouse (# 130-092-613) for flow cytometric analysis.

- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for flow cytometric exclusion of dead cells.

- Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.

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 (30 µm), # 130-041-407) to remove cell clumps which may block the column. Moisten 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.
6. Proceed to magnetic labeling of $CD105^+$ cells (2.2).

2.2 Magnetic labeling of $CD105^+$ cells

▲ 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 2×10^8 total cells. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 4×10^8 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 (30 µm), # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

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

▲ 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. 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 2×10^8 total cells. Add 400 µL of CD105-PE and 40 µL of Anti-Sca-1-VioBright 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 2×10^8 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 2×10^8 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 2×10^8 cells in 1 mL of buffer.

    ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
11. Proceed to magnetic separation of $CD105^+$ cells (2.3).

2.3 Magnetic separation of $CD105^+$ cells

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

▲ Always wait until the column reservoir is empty before proceeding to the next step.

1. Place an LS Column in the magnetic field of a MidiMACS™ Separator. For details refer to the LS Column data sheet.
2. Prepare LS Column by rinsing with 3 mL of buffer.

3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
4. Wash column with 3×3 mL 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 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$^+$ 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$^+$ cells

▲ Volumes for magnetic labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 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 2×10^7 total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ 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. 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 espression (<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 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. Resuspend cells at a final concentration of 10^7 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$^+$ Sca-1$^+$ 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$^+$ cells (2.5).

2.5 Magnetic separation of Sca-1$^+$ cells

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

▲ Always wait until the column reservoir is empty before proceeding to the next step.

1. Place an MS Column in the magnetic field of a MiniMACS™ Separator. For details refer to the MS Column data sheet.
2. Prepare MS Column by rinsing with 500 µL of buffer.
3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
4. Wash column with 3×500 µL of buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 3.

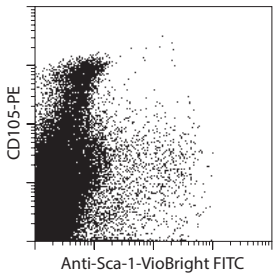
   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
5. Remove MS Column from the MiniMACS Separator and place it on a suitable collection tube.

   ▲ **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$^+$ Sca-1$^+$ 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.

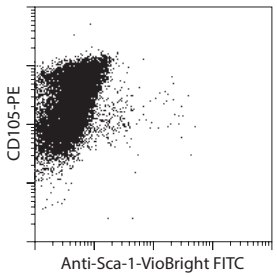
3. Example of a separation using the CD105 MultiSort Kit (PE), mouse, and the Anti-Sca-1 MicroBead Kit (VioBright™ FITC), mouse

Separation of CD105$^+$ Sca-1$^+$ 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 (VioBright™ FITC), mouse, and two MS Columns in the second positive selection step.

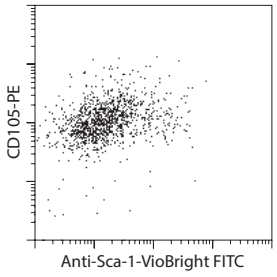
Before separation



CD105$^+$ cells



CD105$^+$Sca-1$^+$ LTR-HSCs



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^{positive} Sca-1^{positive} rhodamine^{low} phenotype defines a hear-homogeneous population of long-term repopulating stem cells. Immunity 19: 525–533.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols.

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