Miltenyi Biotec

Tumor-Associated Fibroblast Isolation Kit
mouse

Order no. 130-116-474

Contents

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.

1. Description

This product is for research use only.

Components	**1 mL Non–Tumor-Associated Fibroblast Depletion Cocktail, mouse** **1 mL CD90.2 (Tumor-Associated Fibroblast) MicroBeads, mouse:** MicroBeads conjugated to monoclonal anti-CD90.2 antibody (isotype: rat IgG2b).
Capacity	For up to 5×10^8 total cells (excluding red blood cells), up to 50 separations.
Product format	All components are supplied in buffer containing stabilizer and 0.05% sodium azide.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Principle of the MACS® Separation

The isolation of tumor-associated fibroblasts is performed in a two-step procedure. First, the non–tumor-associated fibroblasts are directly magnetically labeled with the Non–Tumor-Associated Fibroblast Depletion Cocktail, a cocktail of monoclonal antibodies conjugated to MACS® MicroBeads. The magnetically labeled non-target cells are subsequently depleted by separation over a MACS Column, which is placed in the magnetic field of a MACS Separator. In the second step, the fibroblasts are directly labeled with CD90.2 (Tumor-Associated Fibroblast) MicroBeads and isolated by positive selection from the pre-enriched fraction by separation over a MACS Column, which is placed in the magnetic field of a MACS Separator.
After removing the column from the magnetic field, the magnetically retained target cells can be eluted as the positively selected cell fraction.

Mouse tumor: Depletion of non–tumor-associated fibroblasts

1. Direct magnetic labeling of non-target cells with the Non–Tumor-Associated Fibroblast Depletion Cocktail.
2. Magnetic separation using an LD Column or an autoMACS® Column (program "Depl05").

Pre-enriched tumor-associated fibroblasts (flow-through fraction):
Positive selection of tumor-associated fibroblasts

1. Direct magnetic labeling of tumor-associated fibroblasts with CD90.2 (Tumor-Associated Fibroblast) MicroBeads.
2. Magnetic separation using an MS Column or an autoMACS Column (program "Posseld").

Tumor-associated fibroblasts (eluted fraction)
Non–tumor-associated fibroblasts (flow-through fraction)

1.2 Background information

The Tumor-Associated Fibroblast Isolation Kit, mouse has been developed for the isolation of tissue-derived, tumor-associated fibroblasts from dissociated mouse tumors.
Tumor-associated fibroblasts have been shown to play an important role in tumor development, progression, and function. However, they may only make up about 0.5–5% of the total number of cells found in tumors. Applying a two-step strategy, tumor-associated fibroblasts are first pre-enriched by removing non-target cells, followed by isolation using the general fibroblast marker CD90.2. This allows reliable isolation from different syngeneic mouse tumor models.
For optimal results, the Tumor-Associated Fibroblast Isolation Kit, mouse should be used in combination with the Tumor Dissociation Kit, mouse and the gentleMACS™ Dissociators.

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1.3 Applications

- Isolation of mouse tumor-associated fibroblasts from induced mouse tumors for further phenotypical or functional characterization, for example, culture or direct use for biochemical, physiological, and morphological studies.
- Isolation of healthy mouse fibroblasts.

1.4 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. Do **not use** autoMACS Running Buffer or MACSQuant® Running Buffer as they contain a small amount of sodium azide that could affect the results.

  ▲ **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.

- MACS Columns and MACS Separators: Depletion of non–tumor-associated fibroblasts can be performed on an LD Column. The subsequent positive selection of tumor-associated fibroblasts can be performed on an MS Columns. Positive selection and depletion can also be performed by using the autoMACS Pro Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, SuperMACS II
Depletion			
LD	10^8	5×10^8	MidiMACS, QuadroMACS, SuperMACS II
Positive selection or depletion			
autoMACS	2×10^8	4×10^9	autoMACS Pro

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

- (Optional) Tumor Dissociation Kit, mouse (# 130-096-730) for the generation of single-cell suspension from tumor tissues.
- (Optional) gentleMACS Dissociator (# 130-093-235), gentleMACS Octo Dissociator (# 130-095-937), or gentleMACS Octo Dissociator with Heaters (# 130-096-427)
- (Optional) gentleMACS C Tubes (# 130-093-237, # 130-096-334)
- (Optional) MACS SmartStrainers (70 µm) (# 130-098-462) to remove cell clumps after dissociation.
- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD45-PE-Vio® 770, CD90.2-APC, and CD140b-PE. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for flow cytometric exclusion of dead cells.
- (Optional) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- (Optional) Pre-Separation Filters (70 µm) (# 130-095-823) to remove cell clumps.

2. Protocol

2.1 Sample preparation

For preparation of a single-cell suspension from mouse tumors use the Tumor Dissociation Kit, mouse (# 130-096-730) in combination with the gentleMACS Dissociators.

For details refer to www.gentleMACS.com/protocols.

▲ Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).

2.2 Magnetic labeling of non-tumor-associated fibroblasts

▲ 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 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).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters (70 µm), # 130-095-823) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ 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 80 µL of buffer per 10^7 total cells (without red blood cells).

   ▲ **Note:** Always use freshly prepared buffer.
4. Add 20 µL of Non–Tumor-Associated Fibroblast Depletion Cocktail per 10^7 total cells.
5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
6. Adjust volume to 500 µL using buffer for up to 10^7 total cells.

   ▲ **Note:** Up to 3×10^7 total cells can be processed on one LD Column. If more cells are used split the sample onto multiple LD Columns.
7. Proceed to magnetic separation (2.3).

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2.3 Magnetic separation: Depletion of non–tumor-associated fibroblasts

▲ Choose an appropriate MACS® Column and MACS Separator according to the number of total cells and the number of non–tumor-associated fibroblasts. For details refer to table in section 1.4.

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

Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details refer to the LD Column data sheet.
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 2×1 mL of buffer. Collect total effluent; this is the unlabeled pre-enriched tumor-associated fibroblasts fraction. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty.
5. Proceed to 2.4 for the labeling of tumor-associated fibroblasts.

Magnetic separation with the autoMACS® Pro Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:

   Depletion:Depl05
   Collect negative fraction in row B of the tube rack.
4. Proceed to 2.4 for the labeling of tumor-associated fibroblasts.

2.4 Magnetic labeling of tumor-associated fibroblasts

▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to 10^7 total cells. For higher initial cell numbers, scale up all volumes accordingly.

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 80 µL of buffer.
3. Add 20 µL of CD90.2 (Tumor-Associated Fibroblast) MicroBeads.
4. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
5. (Optional) Add staining antibodies, e.g., CD45-PE-Vio® 770, CD90.2-APC, and CD140b-PE, and incubate according to manufacturer's recommendation.
6. Add buffer to a final volume of 500 µL.

   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
7. Proceed to magnetic separation (2.5).

2.5 Magnetic separation: Positive selection of tumor-associated fibroblasts

Positive selection with MS Columns

1. Place MS Column in the magnetic field of a suitable MACS Separator. For details refer to MS Column data sheet.
2. Prepare 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 effluent from step 3.

   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette 1 mL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

Magnetic separation with the autoMACS® Pro Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:

   Positive selection: Posseld
   Collect positive fraction in row C of the tube rack. This is the enriched tumor-associated fibroblasts fraction.

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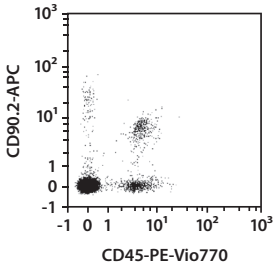
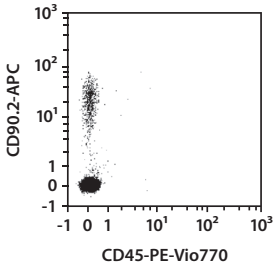
3. Example of a separation using the Tumor-Associated Fibroblast Isolation Kit

Mouse tumor-associated fibroblasts were purified from a mouse B16-F10 tumor (melanoma) using the Tumor-Associated Fibroblast Isolation Kit, mouse. For pre-enrichment, an LD Column and a QuadroMACS™ Separator were used, subsequent isolation was performed on a MS Column and a MiniMACS™ Separator. The cells were fluorescently stained with CD45-PE-Vio® 770 and CD90.2-APC and analyzed by flow cytometry using the MACSQuant® Analyzer. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

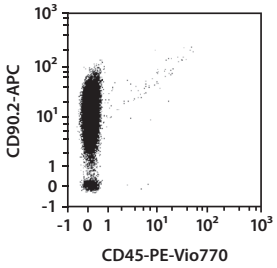
Before separation



Pre-enriched tumor-associated fibroblasts



Isolated tumor-associated fibroblasts



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