Miltenyi Biotec

Anti-FITC MultiSort Kit

Order no. 130-058-701

Contents

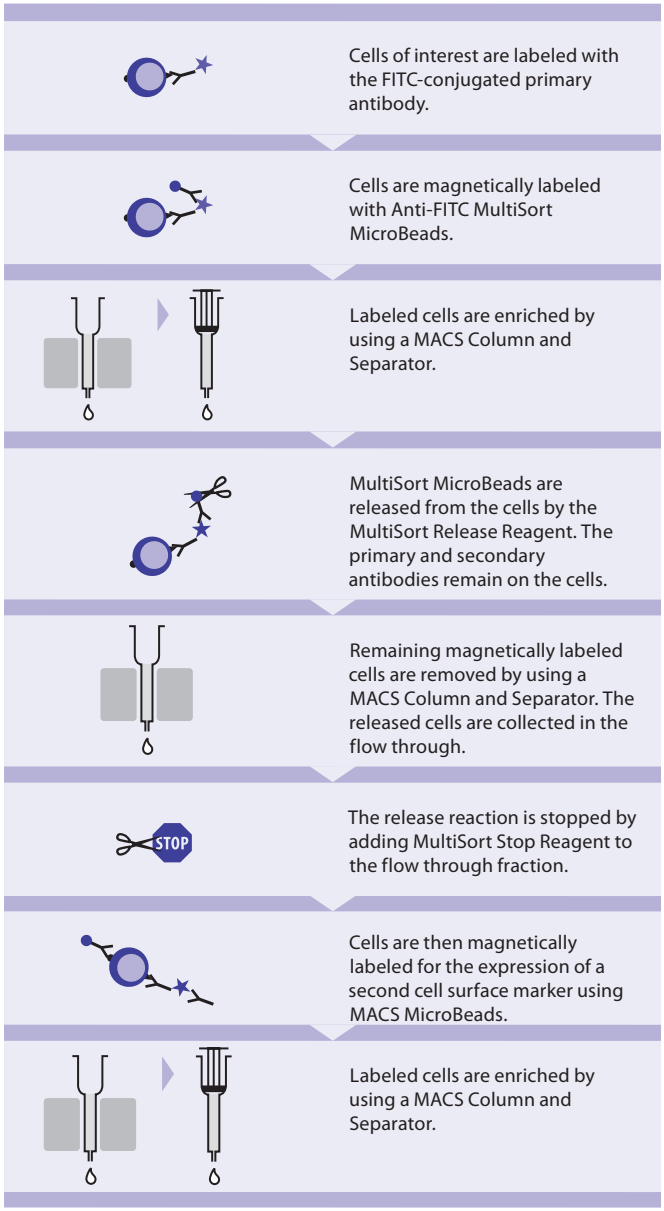
1. Description

Components	**2 mL Anti-FITC MultiSort MicroBeads:** MultiSort MicroBeads conjugated to monoclonal mouse anti-FITC (fluorescein isothiocyanate) antibodies (isotype: mouse IgG1). **1 mL MultiSort Release Reagent** **2 mL MultiSort Stop Reagent**
Capacity	For 1–5×10^9 total cells.
Product format	Anti-FITC MultiSort MicroBeads, the MultiSort Release Reagent, and the MultiSort Stop Reagent 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 Background information

The Anti-FITC MultiSort Kit is an indirect magnetic labeling system that allows the sorting of cells according to multiple surface markers. Cells are first stained with an FITC-conjugated primary antibody followed by magnetic labeling with Anti-FITC MultiSort MicroBeads. Following the enrichment of the FITC-labeled cells, using a MACS® Column and Separator, the magnetic particles are removed from the cells by using MultiSort Release Reagent. This allows a second magnetic labeling and separation of the cells for another surface marker of interest. Magnetic labeling for the second marker is achieved by using either direct or indirect magnetic labeling with MACS MicroBeads. For indirect labeling, Anti-Biotin MicroBeads, Anti-PE MicroBeads, or Anti-APC MicroBeads are recommended.

1.2 Principle of the MACS® Separation



1.3 Applications

For positive selection of a cell population according to two cell surface antigens when the first selection marker is stained with an FITC-conjugated antibody. Some examples are:

- Isolation of subsets of $CD4^+$ T helper cells, such as integrin alpha E^+CD4^+, and integrin alpha E^-CD4^+ T helper cells from murine spleen or lymph nodes[1].
- Isolation of $CCR5^+CD4^+$ T helper cells from synovial fluid of patients with rheumatoid arthritis.[2]
- Isolation of subsets of $CD8^+$ cytotoxic T cells.[3,4]

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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.

  ▲ **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 human serum albumin, human serum, or fetal bovine serum. Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- MACS Columns and MACS Separators: Cells can be enriched (positive selection) by using MS or LS Columns. Positive selection can also be performed by using the autoMACS or the autoMACS Pro Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
autoMACS	2×10^8	4×10^9	autoMACS, autoMACS Pro

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

- FITC-conjugated antibody, peptide, or ligand.

  ▲ **Note:** Use primary reagents conjugated with FITC Isomer-1 only. Most commercially available antibodies are conjugated to FITC Isomer-1.

  ▲ **Note:** Magnetic labeling with Anti-FITC MultiSort MicroBeads may reduce the fluorescence intensity of the FITC staining.

- (Optional) FcR Blocking Reagent, human (# 130-059-901)/ mouse (# 130-092-575) to avoid Fc receptor–mediated antibody labeling when using human or mouse samples.

- (Optional) Fluorochrome-conjugated antibody for flow cytometric analysis. For information about fluorochrome-conjugates see www.miltenyibiotec.com.

- (Optional) MACS MicroBeads for direct or indirect labeling of a second cell surface marker.

- (Optional) Propidium iodide (PI) or 7-AAD 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 (# 130-041-407) to remove cell clumps.

2. Protocol

2.1 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™. For details see the General Protocols section of the respective separator user manual. The General Protocols are also available at www.miltenyibiotec.com/protocols.

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

When working with tissues or lysed blood, prepare a single-cell suspension using standard preparation methods. For details see the General Protocols section of the respective separator user manual. The General Protocols are also available at www.miltenyibiotec.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 First magnetic labeling

▲ 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 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.

▲ Primary FITC-conjugated antibodies should be titrated to determine the optimal staining dilution. Staining should not increase fluorescence intensity of the negative population.

▲ The concentration of Anti-FITC MultiSort MicroBeads used to achieve an optimal magnetic separation and release of MultiSort MicroBeads is primarily dependent on the intensity of FITC-conjugated antibody staining and, to some degree, also on the frequency of target cells in suspension. Dimly FITC-stained target cells require a higher concentration of Anti-FITC MultiSort MicroBeads to achieve optimal magnetic labeling and separation. Target cells with a high frequency (>50%) may also require a higher concentration of Anti-FITC MultiSort MicroBeads than target cells with lower frequencies. For details see table on the next page.

▲ The optimal relative centrifugal force (RCF) and centrifugation time may be different depending on the cell sample.

▲ The recommended incubation temperature is 2–8°C. 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. (Optional) Add FcR Blocking Reagent, human or mouse in appropriate ratio. For details see the respective FcR Blocking Reagent data sheet.
4. Stain cells with primary FITC-conjugated antibody according to the manufacturer's recommendations. For MACS FITC-conjugated antibodies, resuspend 10^7 total cells in 100 µL of buffer and add 10 µL of FITC-conjugated antibody.

   ▲ **Note:** If the second parameter sorting is to be performed using indirect MicroBeads, we recommend to simultaneously label cells with the FITC-conjugated primary antibody as well as the primary antibody conjugate to be used in the second parameter. Reduce the volume of the buffer accordingly to accomodate both antibodies in their optimal staining concentration.

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5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C) or according to the manufacturer's recommendations.
6. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. (Optional) Repeat washing step.
8. Resuspend cell pellet in buffer and add Anti-FITC MultiSort MicroBeads according to recommendations in the table below. For more details see also section 2.5.

Buffer and MicroBead volumes per 10^7 total cells

Antigen staining	Buffer	Anti-FITC MultiSort MicroBeads
"dim"	80 µL	20 µL
"intermediate"	**90 µL**	**10 µL**
"bright"	96 µL	4 µL

▲ **Note:** A too strong dilution of the Anti-FITC MultiSort MicroBeads may result in poor retention of the FITC-labeled target cells. A too low dilution may result in less efficient release of MicroBeads.

9. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
10. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
11. Resuspend up to 10^8 cells in 500 µL of buffer.
    ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
12. Proceed to magnetic separation (2.3).

2.3 Magnetic separation

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of magnetically labeled cells. For details see table in section 1.4.

Magnetic separation with MS or LS Columns

1. Place column in the magnetic field of a suitable MACS Separator. For details see the respective MACS Column data sheet.
2. Prepare column by rinsing with the appropriate amount of buffer:
   MS: 500 µL LS: 3 mL
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with the appropriate amount 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.
   MS: 3×500 µL LS: 3×3 mL
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
   MS: 1 mL LS: 5 mL
7. (Optional) To increase purity of the magnetically labeled cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.
8. Proceed to removal of MultiSort MicroBeads (2.4).

Magnetic separation with the autoMACS™ Separator or the autoMACS™ Pro Separator

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

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

▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the respective user manual.

Magnetic separation with the autoMACS™ Separator

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 at the uptake port and the fraction collection tubes at port neg1 and port pos1.
3. For a standard separation choose one of the following programs:
   Positive selection: possel
   Collect positive fraction from outlet port pos1.
4. Proceed to removal of MultiSort MicroBeads (2.4).

Magnetic separation with the autoMACS™ Pro Separator

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 fraction collection tubes in rows B and C.
3. For a standard separation choose one of the following programs:
   Positive selection: possel
   Collect positive fraction in row C of the tube rack.
4. Proceed to removal of MultiSort MicroBeads (2.4).

2.4 Removal of MultiSort MicroBeads and second magnetic labeling and separation

▲ The recommended incubation temperature is 2–8°C. 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 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).

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4. (Optional) To remove any residual magnetically labeled cells, repeat the magnetic separation procedure as described in 2.3. Separate cells over a new column of the same type (MS or LS Column) or use the same autoMACS™ or autoMACS Pro program to be used in the second parameter separation. Collect magnetic (unreleased) and non-magnetic (released) cell fractions to determine the efficiency of the release reaction. For details 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 the first parameter sorting with Anti-FITC MultiSort MicroBeads (<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 in buffer at a final concentration of 10^7 total cells per 50 µL of buffer.

7. Add 30 µL of MultiSort Stop Reagent per 10^7 total cells and mix well.

8. Add the recommended amount of direct or indirect MACS MicroBeads (see the respective MACS MicroBead data sheet) to magnetically label the cells for the second marker. Adjust to 100 µL total volume by adding buffer.

   ▲ **Note:** The Anti-FITC antibody is of mouse IgG1 isotype. Thus, Goat Anti-Mouse IgG MicroBeads or Rat Anti-Mouse IgG1 MicroBeads cannot be used for second parameter sorting. When using other Anti-Immunoglobulin MicroBeads for the second parameter sorting, any reactivity with the isotype of the primary antibody of the first parameter sorting must be avoided.

9. Mix well and incubate as recommended in the respective data sheet in the refrigerator (2–8 °C).

10. Proceed to magnetic separation (2.3). For details see the respective MACS MicroBeads data sheet.

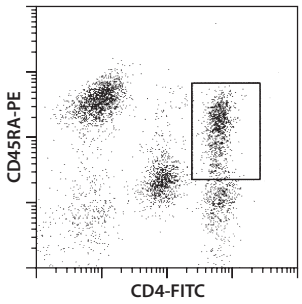
2.5 Recommendations for the optimization of cell isolation using the Anti-FITC MultiSort Kit

For optimization of MultiSort Kit isolations please refer to the corresponding FAQ section at www.miltenyibiotec.com.

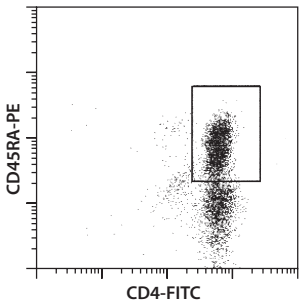
3. Example of a separation using the Anti-FITC MultiSort Kit

$CD4^+CD45RA^+$ cells were separated from human PBMCs using CD4-FITC, the Anti-FITC MultiSort Kit, and MS Columns for the first positive selection, followed by CD45RA MicroBeads and an MS Column in the second positive selection step. Cells are fluorescently stained with CD45RA-PE (# 130-092-248). Cell debris and dead cells are excluded from the analysis based on scatter signals and PI fluorescence.

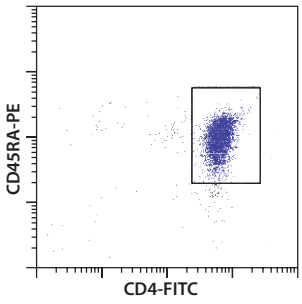
PBMCs before separation



$CD4^+$ cells



$CD4^+CD45RA^+$ cells



4. References

1. Lehmann, J. *et al.* (2002) Expression of the integrin $a_E b_7$ identifies unique subsets of $CD25^+$ as well as $CD25^-$ regulatory T cells. Proc. Natl. Acad. Sci. 99: 13031–13036. [2370]
2. Suzuki, N. *et al.* (1999) Selective accumulation of $CCR5^+$ T lymphocytes into inflamed joints of rheumatoid arthritis. Int. Immunol. 11: 553–559. [808]
3. Harrison L. C. *et al.* (1996) Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. J. Exp. Med. 184: 2167–2174. [417]
4. Holtappels, R. *et al.* (2000) Enrichment of Immediate-Early 1 (*m123*/pp89) Peptide-Specific CD8 T Cells in a Pulmonary $CD62L^{lo}$ Memory-Effector Cell Pool During Latent Murine Cytomegalovirus Infection of the Lungs. J. Virol. 24: 11495–11503. [923]

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com/local to find your nearest Miltenyi Biotec contact.

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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