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1. Description

Components	<p>2 mL Non-DC Depletion Cocktail, human: Cocktail of monoclonal biotin-conjugated antibodies against human CD1c (BDCA-1) (clone: AD5-8E7; isotype: mouse IgG2a), and MicroBeads conjugated to monoclonal antibodies against human CD14 (isotype: mouse IgG2a) and human CD19 (isotype: mouse IgG1).</p> <p>2 mL DC Enrichment Cocktail, human: MicroBeads conjugated to monoclonal antibodies against human CD304 (BDCA-4/Neuropilin-1) (isotype: mouse IgG1), human CD141 (BDCA-3) (isotype: mouse IgG1), and Biotin (isotype: mouse IgG1)</p> <p>2 mL FcR Blocking Reagent: human IgG</p>
Capacity	For 2×10^9 total cells, up to 20 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 Blood Dendritic Cell Isolation Kit II is developed for the isolation of dendritic cells from peripheral blood mononuclear cells (PBMCs). Isolation of dendritic cells is performed in a two-step procedure. First, PBMCs are labeled with the Non-DC Depletion Cocktail, comprising CD14 and CD19 MicroBeads for magnetic labeling

of monocytes and B cells as well as a biotin-conjugated antibody against CD1c (BDCA-1) for biotin-labeling of CD1c (BDCA-1)⁺ myeloid dendritic cells. Upon subsequent magnetic separation of the cells over a MACS® Column, which is placed in a magnetic field of a MACS Separator, the magnetically labeled monocytes and B cells are depleted. The flow-through fraction with the pre-enriched dendritic cells is then incubated with the DC Enrichment Cocktail, comprising CD304 (BDCA-4/Neuropilin-1) and CD141 (BDCA-3) MicroBeads for magnetic labeling of plasmacytoid dendritic cells and CD141 (BDCA-3)⁺ myeloid dendritic cells as well as Anti-Biotin MicroBeads for magnetic labeling of biotin-labeled CD1c (BDCA-1)⁺ myeloid dendritic cells. Upon subsequent magnetic separation, the magnetically labeled dendritic cells are retained on the column and are eluted after removal of the column from the magnetic field. To achieve highest purities, the positively selected cell fraction containing the enriched dendritic cells is separated over a second MACS Column.

1.2 Background information

Dendritic cells are most effective antigen-presenting cells and play a key role in initiating and directing immune responses. In blood, there are at least three different subsets: plasmacytoid dendritic cells (PDCs) and two distinct subsets of myeloid dendritic cells (MDC1s and MDC2s).^{1,2} The subsets show phenotypic and functional differences.^{2–5} The Blood Dendritic Cell Isolation Kit II allows the concurrent isolation of PDCs, MDC1s, and MDC2s. Three markers are used for immunomagnetic labeling of all dendritic cell subsets: CD304 (BDCA-4/Neuropilin-1), CD1c (BDCA-1), and CD141 (BDCA-3).^{2,6} CD304 (BDCA-4/Neuropilin-1) expression is strictly confined to PDCs.^{2,7} CD1c (BDCA-1) is expressed on MDC1s, which are CD11c^{high} CD123^{low}. CD141 (BDCA-3) is expressed at high levels only on MDC2s, which are CD11c^{low} and CD123⁺. In blood, CD1c (BDCA-1) is also expressed on B cells, and CD141 (BDCA-3) is expressed at low levels also on monocytes. Monocytes and B cells are, thus, depleted prior to positive selection of plasmacytoid and myeloid dendritic cells by magnetic labeling for CD304 (BDCA-4), CD1c (BDCA-1) and CD141 (BDCA-3).

In healthy donors, dendritic cells represent about 1% of all PBMCs, of which approximately 0.37% are PDCs, 0.60% are MDC1s, and 0.03% are MDC2s.

1.3 Applications

- Analysis of gene expression in blood dendritic cells.
- Elucidation of specific signal transduction pathways.
- Analysis of antigen uptake, processing, and presentation.
- Stimulation of primary T cell responses by antigen-pulsed blood dendritic cells.

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:** Depletion of monocytes and B cells is performed on an LD Column. The subsequent positive selection of dendritic cells is performed on two MS Columns. Depletion and positive selection can also be performed by using the autoMACS Pro or the autoMACS Separator.

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

▲ **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) The Blood Dendritic Cell Enumeration Kit (# 130-091-086; # 130-091-324) for evaluation of dendritic cell purity and identification of the distinct dendritic cell subset. To determine only the purity of enriched dendritic cells, a cocktail of fluorochrome-conjugated antibodies, e.g., CD14-FITC (# 130-080-701), CD19-FITC (# 130-091-328), CD1c (BDCA-1)-PE (# 130-090-508), CD303 (BDCA-2)-PE (# 130-090-511), and CD141 (BDCA-3)-PE (# 130-090-514) can be used.
- (Optional) Propidium Iodide Solution (# 130-093-233) 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, 30 μm (# 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™.

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at $200 \times g$ 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 methods.

For details refer to the protocols section 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 Magnetic labeling of non-dendritic 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 10^8 total cells. When working with fewer than 10^8 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 2×10^8 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. Wet 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 $300 \times g$ for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 300 μL of buffer per 10^8 total cells.
4. Add 100 μL of FcR Blocking Reagent and 100 μL of Non-DC Depletion Cocktail per 10^8 total cells.
5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
6. Wash cells by adding 5–10 mL of buffer per 10^8 cells and centrifuge at $300 \times g$ for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in buffer:
Depletion with LD Column: 500 μL for up to 1.25×10^8 cells
Depletion with autoMACS or autoMACS Pro Separator: 500 μL for up to 1×10^8 cells
8. Proceed to magnetic separation (2.3).



2.3 Magnetic separation: Depletion of non-dendritic cells

Depletion with LD Column

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details see 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 dendritic cell 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 isolation of dendritic cells.

Depletion with the autoMACS Pro® Separator or the autoMACS® Separator

- ▲ Refer to the respective user manual for instructions on how to use the autoMACS Pro® Separator or the autoMACS Separator.
- ▲ Buffers used for operating the autoMACS Pro Separator or the autoMACS Separator should have a temperature of $\geq 10^\circ\text{C}$.
- ▲ 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® 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 the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:
Depletion: "Depletes"
Collect negative fraction in row B of the tube rack.

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.
3. For a standard separation choose the following program:
Depletion: "Depletes"
Collect negative fraction from outlet port neg1.



2.4 Magnetic labeling of pre-enriched dendritic cells

- ▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to 10^8 cells (step 2.2). For larger starting cell numbers, scale up volumes accordingly.
 - ▲ The recommended incubation temperature is $2-8^\circ\text{C}$. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.
1. Centrifuge cell suspension at $300\times g$ for 10 minutes. Aspirate supernatant completely.
 2. Resuspend cell pellet in 400 μL of buffer.
 3. Add 100 μL of **DC Enrichment Cocktail**.
 4. Mix well and incubate for 15 minutes in the refrigerator ($2-8^\circ\text{C}$).
 5. Wash cells by adding 5–10 mL of buffer and centrifuge at $300\times g$ for 10 minutes. Aspirate supernatant completely.
 6. Resuspend up to 10^8 cells in 500 μL of buffer.
 7. Proceed to magnetic separation (2.5).



2.5 Magnetic separation: Positive selection of dendritic cells

Positive selection with MS Columns

1. Place MS Column in the magnetic field of a suitable MACS Separator. For details see the 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 $3\times 500 \mu\text{L}$ 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 only when the column reservoir is empty.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette 500 μL 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 dendritic 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.

Positive selection with the autoMACS® Pro Separator or the autoMACS® Separator

- ▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator or the autoMACS Separator.
- ▲ Buffers used for operating the autoMACS Pro Separator or the autoMACS Separator should have a temperature of $\geq 10^\circ\text{C}$.
- ▲ 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® 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 the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:
Depletion: "Posseld2"
Collect positive fraction in row C of the tube rack.

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 pos2.
3. For a standard separation choose the following program:
Positive selection: "Posseld2"
Collect positive fraction from outlet port pos2.

2.6 Evaluation of dendritic cell purity

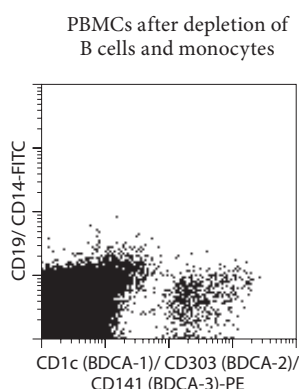
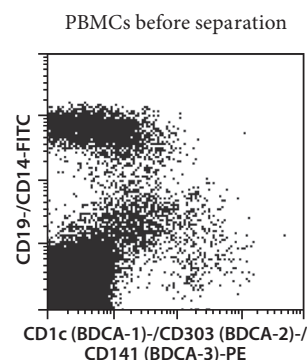
The purity of the isolated blood dendritic cells can be evaluated by flow cytometry or fluorescence microscopy. Due to the lack of a surface antigen specifically expressed by all dendritic cells, staining with a cocktail of fluorochrome-conjugated antibodies is required. The cocktail should contain antibodies specific for monocytes (e.g. CD14) and B cells (e.g. CD19) as well as antibodies against the dendritic cell subset markers CD1c (BDCA-1), CD141 (BDCA-3), and CD303 (BDCA-2). Comparable to CD304 (BDCA-4/Neuropilin-1), CD303 (BDCA-2) is a specific marker for PDCs in blood. Staining should be performed on aliquots of each cell fraction after magnetic separation. Monocyte and B cell specific antibodies might be coupled to fluorochrome 1, and antibodies against the dendritic cell subsets might be coupled to fluorochrome 2 (see flow cytometric data 3. A).

3. Example of a separation using the Blood Dendritic Cell Isolation Kit II

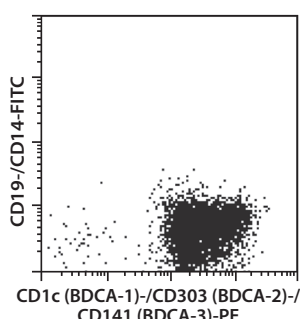
Dendritic cells were isolated from PBMCs using the Blood Dendritic Cell Isolation Kit II, an LD and two MS Columns, a MidiMACS™, and a MiniMACS™ Separator.

A) Evaluation of MACS® Separation

Aliquots of the different cell fractions were stained with FITC-conjugated antibodies against monocytes (CD14-FITC) and B cells (CD19-FITC) as well as PE-conjugated antibodies against PDCs (CD303 (BDCA-2)-PE), MDC1s (CD1c (BDCA-1)-PE) and MDC2s (CD141 (BDCA-3)-PE). Cell debris and dead cells were excluded from analysis based on scatter signals and PI fluorescence.

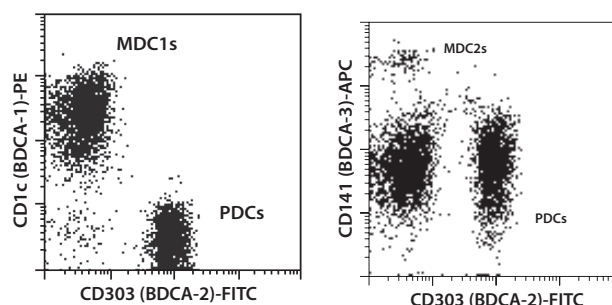


Isolated blood dendritic cells



B) Identification of distinct dendritic cell subsets

An aliquot of the enriched blood dendritic cells was stained with the Blood Dendritic Cell Enumeration Kit. Cell debris, monocytes, B cells, and dead cells were excluded from the analysis based on scatter signals, PE-Cy5, and Dead Cell Discriminator fluorescence. PDCs are identified by CD303 (BDCA-2), MDC1s by CD1c (BDCA-1), and MDC2s by CD141 (BDCA-3) expression.



4. References

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- Dzionek, A. *et al.* (2002) Plasmacytoid dendritic cells: from specific surface markers to specific cellular functions. *Hum. Immunol.* 63: 1133–1148.

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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