Miltenyi Biotec

REAlease® CD279 MicroBead Kit
human

Order no. 130-132-979

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3. Example of a separation using the REAlease CD279 MicroBead Kit

1. Description

This product is for research use only.

Components	**1 mL REAlease CD279-Biotin, human**
	5 mL REAlease Anti-Biotin MicroBeads (CD279, human)
	4 mL REAlease Bead Release Reagent (50×)
	4 mL REAlease Release Reagent
	4 mL REAlease Stop Reagent
Capacity	For 10^9 total cells, up to 100 separations.
Product format	REAlease Stop Reagent is supplied in buffer containing 0.05% sodium azide. All other reagents are supplied in buffer containing stabilizer and 0.05% sodium azide.
Storage	Store protected from light at +2 to +8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Principle of the REAlease MACS Separation

The REAlease Technology relies on recombinantly engineered antibody fragments to label specific cell surface markers. The fragments are developed to have low affinity for epitopes. However, when fragments are multimerized as a REAlease Biotin Complex (i.e., REAlease CD279-Biotin, human) they bind to epitopes with high avidity. REAlease Technology can control the multimer/monomer state of antibody fragments. With this technology a controlled label release is possible where monomerized antibody fragments dissociate from the cell surface, enabling users to obtain bead- and label-free cells.
First, the target cells in a peripheral blood mononuclear cell (PBMC) population are labeled with REAlease CD279-Biotin (REAlease Biotin Complex). Subsequently, REAlease Anti-Biotin MicroBeads (CD279, human) bind to the REAlease Biotin Complex. Then, the cell suspension is loaded onto a MACS Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled cells are retained within the column. The unlabeled non-target cells flow through; this cell fraction is thus depleted of $CD279^+$ cells. To increase purity, a second column run is needed. Therefore, cells are eluted directly from the first onto the second, equilibrated column. After washing the column again and removing the column from the magnetic field, the target cells are eluted using the REAlease Bead Release Reagent, which simultaneously removes the MicroBeads from the cells. Finally, during the subsequent incubation with the REAlease Release Reagent, the REAlease Biotin Complex monomerizes and dissociates from the cell surface leaving the cells free of all labels.

1.2 Background information

The REAlease CD279 MicroBead Kit has been developed for the isolation of $CD279^+$ cells from PBMCs.
The REAlease CD279 MicroBead Kit is an indirect magnetic labeling system that allows to obtain cells free of MicroBeads and REAlease Biotin Complex.
CD279, also known as PD-1 (programmed cell death 1), is expressed on T cells, B cells, NK cells, activated myeloid cells, and dendritic cells. The ligands PD-L1 (B7-H1;CD274) and PD-L2 (B7-DC; CD273) belong to the B7 immunglobulin superfamily. PD-1 and its ligands mediate inhibitory signals that regulate the balance between T cell activation and tolerance in infections, autoimmunity, and tumor. PD-1 upregulation on human immunodificiency virus (HIV)– specific $CD8^+$ T cells correlates with high viral load. Differential expression of PD-1 on HIV–, cytomegalo virus (CMV)–, ebstein-barr-virus (EBV)–, and varizella zosta virus (VZV)–specific T cells from the same subjects provides evidence for differential regulation of virus-specific T cell function during chronic viral infections.
In Hepatitis C virus (HCV) infection PD-1 expression was reported to be high on HCV-specific $CD4^+$ and $CD8^+$ T cells early in acute infection, but also to be continued in resolved and chronic stages of infection. Tumor antigen-specific $CD8^+$ T cells infiltrating the tumor were shown to express high levels of PD-1 and to be functionally impaired.

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1.3 Applications

- Isolation of CD279$^+$ label free cells. The selected CD279$^+$ cells can be sorted according to a second marker of interest, for example, CD8, CD4, CD137 (4-1BB), CD223 (LAG-3), or CD366 (TIM-3).

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 to +8 °C). Degas buffer before use, as air bubbles could block the column.

  ▲ **Note:** BSA can be replaced by other proteins such as human serum albumin, human serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- REAlease Bead Release buffer: Prepare a 1:50 dilution of REAlease Bead Release Reagent (50×), e.g., for 1 mL add 20 µL of REAlease Bead Release Reagent to 980 µL of separation buffer.

  ▲ **Note:** Use freshly prepared buffer the same day. Store at room temperature.

  ▲ **Note:** Prepare 1 mL per MS Column and 5 mL per LS Column.

- MACS Columns and MACS Separators: CD279$^+$ cells can be enriched by using MS or LS. Positive selection or depletion can also be performed by using the autoMACS Columns on the autoMACS NEO Separator.

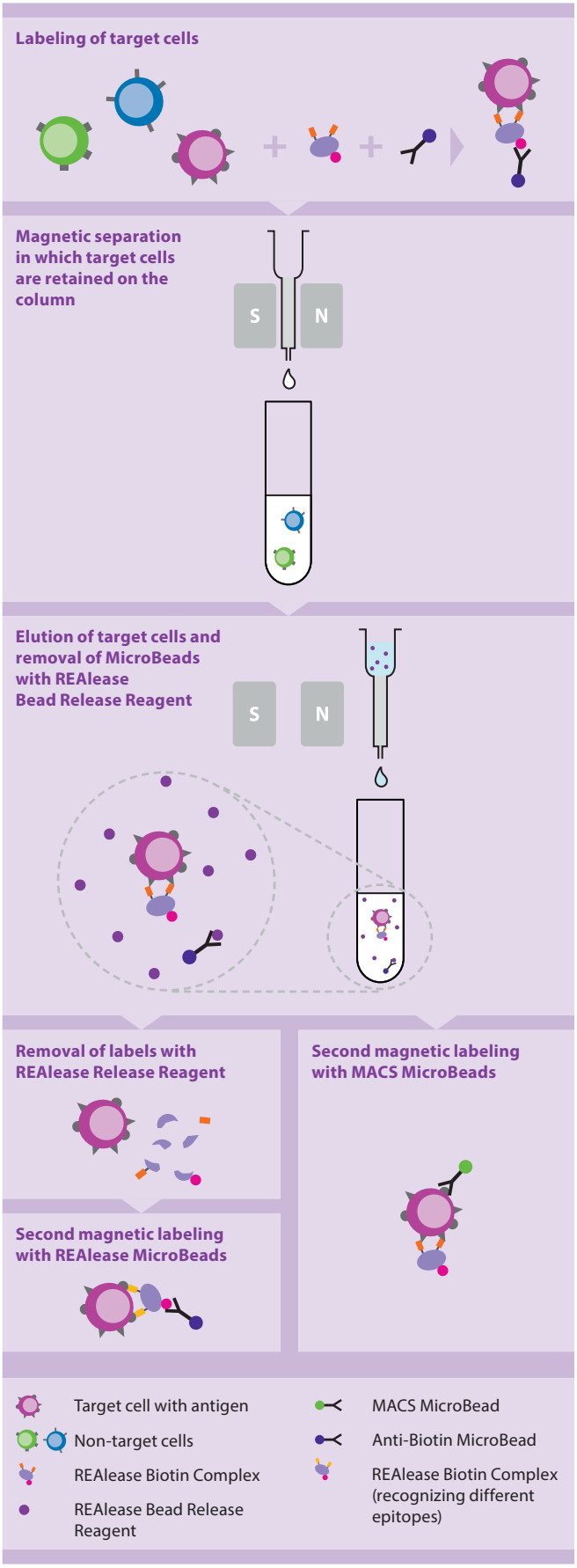
Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, SuperMACS II
LS	10^8	2×10^9	MidiMACS, QuadroMACS, SuperMACS II
autoMACS	2×10^8	4×10^9	autoMACS NEO Separator,

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

- (Optional) Fluorochrome-conjugated CD3 antibodies for flow cytometric analysis, e.g., CD3 Antibody, anti-human, VioBlue®. 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) or Viobility™ Fixable Dyes (# 130-109-812, # 130-109-814, # 130-109-816) 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 Protocol overview



2.2 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by using a MACS PBMC Isolation Kit or 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×g for 10–15 minutes at +20 °C. Carefully aspirate supernatant. Repeat washing step.

For details refer to the data sheet or the protocols section at www.miltenyibiotec.com/protocols.

When working with tissues, prepare a single-cell suspension using the gentleMACS™ Dissociator.

For details refer to www.miltenyibiotec.com/gentlemacs.

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



2.3 Magnetic labeling

▲ Cells can be labeled with MACS MicroBeads using the autolabeling function of the autoMACS NEO Separator. For more information refer to section 2.5.

▲ 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.

▲ 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 (30 µm), # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ 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 +19 to +25 °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 40 µL of separation buffer per 10^7 total cells.
4. Add 10 µL of REAlease CD279-Biotin per 10^7 total cells.
5. Mix well and incubate for 5 minutes.
6. Add 50 µL of REAlease Anti-Biotin MicroBeads (CD279, human) per 10^7 total cells.
7. Mix well and incubate for 5 minutes.
8. (Optional) Add staining antibodies, e.g., C3 Antibody, anti-human, VioBlue, and incubate according to manufacturer's recommendation.

   ▲ **Note:** These staining antibodies cannot be removed from the cells.
9. Dilute up to 5×10^7 cells in a total volume of 500 µL with separation buffer.

   ▲ **Note:** For volumes larger than 500 µL a dilution is not needed.
10. Proceed to magnetic separation (2.4).



2.4 Magnetic separation and removal of magnetic labeling

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of $CD279^+$ cells. For details refer to the table in section 1.4.

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

▲ The recommended incubation temperature is +19 to +25 °C.

Magnetic separation with MS or LS Columns

1. Prepare two columns by rinsing with the appropriate amount of separation buffer:

   MS: 500 µL LS: 3 mL
2. Place the first column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
3. Apply cell suspension onto the first column. Collect flow-through containing unlabeled cells.
4. Wash the first column with the appropriate amount of separation buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 3.

   MS: 3×500 µL LS: 3×3 mL

   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
5. Remove the first column from the separator.
6. Place the second column in the MACS Separator and put the first column on top of the second column.
7. Add the appropriate amount of separation buffer onto the first column. Immediately flush out target cells directly onto the second column by firmly pushing the plunger into the first column.

   MS: 1 mL LS: 5 mL
8. Remove the first column from the second column.
9. Wash second column with the appropriate amount of separation buffer.

   MS: 3×500 µL LS: 3×3 mL

   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
10. Remove the second column from the separator and place it on a suitable collection tube.
11. (Optional) If removal of MicroBeads is not required, add the appropriate amount of separation buffer onto the second column. Immediately flush out target cells by firmly pushing the plunger into the column. Eluted cells are ready for downstream applications, e.g., flow cytometric analysis.

    MS: 1 mL LS: 5 mL
12. For removal of MicroBeads proceed with step 13.
13. Add the appropriate amount of prepared REAlease Bead

Release buffer (refer to chapter 1.4) onto the second column. Immediately flush out target cells by firmly pushing the plunger into the column.

MS: 1 mL LS: 5 mL

14. Mix well and incubate for 10 minutes.
15. Cells are now free from MicroBeads and ready for analysis and downstream applications.
16. (Optional) Proceed either to
    - 2.6 Removal of REAlease Complex and second magnetic labeling with REAlease MicroBeads

    or proceed to
    - 2.7 Second magnetic labeling with MACS MicroBeads.

2.5 Magnetic labeling and separation using autoMACS Separators

▲ Refer to the user manual and the short instructions for instructions on how to use the autoMACS Separators.

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

▲ Place tubes in the following Chill Rack positions:

position A = sample, position B = unlabeled (negative) fraction,

position C = labeled (positive) fraction.

2.5.1 Magnetic labeling and separation using the autoMACS NEO Separator

▲ The autoMACS NEO Separator enables stage loading to extend column capacity for selected reagents, minimizing the need to divide larger samples.

▲ For more information on selecting alternative separation programs, stage loading–compatible reagents, autolabeling-compatible reagents, and the minimal and maximal volumes for each reagent and Chill Rack, refer to www.miltenyibiotec.com/automacs-neo-sample-processing.

Fully automated magnetic labeling and separation

1. Prepare and prime the instrument.
2. Place the Chill Rack and MACS Reagent Rack 8 on the MACS MiniSampler S.
3. Select the same Chill Rack and MACS Reagent Rack 8 in the **Experiment** tab. An experiment is created automatically.
4. Tap to select sample position(s).
5. To assign a reagent to each sample, tap **Scan reagent** and scan the reagent barcode. Alternatively, tap on a free position of the MACS Reagent Rack 8 for selection out of the reagent list.
6. Unscrew the lids from the reagent vials and place the vials onto the designated positions on the MACS Reagent Rack 8.
7. Tap **Place reagent(s) on reagent rack** button in the dialog box.
8. Automated labeling is set automatically if autolabeling is supported and a reagent rack is selected. Alternatively, tap **Labeling** in the reagent placement dialog and select **Auto**.
9. Tap **Sample volume** in the **Sample process** pane and enter the sample volume. Tap the return key.
10. Tap **Run** to start the separation process.

2.6 (Optional) Removal of the REAlease Complex and second magnetic labeling with REAlease MicroBeads

▲ The recommended incubation temperature is +19 to +25 °C.

▲ For second magnetic labeling with MACS Anti-Biotin MicroBeads proceed through all steps of chapter 2.5 to remove the REAlease Biotin Complex.

2.6.1 Removal of the REAlease Complex

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in appropriate amount of separation buffer:

   MS: 1 mL LS: 5 mL
3. Add an appropriate amount of REAlease Release Reagent:

   MS: 20 µL LS: 100 µL
4. Mix well and incubate for 5 minutes.
5. Cells are now free from REAlease Complex and MicroBeads and are ready for analysis or downstream applications.
6. (Optional) For second magnetic labeling with REAlease MicroBeads continue with 2.5.2.

2.6.2 Second magnetic labeling with REAlease MicroBeads

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 40 µL of REAlease Stop Reagent per 10^7 total cells.
3. Mix well.
4. For a second magnetic labeling follow the labeling protocol in the respective REAlease MicroBead Kit data sheet.

   ▲ **Note:** For best recovery and purity of cells, the amount of MACS MicroBeads for the second positive labeling may need optimization as the starting frequency of target cells may be different from a PBMC sample.

2.7 (Optional) Second magnetic labeling with MACS MicroBeads

▲ For second magnetic labeling with MACS Anti-Biotin MicroBeads proceed through all steps of chapter 2.5 to remove the REAlease Biotin Complex.

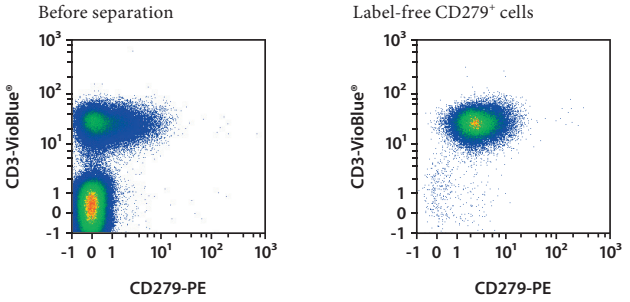
1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Add the recommended amount of MACS MicroBeads to label the cells magnetically for the second marker. For details refer to the respective MACS MicroBeads data sheet.

   ▲ **Note:** For best recovery and purity of cells, the amount of MACS MicroBeads for the second positive labeling may need optimization as the starting frequency of target cells may be different from a PBMC sample.

3. Example of a separation using the REAlease CD279 MicroBead Kit

CD279$^+$ cells were isolated from human PBMC using the REAlease CD279 MicroBead Kit, MS Columns, and a MiniMACS™ Separator. Cells were fluorescently stained with CD279-PE and CD3-VioBlue and analyzed by flow cytometry using the MACSQuant® Analyzer X. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

A) Cell purity

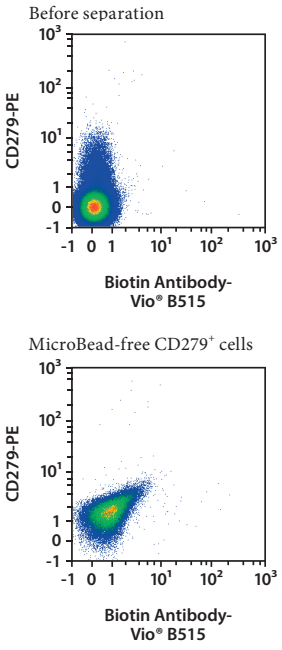


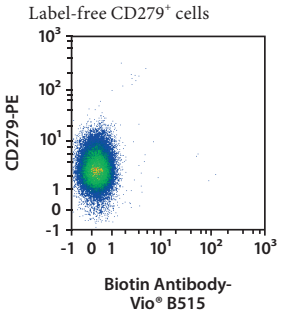
B) Bead-free cells: efficiency of REAlease Anti-Biotin MicroBeads release

Release efficiency was higher than 99% for the REAlease Anti-Biotin MicroBeads (CD279). The efficiency was determined by re-applying the isolated cells to a second MACS Column. The ratio between the numbers of cells in the flow-through and the total number of cells applied to the second column allowed us to calculate the efficiency of magnetic labeling removal.

C) Label-free cells: REAlease Biotin Complex release

The efficient removal of all labels was shown by using Anti-Biotin-APC to analyze the cells by flow cytometry for the presence of REAlease Biotin Complex. Directly after isolation, the cells showed staining of biotin ("MicroBead-free CD279$^+$ cells"), whereas the label-free CD279$^+$ cells after the REAlease Biotin Complex release were negative for biotin similar to the non-labeled cells before separation.





Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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