Miltenyi Biotec

Ova Antigen Delivery Module Set

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Miltenyi Biotec

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1. Description

Components

200 µL Ova Antigen Delivery Reagent: monoclonal Anti-Biotin antibodies (isotype: mouse IgG1) conjugated to ovalbumin (Ova) and fluorescein isothiocyanate (FITC).

2 mL CD11c MicroBeads, mouse: MicroBeads conjugated to monoclonal anti-mouse CD11c antibodies (isotype: hamster IgG).

1 mL CD205 (DEC205)-Biotin, mouse: monoclonal CD205 (DEC205) antibody conjugated to biotin (isotype: rat IgG2a).

1 mL Anti-MHC Class II-PE, mouse: monoclonal Anti-MHC Class II antibody conjugated to R-phycoerythrin (PE) (isotype: rat IgG2b).

Capacity

For targeting up to 2×10^7 cells with Ova Antigen Delivery Reagent.

Product format

Ova Antigen Delivery Reagent is supplied in buffer containing stabilizer. Endotoxin content <2.5 EU/mL as determined by LAL assay.

CD11c MicroBeads, CD205 (DEC205)-Biotin, and Anti-MHC Class II-PE 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 dates are indicated on the vial labels.

1.1 Background information

Antigen targeting to antigen-presenting cells (APCs) via specific receptors has been used to study vaccination strategies to induce effective antigen-specific T cell responses. Ovalbumin is widely used as a model antigen for the characterization of antigen uptake, processing, and presentation by mouse APCs. Especially the induction of $CD8^+$ T cell responses after targeting antigen via antigen uptake receptors to dendritic cells (DCs), commonly termed cross-priming, has raised major interest. For functional studies of antigen presentation ovalbumin T cell receptor-transgenic $CD4^+$ and $CD8^+$ T cells from DO11.10, OT-II, and OT-I mouse strain are often used.[1–5]

The Ova Antigen Delivery Reagent has been developed for the *in vitro* targeting of ovalbumin to APCs, analysis of antigen uptake, and detection of antigen routes during antigen processing. It is a monoclonal anti-biotin antibody conjugated to ovalbumin and FITC. In combination with an appropriate biotinylated anti-receptor antibody any desired antigen uptake receptor can be targeted. This allows the functional characterization of new receptors on APCs for comparison with well-characterized ones, such as CD205 (DEC205) or DCIR2 (33D1). The Ova Antigen Delivery Reagent is also well suited for the analysis of antigen uptake and trafficking by fluorescent or confocal laser scanning microscopy.

1.2 Applications

The Ova Antigen Delivery Reagent is well suited for

- analysis of antigen-uptake by antigen-presenting cells, i.e., DCs, macrophages, and B cells
- analysis of antigen-processing and intracellular trafficking of endocytotic receptors
- research on cross-presentation of antigens
- research on protocols of antigen-delivery for DC vaccination

by using, for example, confocal laser scanning microscopy, flow cytometry, or downstream applications, such as CFSE-labeling, or ^{3}H-incorporation for the detection of T cell proliferation.

1.3 Reagent and instrument requirements

- Phosphate-buffered saline (PBS), sterile
- PE buffer with 0.5% fetal bovine serum (FBS), sterile
- Ethylendiamin-tetraacetat (EDTA)
- Buffer for cell separation: Prepare a sterile solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% fetal bovine serum (FBS), and 2 mM EDTA. Keep buffer cold (2–8 °C).

  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD).

- Carboxyfluorescein diacetate succinimidyl ester (CFSE)
- Dimethylsulfoxid (DMSO)
- (Optional) $CD4^+$ T Cell Isolation Kit, mouse (# 130-090-860) for negative selection of mouse T helper cells.
- (Optional) Pam3CysK4
- Appropriate cell culture containers like culture dishes.
- (Optional) $CD8a^+$ T Cell Isolation Kit, mouse (# 130-090-859) for negative selection of cytotoxic mouse T cells.
- (Optional) Pan DC MicroBeads, mouse (# 130-092-465) for separation of $CD11c^+$ mouse dendritic cells.
- (Optional) FcR Blocking Reagent, mouse (# 130-092-575) to avoid Fc receptor–mediated antibody labeling.
- Cell culture medium, e.g., RPMI 1640 containing 5% mouse serum.

2. Protocols

▲ Work under sterile conditions throughout the whole experiment.

2.1 Sample preparation

▲ For receptor-mediated targeting of Ova antigen to mouse DCs, best results are achieved with freshly isolated mouse spleen, lymph node, or bone marrow. Magnetic cell separation using MACS® Technology requires single-cell suspensions. For preparing a single-cell suspension please refer to our gentleMACS™ Protocols section at www.miltenyibiotec.com/protocols.

2.2 Magnetic labeling of $CD11c^+$ DCs

▲ 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 labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ 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 200×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 400 µL of buffer per 10^8 total cells.
   ▲ **Note:** To obtain high purities of DCs (≥90%), Fc receptor-mediated magnetic labeling should be blocked by adding FcR Blocking Reagent (CD16/32 antibody) or mouse immunoglobulin (1 mg per 500 µL labeling volume) to the cell suspension before adding CD11c MicroBeads.
4. Add 100 µL of CD11c MicroBeads per 10^8 total cells.
5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
6. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 200×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend up to 10^8 cells in 500 µL of buffer.
   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
8. Proceed to magnetic separation of CD11c$^+$ DCs (2.3).

2.3 Magnetic separation of CD11c$^+$ DCs

▲ Choose an appropriate MACS® Column and MACS Separator according to the number of total cells and the number of CD11c$^+$ cells. For details see following table.

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

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

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

   ▲ **Note:** Staining of some antigen uptake receptors, e.g., members of the c-type lectin family, require the presence of Ca^{2+} ions. Please use buffers recommended by the manufacturer for staining of those receptors with monoclonal antibodies.

3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
4. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the effluent from step 3.
   MS: 3×500 µL LS: 3×3 mL

   ▲ **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 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. To increase the purity of CD11c$^+$ 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.

Magnetic separation 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 ≥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® 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 one of the following programs:
   Positive selection: "Posseld"
   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 one of the following programs:
   Positive selection: "Posseld"
   Collect positive fraction from outlet port pos2.

2.4 Staining with CD205 (DEC-205)-Biotin antibodies

▲ Volumes for antibody labeling given below are for **up to 10^7** nucleated 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 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For the analysis of antigen uptake receptors other than CD205 (DEC-05), specific antibodies conjugated to biotin should be used in direct comparison to CD205 (DEC-205)-Biotin by using the One-Step Antibody Biotinylation Kit (# 130-093-385).

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

3. Resuspend up to 10^7 nucleated cells per 100 µL of buffer.
4. Add 10 µL of CD205 (DEC205)-Biotin.
5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
   ▲ **Note:** Working on ice requires increased incubation times. Higher temperatures and/or longer incubation times lead to non-specific cell labeling.
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. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.

2.5 *In vitro* targeting using Ova Antigen Delivery Reagent

▲ Volumes given below are for **up to 10^6** nucleated cells. When working with fewer than 10^6 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 2×10^6 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For accurate analysis of antigen targeting a negative control sample should always be included, for example, by omitting the antigen uptake receptor–specific biotin-conjugated antibody.

▲ Positive control, such as the sample labeled with CD205 (DEC-205)-Biotin should also be included.

1. Determine cell number.
2. Resuspend up to 10^6 nucleated cells per 100 µL of cold buffer.
3. Add 10 µL of the Ova Antigen Delivery Reagent.
4. Mix well and incubate for 10 minutes on ice.
   ▲ **Note:** Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.
5. Wash cells by adding 1–2 mL of buffer or medium and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. For subsequent analysis by flow cytometry resuspend cell pellet in a suitable amount of buffer or for culture in medium.

   Recommendation: The ovalbumin antigen presentation can be detected by proliferation of ovalbumin-specific transgenic T cells derived from OT-I, OT-II, or DO11.10 mouse strains.

   For more information about downstream analysis of antigen presentation refer to www.miltenyibiotec.com/protocols.

2.6 Staining with Anti-MHC Class II-PE antibodies

▲ Volumes given below are for **up to 10^7** nucleated 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 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^7 nucleated cells per 100 µL of buffer.
4. Add 10 µL of Anti-MHC Class II-PE.
5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
   ▲ **Note:** Working on ice requires increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.
6. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.

2.7 Cell culture *in vitro* maturation of DCs

1. Determine cell number.
2. Wash cells once in culture medium, centrifuge at 300×g for 10 minutes. Aspirate supernatant. Suspend DCs to 10^5 cells/mL in medium. Mix well and plate cells in appropriate cell culture dish at 5×10^6 cells/cm², e.g., 2 mL of cell suspension in one well of a 24 well plate.

   ▲ **Note:** Take into consideration the number of T cells that need to be added to the culture after the *in vitro* maturation of DCs the following day, e.g., add 5×10^5 and 1×10^6 CFSE-labeled T cells to 1×10^5 *in vitro* matured DCs, respectively.

   ▲ **Note:** Particulary T cells should be plated at 5×10^6 cells/cm².
3. Add appropriate stimulus of maturation of choice to the DC culture, e.g., Pam3CysK4 at a concentration of 500 ng/mL.
4. Incubate cells at 37 °C, 5–7.5 % CO_2 over night.

2.8 (Optional) Magnetic labeling of CD4⁺ T 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 10^7 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, # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ 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 200×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 40 µL of buffer per 10^7 total cells.
4. Add 10 µL of Biotin-Antibody Cocktail per 10^7 total cells.
5. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
6. Add 30 µL of buffer and 20 µL of Anti-Biotin MicroBeads per 10^7 total cells.
7. Mix well and incubate for additional 15 minutes in the refrigerator (2–8 °C).
8. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
9. Resuspend up to 10^8 cells in 500 µL of buffer.

   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
10. Proceed to magnetic separation of CD4⁺ T cells (2.9).

2.9 (Optional) Magnetic separation of CD4⁺ T cells

▲ In case T cells are not magnetically isolated with the CD4⁺ T Cell Isolation Kit, mouse (# 130-090-860), please follow the instructions of the respective manufacturer.

▲ Choose an appropriate MACS® Column and MACS Separator according to the number of total cells and the number of CD4⁺ cells. For details see following table.

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

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

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. Collect flow-through containing unlabeled cells, representing the enriched CD4⁺ T cell fraction.
4. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the effluent from step 3.
   MS: 3×500 µL LS: 3×3 mL

   ▲ **Note:** Perform washing steps by adding buffer aliquots only when the column reservoir is empty.

Magnetic separation 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 ≥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® 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: "Deplete"
   Collect negative fraction, representing the enriched $CD4^+$ T cell 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 and port pos1.
3. For a standard separation choose the following program:
   Depletion: "Deplete"
   Collect negative fraction, representing the enriched $CD4^+$ T cell fraction, from outlet port neg1.

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(Optional) Evaluation of $CD4^+$ T cell purity

The purity of the enriched $CD4^+$ T cells can be evaluated by flow cytometry or fluorescence microscopy. Stain aliquots of the cell fractions with a fluorochrome-conjugated antibody against CD4 as recommended by the manufacturer and analyze cells by flow cytometry or fluorescence microscopy. To determine if any cells in the enriched fraction are $CD4^-$ T cells or $CD4^-$ non-T cells, counterstain aliquots of the fractions with an antibody against a T cell marker (e.g. CD3ε) coupled to another fluorochrome.

2.10 CFSE-labeling of T cells

1. Determine cell number.
2. Wash cells in room temperature PBS buffer.
3. Resuspend cells in PBS containing 0.5% FBS at 2×10^7 cells/mL. Add the same volume of a 10 µM CFSE/PBS solution. Mix well and incubate for 5 minutes at room temperature.
   ▲ **Note:** Optimal CFSE concentration and incubation time may vary and may need to be adjusted. CFSE concentration usually ranging from 1–10 µM CFSE.
4. Wash cells in PBS or medium containing serum. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
5. Repeat step 4.
6. Resuspend T cells in medium ad 2×10^7/mL.

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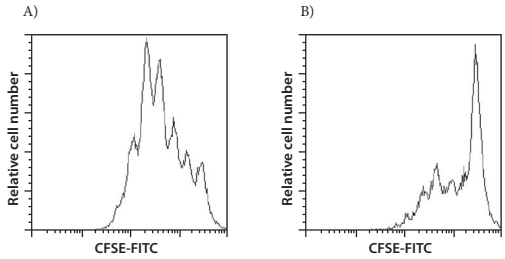
2.11 CFSE-proliferation assay

1. Add desired number of T cells to the DC culture.
   ▲ **Note:** When using T cell proliferation assays as a read-out system for efficient antigen presentation, the number of DCs to T cell ratio is usually titrated, e.g., one dendritic cell to 5 or 20 T cells.
2. Incubate cells at 37 °C, 5–7.5% CO_2.
   ▲ **Note:** If at any time point the indicator of the medium turns yellow, remove about 70% of the medium with a pipette without suspending the cells. Replace the volume with medium. After two to three days antigen-specific T cells have usually proliferated in case of the CD205 (DEC-205)-Biotin labeling. For other antigen uptake receptors the suitable time-point has to be determined.
3. For flow cytometric analysis of T cell proliferation after CFSE labeling please refer to the protocols of the chosen manufacturer.

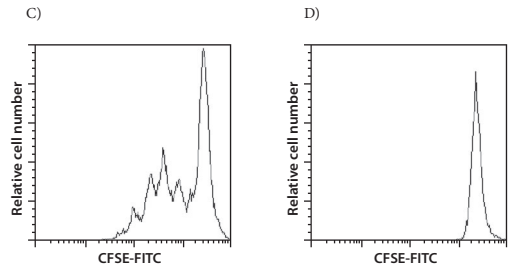
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3. Example of using the Ova Antigen Delivery Module Set

Dendritic cells (DCs) were isolated using CD11c MicroBeads, mouse. Ovalbumin was targeted to DCs via DCIR2 (33D1) (A), CD205 (DEC205) (B), or CD36 (C), using biotin-conjugated antibodies against the respective antigen uptake receptors or for control without a biotin-conjugated antibody (D) in combination with the Ova Antigen Delivery Reagent. Cells were cultured in the presence of Pam3CysSK4 for 24 h that induces DC maturation via toll-like receptors (TLR). Subsequently, cells were cultured with CFSE-labeled $CD4^+$ T cells from OT-II mice at a ratio of 1:5 (DC:T cell). After three days proliferation of T cells was analyzed by flow cytometry.



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4. References

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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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