Miltenyi Biotec

Detection and enrichment of cytokine-secreting cells from whole blood

Contents

The following special protocol can be used in combination with one of the Cytokine Secretion Assay - Cell enrichment and Detection Kits for human cells.

1. Reagent and instrument requirements

- **Cytokine Secretion Assay Kit**, for example:

  IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-054-201)

  IL-2 Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-090-488)

  IL-4 Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-054-101)

  IL-10 Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-090-435)

- **Anticoagulant**: sodium heparin
- 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 (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- Culture medium, e.g., RPMI 1640, containing 5% human serum (do not use BSA or FBS because of non-specific stimulation!).
- **Erythrocyte lysing solution (1×):**

  Prepare freshly from Red Blood Cell Lysis Solution 10× (130-094-183).

- (Optional) **Staining reagents**: CD4-FITC or CD8-FITC and CD14-PerCP.

  ▲ **Note:** Do not use PE-tandem conjugates. They may also be recognized by Anti-PE MicroBeads UltraPure.

  ▲ **Note:** Upon activation of T cells, TCR and some associated molecules, like CD3, might be down-regulated.

  ▲ **Note:** For optimal sensitivity, we recommend labeling of undesired non-T cells such as monocytes with antibodies conjugated to PerCP, e.g. CD14-PerCP (# 130-094-969). These cells can then be excluded together with PI-stained dead cells by gating.

- **Propidium Iodide Solution** or **7-AAD** to exclude dead cells from analysis.
- MACS Columns and MACS Separators:

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

  ▲ **Note:** Column adapters are required to insert certain columns into VarioMACS™ Separator or SuperMACS™ Separator. For details, see MACS Separator data sheets.

- (Optional) Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753)
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.

2. Protocol

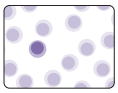
2.1 (Antigen-specific) in vitro stimulation

▲ The peripheral blood should not be older than 20 hours and should be supplemented with anticoagulant **sodium heparin**. **Do not use EDTA, or ACD**. Lymphocyte activation and secretion of cytokines requires calcium, and is consequently inhibited by chelating anticoagulants.

▲ **Note:** Whole blood may be stored overnight at **room temperature**.

▲ Always include a **negative control** sample in the experiment. A **positive control** with e.g. Staphylococcal Enterotoxin B (SEB) may be included in the experiment (see also detailed protocol provided with the Cytokine Secretion Assay Kits).

▲ Do **not use** media containing any **non-human** proteins, like BSA or FBS because of non-specific stimulation.



Protocol for *in vitro* stimulation

1. Start with **5 mL of fresh, sodium heparinized, human blood** (containing about 10^7 lymphocytes) in a 50 mL conical polypropylene tube.
2. Add the antigen or, as a positive control, 1 µg/mL SEB for 3–16 hours at 37 °C, 5–7% CO_2 (for details on the kinetics of cytokine secretion and on concentrations of antigen to add, refer to Cytokine Secretion Assay data sheet, 3.1–3.2).

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3. A negative control sample, treated exactly the same as the antigen-stimulated sample but without addition of antigen, should always be included in the experiment.
4. (Optional) Costimulatory agents like CD28 and CD49d antibodies may be added.

2.2 Cytokine Secretion Assay

▲ This protocol is optimized for cell samples containing <5% of total cytokine-secreting cells. If ≥5% of cytokine-secreting cells are expected, it is necessary to dilute the cells further during the cytokine secretion period, and therefore a larger test tube will be needed. The dilution avoids non-specific staining of cells not secreting cytokines during this period.

▲ For each sample with 5 mL whole blood prepare:

100 mL of **cold buffer** (4–8 °C)
200 µL of **cold medium** (4–8 °C)
7 mL of **warm medium** (37 °C)
45 mL of **erythrocyte lysing solution** (room temperature).

▲ Work fast, keep the cells cold, use pre-cooled solutions which will prevent capping of antibodies on the cell surface and a non-specific cell labeling (exception: warm medium during secretion period and room temperature during lysing step).

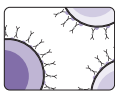
▲ Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes. Pipette off or aspirate supernatant.

▲ Dead cells may bind non-specifically to MACS® MicroBeads or antibodies. Therefore, when working with cell preparations containing large amounts of dead cells, they should be removed before starting the Cytokine Secretion Assay, e.g. by density gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

▲ Higher temperatures and longer incubation times for staining should be avoided. This will lead to non-specific cell labeling.

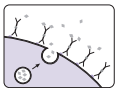
Lysis of erythrocytes

1. After stimulation add 45 mL of erythrocyte lysing solution to 5 mL whole blood sample.
2. Mix gently and incubate for 10 minutes at **room temperature**. Rotate tube continuously using the MACSmix™ Tube Rotator (# 130-090-753), or turn tube several times during incubation.
3. Centrifuge cells at 300×g for 10 minutes at **room temperature**, remove supernatant **completely**.



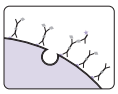
Labeling cells with Cytokine Catch Reagent

1. Resuspend cell pellet in 15 mL of **cold buffer**, and transfer into a 15 mL conical propylene tube.
2. Centrifuge at 300×g for 10 minutes at **2–8 °C**. Pipette off supernatant completely.
3. Resuspend pellet in 160 µL of **cold medium**.
4. Add 40 µL of **Cytokine Catch Reagent**, mix well and incubate for 5 minutes **on ice**.



Cytokine secretion period

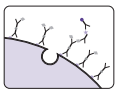
1. Add 7 mL of **warm medium** (37 °C) to dilute the cells.
   ▲ **Note:** For frequencies of cytokine-secreting cells ≥ 5 % the cells need to be further diluted, e.g. by a factor of 5.
2. Incubate cells in a closed tube for 45 minutes at **37 °C** under slow continuous rotation using the MACSmix Tube Rotator, or turn tube every 5 minutes to resuspend settled cells.
   ▲ **Note:** During this step it is crucial to prevent contact of cells to avoid cross contamination with cytokines.



Labeling cells with Cytokine Detection Antibody

1. Put the tube **on ice**.
2. Wash cells by adding 8 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at **2–8 °C**. Pipette off supernatant completely.
3. Resuspend cell pellet in 160 µL of **cold buffer**.
4. Add 40 µL of **Cytokine Detection Antibody (PE)**.
5. (Optional) Add additional staining reagents, e.g. 20 µL of CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP (# 130-094-969).
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at **2–8 °C**. Pipette off supernatant completely.

2.3 Magnetic labeling



Magnetic labeling with Anti-PE MicroBeads UltraPure or Anti-PE MicroBeads

1. Resuspend cell pellet in 160 µL of **cold buffer**.
2. Add 40 µL of **Anti–PE MicroBeads UltraPure**, mix well and incubate for 15 minutes at **2–8 °C**.
   ▲ **Note:** Incubate in refrigerator at 2–8 °C; do not work on ice during this step.
3. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at 2–8 °C. Pipette off supernatant completely.
4. Resuspend cell pellet in 500 µL of **cold buffer**.
5. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.
6. Proceed to magnetic separation.

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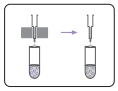
2.4 Magnetic separation



Magnetic separation using MS Columns

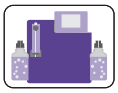
▲ To achieve highest purities, perform two consecutive column runs.

1. Prepare **two MS Columns** per sample by rinsing with 500 µL **cold buffer**, discard effluent.
2. Place first column into the magnetic field of a suitable MACS® Separator. For details refer to MS Column data sheet.
3. (Optional) Pass cells through Pre-Separation Filters (30 µm) (# 130-041-407) to remove clumps.
4. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
5. Wash column with 3×500 µL of cold buffer. Collect unlabeled cells that pass through and combine with the effluent from step 4.

   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
6. Remove first column from separator, place second column into the separator, and put the first column on top of the second one.
7. Pipette 1 mL of cold buffer on top of the first column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger; directly onto the second column. Collect unlabeled cells that pass through.
8. Wash second column with 3×500 µL of cold buffer. Collect unlabeled cells that pass through and combine with the effluent from step 5.

   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
9. Remove second column from separator, place column on a suitable collection tube.
10. Pipette 500 µL of cold buffer on top of the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger.

    ▲ **Note:** For subsequent cell culture, the cells can also be eluted with medium. If part of the cells are analyzed by flow cytometry, the medium should **not contain** phenol red.
11. Proceed to flow cytometric analysis (see detailed protocol), cell culture, or other subsequent experiment.



Magnetic separation using the autoMACS® Pro Separator

▲ Refer to the 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.

▲ 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 user manual.

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.
4. Proceed to flow cytometric analysis (see detailed protocol), cell culture or other subsequent experiment.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols.