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140-000-592.04

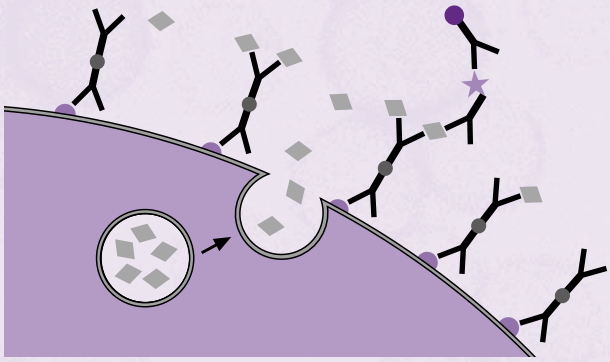
Cytokine Secretion Assays

IL-2 Secretion Assay

Cell Enrichment and Detection Kit (PE*) human

For 50 tests with 10^7 cells

Order No. 130-090-488



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1. Description

Components	1 ml **IL-2 Catch Reagent**: anti-IL-2 monoclonal antibody (mouse IgG1) conjugated to cell surface specific monoclonal antibody (mouse IgG2a). 1 ml **IL-2 Detection Antibody**: anti-IL-2 monoclonal antibody (mouse IgG2a) conjugated to PE (phycoerythrin). 1 ml **Anti-PE MicroBeads**: colloidal super-paramagnetic MicroBeads conjugated to monoclonal mouse anti-PE antibody (mouse IgG1).
Size	For 50 tests with 10^7 cells
Product format	All components are supplied as a suspension containing 0.1% gelatine and 0.05% sodium azide.
Storage	Store protected from light at 4-8°C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Principle of the IL-2 Secretion Assay

Antigen-specific T cells are analyzed and isolated using the IL-2 Secretion Assay starting from whole blood, PBMC or other leukocyte containing single cell preparations. The cells are restimulated for a short period of time with specific peptide, protein or other antigen preparations.

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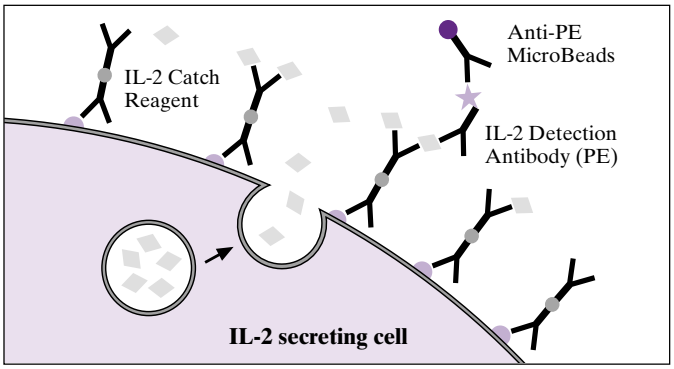


Subsequently, an IL-2-specific **Catch Reagent** is attached to the cell surface of all leukocytes. The cells are then incubated for a short time at 37°C to allow cytokine secretion. The secreted IL-2 binds to the IL-2 Catch Reagent on the positive, secreting cells. These cells are subsequently labeled with a second IL-2-specific antibody, the **IL-2 Detection Antibody** conjugated to phycoerythrin (PE) for sensitive detection by flow cytometry.

The IL-2 secreting cells can now be magnetically labeled with **Anti-PE MicroBeads** and enriched over a MACS Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled cells are retained in the MACS Column while the unlabeled cells run through. After the column has been removed from the magnetic field, the magnetically retained cells can be eluted as positively selected cell fraction, enriched for cytokine secreting cells. The cells can now be used for cell culture or analysis. Since viable cells are analyzed, non-specific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Background and product applications

The IL-2 Secretion Assay - Cell Enrichment and Detection Kit is designed for the detection, isolation and analysis of viable IL-2 secreting leukocytes. It is specially developed for the **detection and isolation of antigen-specific T cells**. After restimulation with specific antigen in vitro secretion of IL-2 is induced. IL-2 is rapidly secreted by naive T helper cells and by certain subsets of memory T cells upon activation. It promotes growth and differentiation of T cells and has pleiotropic effects on many other leukocytes.

Quantitative analysis of antigen-specific T cell populations can provide important information on the natural course of immune responses. MACS enrichment of the antigen-specific T cells increases the sensitivity of analysis, allowing detection of frequencies as low as one in a million cells.

The MACS enrichment also enables further functional characterization of the antigen-specific cells and downstream experiments, as well as the expansion of antigen-specific cells allowing research on potential future immunotherapeutical applications.

Examples of applications

- Detection and enrichment of viable IL-2 secreting leukocytes.
- Detection and enrichment of IL-2 secreting, antigen-specific T cells for enumeration and phenotypic analysis as well as for expansion and functional characterization.

- Monitoring and analysis of antigen-specific T cell immunity, e.g. in infection, autoimmunity, cancer, allergy or alloreactivity.
- Isolation and expansion of antigen-specific T cells for research in immunotherapy.
- Enrichment and analysis of IL-2 secreting cells for determination of functional antigens in disease and for T cell receptor (TCR) epitope mapping.
- Analysis or cloning of TCR repertoire of antigen-specific T cells.

1.3 Reagent and instrument requirements

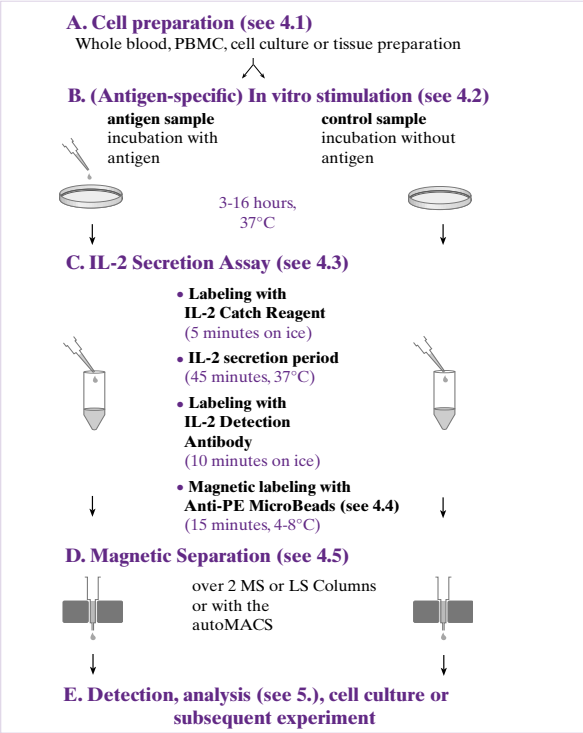
- **Buffer** (degassed): phosphate buffered saline pH 7.2, containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA (e.g. 4 ml of a 0.5 M EDTA stock solution per 1 liter of buffer).
- (Optional) 0.5 M EDTA stock solution: dissolve 56 g sodium hydroxide (NaOH) in 900 ml dd H_2O. Add 146.2 g ethylene-diamine-tetraacetic acid (EDTA), adjust pH to 7.5, fill up to 1000 ml with dd H_2O.
- **Culture medium**, e.g. RPMI 1640 containing 5% human serum, like autologous or AB serum (do **not use BSA** or **FCS** because of non-specific stimulation!).
- **Propidium iodide (PI)** or **7-AAD** to exclude dead cells from analysis.
- (Optional) Staining reagents such as CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP™.
- 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; with Column Adapter: VarioMACS, SuperMACS
LS	10^8	2×10^9	MidiMACS; with Column Adapter: VarioMACS, SuperMACS
autoMACS	2×10^8	4×10^9	autoMACS

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

- Refrigerated centrifuge (4-8°C).
- Rotation device for tubes: MACSmix (# 130-090-753).
- (Optional) Pre-Separation Filter (# 130-041-407).

2. Protocol overview



3. Experimental set-up

3.1 Controls

Negative control

For accurate detection of IL-2 secreting antigen-specific cells, a negative control sample should always be included. This will provide information about IL-2 secretion unrelated to the specific antigen-stimulation, but e.g. due to ongoing in vivo immune responses. The control sample should be treated exactly the same as the antigen-stimulated sample except for the addition of antigen, or by using a control antigen.

Positive control

When setting up a new experiment, it is recommended to include a positive control. As a positive control, a sample stimulated with the superantigen Staphylococcal Enterotoxin B (Sigma) 1 µg/ml for 3-16 hours, may be included in the experiment.

▲ **Note:** Mitogens like PHA or PMA/Ionomycin are not recommended for stimulation of a positive control, as the resulting high frequencies of IL-2 secreting cells do not allow conclusions on the performance (e.g. sensitivity) of the IL-2 Secretion Assay.

3.2 Kinetics of restimulation and proposed time schedule

Peptides

Upon stimulation with peptide, the cells can be analyzed for IL-2 secretion 3-6 hours later.

It is possible to prepare the cells first and take them into culture overnight, but without adding the antigen (see 4.2 step 2.).

Peptide is then added the next morning for 3 hours of stimulation, directly followed by the IL-2 Secretion Assay.

Proteins

Upon stimulation with protein, the cells can be analyzed for IL-2 secretion 6-16 hours later.

It is possible to start the stimulation of the cells late in the afternoon, and to perform the IL-2 Secretion Assay the following morning.

Costimulation

The addition of costimulatory agents like CD28 or CD49d antibody may enhance the response to the antigen. If costimulatory agents are added to the antigen sample, they also have to be included in the control sample.

3.3 Counterstaining of cytokine secreting cells

The IL-2 secreting cells are stained with PE-conjugated IL-2 Detection Antibodies. To identify cells of interest, counterstaining for T cells with e.g. CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) is important.

▲ Do **not use** tandem conjugates of phycoerythrin, like Cy-Chrome® (PharMingen), PE-Cy5 (Serotec), ECD, PC5 (Coulter-Immunotech) etc., they may also be recognized by the Anti-PE MicroBeads.

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

▲ The samples should be stained with propidium iodide (PI) or 7-AAD prior to acquisition, to exclude dead cells from analysis. This will reduce non-specific background staining and increase sensitivity.

▲ For optimal sensitivity, we recommend labeling of undesired non-T cells such as monocytes with antibodies conjugated to PerCP™, e.g. CD14-PerCP™. These cells can then be excluded together with PI stained dead cells by gating.

3.4 Two color cytokine analysis

IL-2 secreting cells can be analyzed simultaneously for IFN-γ or IL-10 production by two color cytokine analysis combining the IL-2 Secretion Assay with the IFN-γ Secretion Assay - Detection Kit (APC) (# 130-090-762), or the IL-10 Secretion Assay - Detection Kit (APC) (# 130-090-761). Detailed protocols are included in the data sheets of the Cytokine Secretion Assay - Detection Kits (APC) and are available from our website www.miltenyibiotec.com.

3.5 Combination with peptide-MHC tetramer staining

IL-2 secreting cells can be analyzed simultaneously for peptide-MHC tetramer staining by combining the IL-2 Secretion Assay (PE) with APC-conjugated peptide-MHC tetramers. For combination with PE-conjugated peptide-MHC tetramers the IL-2 Secretion Assay - Detection Kit (APC) (# 130-090-763) is available. Detailed recommendations for the experimental setup and the procedure are included in the data sheets of the Cytokine Secretion Assay - Detection Kits (APC) and are available from our website www.miltenyibiotec.com.

3.6 Detection without prior enrichment

(Optional) If the sample contains more than 0.01-0.1% of IL-2 secreting cells, you may be able to analyze IL-2 secreting cells without prior enrichment (see also: IL-2 Secretion Assay - Detection Kit (PE), # 130-090-487). The assay can also be performed directly starting from whole blood. A detailed protocol is included in the data sheet of the IL-2 Secretion Assay - Detection Kit (PE) and is available from our website www.miltenyibiotec.com.

4. Protocol for the IL-2 Secretion Assay

4.1 Cell preparation

For the detection and isolation of cytokine secreting cells, best results are achieved by starting the assay with fresh PBMC, or other leukocyte containing single cell preparations from tissues or cell lines. Alternatively, frozen cell preparations can be used.

▲ **Note:** PBMC may be stored over night. The cells should be resuspended and incubated in culture medium as described in 4.2 step 2., but without addition of antigen. The antigen is then added to the culture on the next day.

▲ **Note:** Remove platelets after density gradient separation. Resuspend cell pellet, fill tube with buffer and mix. Centrifuge at 200xg for 10-15 minutes at 20°C. Carefully remove supernatant.

Special protocols for whole blood: You can start the IL-2 Secretion Assay directly from whole blood. For details on the procedure, see 7. Appendix B: **Detection and enrichment of cytokine secreting cells from human whole blood**. This special protocol is also available from our website www. miltenyibiotec.com.

4.2 (Antigen-specific) In vitro stimulation

▲ Always include a **negative control** in the experiment. A **positive control** may also be included (see 3.1).

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

Protocol for in vitro stimulation

1. Wash cells by adding medium, centrifuge at 300xg for 10 minutes.
2. Resuspend cells in culture medium, containing 5% human serum, adjust to 10^7 cells/ml and $5x10^6$ cells/cm^2 (see 7. Appendix A: Flask and dish sizes for stimulation).
3. Add antigen or control reagent:

   peptide: 3-6 hours at 37°C, 5-7% CO_2, e.g. 1-10 µg/ml
   protein: 6-16 hours at 37°C, 5-7% CO_2, e.g. 10 µg/ml
   SEB: 3-16 hours at 37°C, 5-7% CO_2, e.g. 1 µg/ml

   For comparison of different experiments, the stimulation time should always be the same (see 3.2).
4. Collect cells carefully by using a cell scraper, or by pipetting up and down when working with smaller volumes. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary, rinse the dish again.

4.3 Cytokine Secretion Assay

General considerations

▲ The assay is optimized for cell samples containing < 5% of total IL-2 secreting cells. If ≥ 5% of IL-2 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 (see table below). The dilution prevents non-specific staining of cells not secreting IL-2 during this period.

▲ For each test with 10^7 total cells, prepare:

100 ml of **cold buffer** (4-8°C)
100 µl of **cold medium** (4-8°C)
10 ml (or 100 ml; see table below) of **warm medium** (37°C).

▲ 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).

▲ Volumes shown below are for 10^7 total cells. When working with less 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 $2x10^7$ total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ 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 IL-2 Secretion Assay, e.g. by density gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

Labeling cells with IL-2 Catch Reagent

1. Use 10^7 total cells in a 15 ml closable tube per sample.

   ▲ **Note:** For larger cell numbers, scale up all volumes accordingly. For less than 10^7 cells, use same volumes.
2. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**, pipette off supernatant completely.

   ▲ **Note:** Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes.
3. Resuspend cell pellet in 80 µl of **cold medium** per 10^7 total cells.
4. Add 20 µl of **IL-2 Catch Reagent** per 10^7 total cells, mix well and incubate for 5 minutes **on ice**.

IL-2 secretion period

1. Add **warm** (37°C) medium to dilute the cells according to the following table:

Expected number of IL-2 secreting cells	Dilution	Amount of medium to add per 10^7 total cells
< 5 %	10^6 cells/ml	10 ml
≥ 5 %	≤ 10^5 cells/ml	100 ml

▲ **Note:** For frequencies of cytokine secreting cells >> 20% the cells need to be further diluted, e.g. by a factor of 5.

2. Incubate cells in closed tube for 45 minutes at 37°C under slow continuous rotation using the MACSmix (# 130-090-753), 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 IL-2 Detection Antibody

1. Put the tube **on ice**.
2. Wash the cells by filling up the tube with **cold buffer**, and centrifuge at 300xg for 10 minutes at **4-8°C**. Pipette off supernatant completely.

▲ **Note:** If the volume of the cell suspension was higher than the volume of added buffer, repeat wash step.

3. Resuspend cell pellet in 80 µl of **cold buffer** per 10^7 total cells.

4. Add 20 µl of **IL-2 Detection Antibody (PE)** per 10^7 total cells.
5. (Optional) Add additional staining reagents, e.g. 10 µl of CD4-FITC (# 130-080-501) or 10 µl of CD8-FITC (# 130-080-601) and CD14-PerCP™.
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**, pipette off supernatant.

4.4 Magnetic labeling

Magnetic labeling with Anti-PE MicroBeads

1. Resuspend cell pellet in 80 µl of cold buffer per 10^7 total cells.
2. Add 20 µl of **Anti-PE MicroBeads** per 10^7 total cells, mix well and incubate for 15 minutes at **4-8°C**.

▲ **Note:** Incubate in refrigerator at 4-8°C, do not work on ice during this step.

3. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**. Pipette off supernatant.
4. Resuspend cell pellet in 500 µl of **cold buffer**. For higher cell numbers than 5x10^7use a dilution of 10^8 cells/ml.
5. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.
6. Proceed to magnetic separation (see 4.5).

4.5 Magnetic separation

Magnetic separation using MS or LS Columns

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells (see table in 1.3).

▲ When enriching antigen-specific T cells, **always perform two consecutive column runs** to achieve best results.

1. Prepare **two columns** per sample by rinsing with cold buffer:
   MS: 500 µl LS Column: 3 ml
   and discard effluent.
2. Place the first column into the magnetic field of a MACS Separator (use column adapter with VarioMACS or SuperMACS).
3. (Optional) Pass the cells through Pre-Separation Filter (# 130-041-407) to remove clumps.
4. Apply cell suspension onto the column.
5. Collect unlabeled cells which pass through and wash with appropriate amount of cold buffer. Perform washing steps by adding buffer successively once the column reservoir is empty.
   MS: 3x500 µl LS: 3x3 ml
   Collect total effluent. This is the unlabeled cell fraction.
6. Remove the first column from separator, place the second column into the separator, and put the first column on top of the second one.

7. Pipette appropriate amount of cold buffer onto the first column. Immediately flush out the fraction with the magnetically labeled cells by firmly applying the plunger, supplied with the column. directly onto the second column.
   MS: 1 ml LS: 5 ml
8. Collect unlabeled cells that pass through and wash with appropriate amount of cold buffer. Perform washing steps by adding buffer successively once the column reservoir is empty.
   MS: 3x500 µl LS: 3x3 ml
9. Remove the second column from separator, place the column on a suitable collection tube.
10. Pipette appropriate amount of cold buffer onto the column. Immediately flush out the fraction with the magnetically labeled cells by firmly applying the plunger, supplied with the column.
    MS: 500 µl LS: 5 ml

▲ **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 analysis (see section 5.), cell culture or other subsequent experiment.

Magnetic separation using the autoMACS

▲ Refer to the autoMACS User Manual for instructions on how to use the autoMACS Separator.

1. Prepare and prime autoMACS.

2. (Optional) Pass cells through Pre-Separation Filter (#130-041-407) to remove clumps.
3. Place tube containing magnetically labeled cells in autoMACS. Choose separation program "Posseld". Collect the separated fractions from outlet port "pos2".
4. Proceed to analysis (see section 5.), cell culture or other subsequent experiment.

5. Detection and analysis of IL-2 secreting T cells

▲ Add propidium iodide (PI) or 7-AAD to a final concentration of 0.5 µg/ml **just prior** to acquisition for exclusion of dead cells from flow cytometric analysis. Incubating with PI for longer periods will affect the viability of the cells.
Do not fix the cells when using PI or 7-AAD.

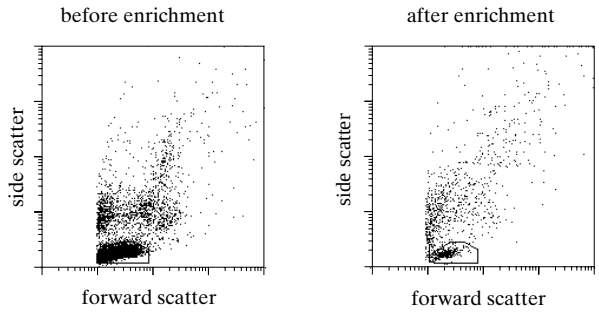
▲ For optimized sensitivity, an appropriate number of viable cells has to be acquired from the antigen stimulated sample as well as from the control sample.

- Acquire $2x10^5$ viable cells from the fraction before enrichment (see 4.4 step 5.).
- For **enumeration** of low frequent IL-2 secreting cells, acquire all of the positive fraction. For **preparative purposes**, acquire an aliquot of the positive fraction to determine the performance of the cell enrichment.

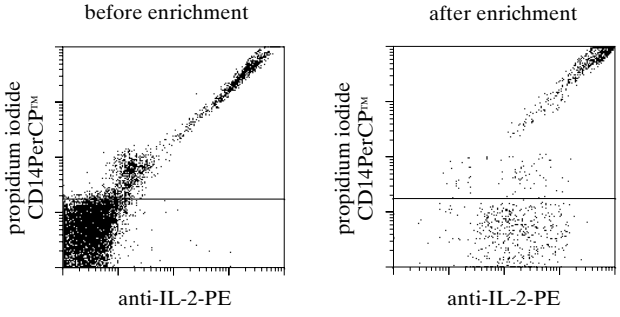
To illustrate the analysis, we describe the detection of IL-2 secreting T cells using the IL-2 Secretion Assay. The detailed description, including how to set gates, should serve as a model for the analysis of your own sample.

1. 10^7 human PBMC of a CMV$^+$ donor have been restimulated for 16 hours with and without CMV-lysate (5 µg/ml; Biowhittaker).
2. The IL-2 Secretion Assay was performed on the stimulated and the unstimulated sample.
3. **Counterstaining of T cells** was performed using CD4-FITC.
4. **Monocytes** were stained with CD14-PerCP™.
5. **Dead cells** were stained with propidium iodide (PI), which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/ml.
6. 200,000 viable cells of the fractions before enrichment and the complete enriched fractions were acquired by flow cytometry, from the stimulated and the unstimulated samples.
7. A **lymphocyte gate** based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude monocytes and debris (see A.).
8. Dead cells and monocytes were excluded according to PI- and CD14-PerCP™-staining in a fluorescence 2 (PE) versus fluorescence 3 plot (PerCP) (see B.).
   - The **dead cell exclusion** is crucial for the analysis of rare antigen-specific T cells, as dead cells may bind non-specifically to antibodies or MicroBeads. This could lead to false positive events.
   - The sensitivity of detection is further enhanced by exclusion of undesired non-T cells, like monocytes which may cause non-specific background staining.
9. Analysis of secreted IL-2 (PE) versus CD4-FITC staining by viable lymphocytes is displayed (see C.).

A. Lymphocyte gate in the forward versus side scatter plot

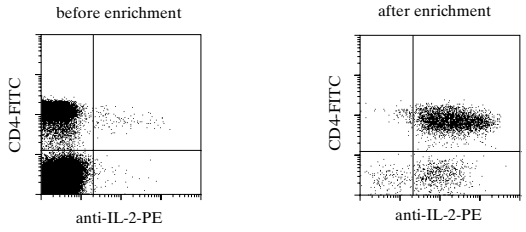


B. Dead cell and monocyte exclusion in FL-2 versus FL-3



C. Antigen-specific CD4$^+$ T cells stained for secreted IL-2

Sample stimulated with CMV lysate



0.350% of the total CD4$^+$ T cell population secrete IL-2 (see formula below).

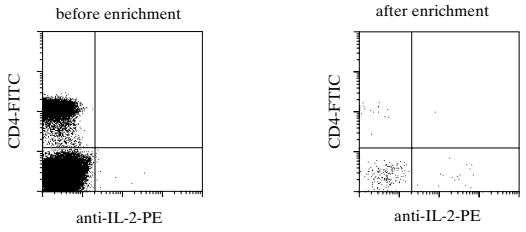
The IL-2 secreting CD4$^+$ T cells have been enriched to 80.4%.

2047 IL-2$^+$CD4$^+$ T cells were enriched from 10^6 CD4$^+$ cells (= 0.205%; see formula below).

$$\%\ \text{IL-2}^+\ \text{cells among CD4}^+ = \frac{\#\ \text{of IL-2}^+\text{CD4}^+\ \text{cells in the analyzed sample}}{\#\ \text{of total CD4}^+\ \text{cells in the analyzed sample}} \times 100$$

$$\%\ \text{IL-2}^+\ \text{cells among CD4}^+ = \frac{\text{abs. \# of IL-2}^+\text{CD4}^+\ \text{cells in the enriched fraction}}{\text{abs. \# of total CD4}^+\ \text{cells before enrichment}} \times 100$$

Unstimulated control sample



0.004% of the total CD4$^+$ T cell population secrete IL-2.

1 IL-2$^+$CD4$^+$ T cell were enriched from 10^6 CD4$^+$ cells (≤ 0.0001%).

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For further information visit our website **www.miltenyibiotec.com.**

7. Appendix:

A: Flask and dish sizes for stimulation

For in vitro stimulation (see 4.2 step 2.) the cells should be resuspended in culture medium, containing 5% of human serum, at 10^7 cells/ml and $5x10^6$ cells/cm². Both the dilution and the cell density are important to assure optimum stimulation.

The following table lists culture plate, dish and flask sizes suitable for different cell numbers. It also indicates the appropriate amount of medium to add.

total cell number	medium volume to add	culture plate	well diameter
0.15×10^7	0.15 ml	96 well	0.64 cm
0.5×10^7	0.5 ml	48 well	1.13 cm
1×10^7	1 ml	24 well	1.6 cm
2×10^7	2 ml	12 well	2.26 cm
5×10^7	5 ml	6 well	3.5 cm

total cell number	medium volume to add	culture dish	dish diameter
4.5×10^7	4.5 ml	small	3.5 cm
10×10^7	10 ml	medium	6 cm
25×10^7	25 ml	large	10 cm
50×10^7	50 ml	extra large	15 cm

total cell number	medium volume to add	culture flask	growth area
12×10^7	12 ml	50 ml	25 cm²
40×10^7	40 ml	250 ml	75 cm²
80×10^7	80 ml	720 ml	162 cm²
120×10^7	120 ml	900 ml	225 cm²

B: Detection and enrichment of cytokine secreting cells from whole blood

B1. Reagent and instrument requirements

B2. Protocol

- **B 2.1 (Antigen-specific) in vitro stimulation**
- **B 2.2 Cytokine Secretion Assay**
- **B 2.3 Magnetic labeling**
- **B 2.4 Magnetic separation**

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

B 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** (degassed): phosphate buffered saline pH 7.2, containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA (e.g. 4 ml of a 0.5 M EDTA stock solution per 1 liter of buffer).
  - (Optional) 0.5 M EDTA stock solution: dissolve 56 g sodium hydroxide (NaOH) in 900 ml dd H_2O. Add 146.2 g ethylene-diamine-tetraacetic acid (EDTA), adjust pH to 7.5, fill up to 1000 ml with dd H_2O .
- **Culture medium**, e.g. RPMI 1640 containing 20% of human serum, like autologous serum or AB serum.

  ▲ **Note**: Do **not use BSA** or **FCS** because of non-specific stimulation.
- **Erythrocyte lysing solution (1x):**
  - prepare freshly from 10x stock solution.
  - **10x stock solution**: 41.4 g NH_4Cl (1.55 M), 5 g $KHCO_3$ (100 mM), 1 ml 0.5 M EDTA (1 mM), adjust pH to 7.3, fill up to 500 ml with dd H_2O.

    ▲ **Note**: Do **not use** FACS Lysing solution™.
- (Optional) **Staining reagents**: CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP™.

  ▲ **Note**: Do **not use** tandem conjugates of phycoerythrin, like Cy-Chrome® (PharMingen), PE-Cy5 (Serotec), ECD, PC5 (Coulter-Immunotech) etc., they may also be recognized by the Anti-PE MicroBeads.

  ▲ **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™. These cells can then be excluded together with PI stained dead cells by gating.
- **Propidium iodide** (PI) or **7-AAD** to exclude dead cells from analysis.

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- MACS Columns and MACS Separators:

Column	max. number of labeled cells	max. number of total cells	Separator
MS	10^7	$2x10^8$	MiniMACS, OctoMACS; with Column Adapter: VarioMACS, SuperMACS
autoMACS	$2x10^8$	$4x10^9$	autoMACS

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

- (Optional) Rotation device for tubes: MACSmix (# 130-090-753)
- (Optional) Pre-Separation Filter (# 130-041-407)

B 2. Protocol

B 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).

▲ 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 (# 130-090-753), or turn tube several times during incubation.

▲ Do **not use** media containing any **non-human** proteins, like BSA or FCS 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).
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.

B 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.

3. Centrifuge cells at 300xg 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 300xg for 10 minutes at **4-8°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, 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 300xg for 10 minutes at **4-8°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™.
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 10 ml of **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**. Pipette off supernatant completely.

B 2.3 Magnetic labeling

Magnetic labeling with Anti-PE MicroBeads

1. Resuspend cell pellet in 160 µl of **cold buffer**.
2. Add 40 µl of **Anti–PE-MicroBeads**, mix well and incubate for 15 minutes at **4-8°C**.
   ▲ **Note**: Incubate in refrigerator at 4-8°C; do not work on ice during this step.
3. Wash cells by adding 10 ml of **cold buffer,** centrifuge at 300xg for 10 minutes at 4-8°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.

B 2.4 Magnetic separation

Magnetic separation using MS Columns

▲ When enriching antigen-specific T cells, **always perform two consecutive MS Columns** to achieve best results.

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 MACS Separator (use column adapter with VarioMACS or SuperMACS).
3. (Optional) Pass cells through Pre-Separation Filter (#130-041-407) to remove clumps.
4. Apply cell suspension onto the column.
5. Collect unlabeled cells which pass through and wash with 3x500 µl of cold buffer. Perform washing steps by adding buffer successively once the column reservoir is empty.
   Collect total effluent. This is the unlabeled cell fraction.
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 fraction with the magnetically labeled cells by firmly applying the plunger, supplied with the column, directly onto the second column.
8. Collect unlabeled cells that pass through and wash with 3x500 µl of cold buffer. Perform washing steps by adding buffer successsively once 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 fraction with the magnetically labeled cells by firmly applying the plunger, supplied with the column.
    ▲ **Note**: For subsequent cell culture, the cells can also be eluted with medium. If part of the cells are analysed 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

▲ Refer to the autoMACS User Manual for instructions on how to use the autoMACS Separator.

1. Prepare and prime autoMACS.
2. (Optional) Pass cells through Pre-Separation Filter (#130-041-407) to remove clumps.

3. Place tube containing magnetically labeled cells in autoMACS. Choose separation program “Posseld”. Collect the separated fractions from outlet port “pos2”.
4. Proceed to flow cytometric analysis (see detailed protocol), cell culture or other subsequent experiment.

Warning

Reagents contain sodium azide. Sodium azide yields hydrazoic acid under acid conditions, which is extremely toxic. Azide compounds should be diluted with running water before being discarded. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

Warranty

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* Phycoerythrin (PE): U.S. Patent 4,520,110; European Patent 76,695; Australian Patent 548,440; Canadian Patent 1,179,942; Japanese Patent 1,594,827.