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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 requirement

- **Cytokine Secretion Assay Kit**, for example:
 - IFN- Secretion Assay - Cell Enrichment and Detection Kit (PE*) (# 130-054-201)
 - IL-2 Secretion Assay - Cell Enrichment and Detection Kit (PE*) (# 130-054-488)
 - IL-4 Secretion Assay - Cell Enrichment and Detection Kit (PE*) (# 130-054-101)
 - IL-10 Secretion Assay - Cell Enrichment and Detection Kit (PE*) (# 130-054-435)
- **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 H₂O. Add 146.2 g ethylenediamine-tetraacetic acid, adjust pH to 7.5, fill up to 1000 ml with dd H₂O .
- **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 NH₄Cl (1.55 M), 5 g KHCO₃ (100 mM), 1 ml 0.5 M EDTA (1 mM), adjust pH to 7.3, fill up to 500 ml with dd H₂O.
 - ▲ **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 R-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.
- MACS Columns and MACS Separators:

Column	max. number of labeled cells	max. number of total cells	Separator
MS	1x10 ⁷	2x10 ⁸	MiniMACS, OctoMACS; with Column Adapter: VarioMACS, SuperMACS
autoMACS	2x10 ⁸	4x10 ⁹	autoMACS

- (Optional) Rotation device for tubes: MACSmix (# 130-090-753).
- (Optional) Pre-Separation Filter (# 130-041-407) or 30 µm nylon mesh.

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 FCS because of non-specific stimulation.



Protocol for in vitro stimulation

1. Start with **5 ml of fresh, sodium heparinized, human blood** (containing about 1x10⁷ 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% CO₂ (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.

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).

▲ Avoid capping of antibodies on the cell surface during staining. **Work fast, keep cells cold, use cold solutions only** (exception: **room temperature** during lysing step and **warm medium** during secretion period).

▲ 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. 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.
2. 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**. Remove supernatant **completely**.
3. Resuspend pellet in 160 µl of **cold medium**, 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**, remove supernatant **completely**.
3. Resuspend cell pellet in 160 µl of **cold buffer** and add 40 µl of **Cytokine Detection Antibody** (PE).

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

2.3 Magnetic labeling



Magnetic labeling with Anti-PE MicroBeads

1. Resuspend cell pellet in 160 µl of **cold buffer**, 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.
2. Wash cells by adding 10 ml of **cold buffer** and centrifuge at 300xg for 10 minutes at 4-8°C, remove supernatant completely.
3. Resuspend cell pellet in 500 µl of **cold buffer**.
4. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.
5. Proceed to magnetic separation.

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 the first column into the magnetic field of a MACS Separator (use column adapter with VarioMACS or SuperMACS).
3. (Optional) Pass the cells through 30 µm nylon mesh (Pre-Separation Filter # 130-041-407) to remove clumps.
4. Apply the magnetically labeled cells to the column, allow the cells to pass through the column. Wash with 3 x 500 µl of **cold buffer**: Collect effluent as unlabeled fraction.
5. Remove the first column from separator, place the second column into the separator, and put the first column on top of the second one. Pipette 1 ml of **cold buffer** on top of the first column.
6. **Firmly** flush out the retained cells from the first column, by using the plunger supplied with the column, directly onto the second column. Allow the cells to pass through the column.
7. Wash with 3 x 500 µl **cold buffer**.
8. Remove the second column from separator, place the column on a suitable collection tube. Pipette 500 µl of **cold buffer** on top of the column.
9. **Firmly** flush out the retained cells by using 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.
10. Proceed to flow cytometric analysis (see detailed protocol), cell culture or other subsequent experiment.



Magnetic separation using the autoMACS

1. Prepare and prime the autoMACS instrument according to the autoMACS User Manual.
2. (Optional) Pass cells through 30 μm nylon mesh or Pre-Separation Filter (# 130-041-407) to remove clumps.
3. Apply magnetically labeled cells to the autoMACS. Choose the separation program "Posseld". Collect the separated fractions.
4. Proceed to flow cytometric analysis (see detailed protocol), cell culture or other subsequent experiment.

PerCP™ Perinidin chlorophyll protein is a trademark of Becton Dickinson.

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