MACS

Special Cytokine Secretion Assay Protocol

Detection of cytokine secreting cells from whole blood

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

1. Reagent and instrument requirement

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

IFN-□Secretion Assay - Detection Kit (PE*)	(# 130-054-202)
IFN-□Secretion Assay - Detection Kit (FITC)	(# 130-090-433)
IFN-□Secretion Assay - Detection Kit (APC*)	(# 130-090-762)
IL-2 Secretion Assay - Detection Kit (PE*)	(# 130-090-487)
IL-2 Secretion Assay - Detection Kit (APC*)	(# 130-090-763)
IL-4 Secretion Assay - Detection Kit (PE*)	(# 130-054-102)
IL-10 Secretion Assay - Detection Kit (PE*)	(# 130-090-434)
IL-10 Secretion Assay - Detection Kit (APC*)	(# 130-090-761)

- **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, adjust pH to 7.5, fill up to 1000 ml with dd H_2O.
- **Culture medium**, e.g. RPMI 1640 containing 10% 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**: 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.
- (Optional) Rotation device for tubes: MACSmix (# 130-090-753).

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 over night 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 **250 µl of fresh, sodium heparinized, human blood** (containing about $5x10^5$ lymphocytes) in a 15 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.

2.2 Cytokine Secretion Assay

▲ This protocol is optimized for cell samples containing < 20% of total cytokine secreting cells. If ≥ 20% 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 prevents non-specific staining of cells not secreting cytokines during this period.

▲ For each sample with 250 µl whole blood prepare:

50 ml of **cold buffer** (4-8°C)

100 µl of **cold medium** (4-8°C)

5 ml of **warm medium** (37°C)

5 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: **warm medium** during secretion period and **room temperature** during lysing step).

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



Labeling cells with Cytokine Catch Reagent

1. Wash cells by adding 10 ml of **cold buffer,** centrifuge at 300xg for 10 minutes at 4-8°C, pipette off supernatant carefully.
   ▲ **Note**: Be careful, leukocytes will appear on top of the loose red cell pellet.
2. Resuspend pellet in 80 µl of **cold medium**. Add 20 µl of **Cytokine Catch Reagent**, mix well and incubate for 5 minutes **on ice**.



Cytokine secretion period

1. Add 5 ml of **warm medium** (37°C) to dilute the cells.
   ▲ **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 by 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 Cytokine Detection Antibody

1. Put the tube **on ice.**
2. Wash cells by adding 10 ml of **cold buffer,** centrifuge at 300xg for 10 minutes at **4-8°C**, pipette off supernatant carefully.
3. Resuspend cell pellet in 80 µl of **cold buffer**. Add 20 µl of **Cytokine Detection Antibody**.
4. (Optional) Add additional staining reagents, e.g. 10 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.



Lysis of erythrocytes

1. Add 5 ml of **erythrocyte lysing solution**. Mix gently and incubate for 10 minutes at room temperature. Rotate tube continuously using the MACSmix, or turn tube several times during incubation.
2. Centrifuge cells at 300xg for 10 minutes at **room temperature**, pipette off supernatant **completely.**
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 the cells in 500 µl of **cold buffer**, and proceed to flow cytometric analysis (see detailed protocol).

2.3 Detection and analysis of cytokine secreting cells

▲ Add **propidium iodide** (PI) or 7-AAD to a final concentration of 0.5 µg/ml **just prior** to acquisition to exclude dead cells from flow cytometric analysis. Incubation 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 2 x 10^5 viable cells from each sample.

▲ For details on analysis please refer to the detailed protocol provided with the Cytokine Secretion Assay Kits.

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

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

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