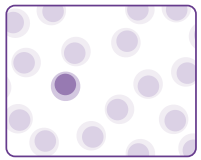
Short protocol

Cytokine Secretion Assay – Cell Enrichment and Detection Kit

For details on the procedure and analysis refer to protocol supplied with the kit.

A In vitro restimulation of the cells



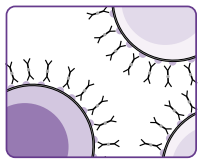
1. Prepare **10^7** cells per sample and wash cells by adding medium, centrifuge at 300xg for 10 minutes.
2. Resuspend 10^7 cells per sample in 1 ml culture medium, containing 5% human or mouse serum.

   Transfer cells in one well of a 24-well-plate per sample.
3. Add respective antigen or control reagent and incubate at 37°C, 5-7% CO_2:

   peptide: e.g. 1-10 µg/ml, 3-6 hours
   protein: e.g. 10 µg/ml, 6-16 hours
   SEB: e.g. 1 µg/ml, 3-16 hours
4. Collect cells carefully by using a cell scraper, or by pipetting up and down. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary, rinse the dish again.

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).

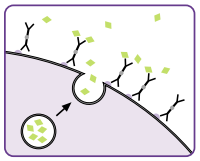
B Labeling cells with Cytokine Catch Reagent



1. Use **10^7** total cells in a 15 ml closable tube per sample.
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:** When working with Mouse Cytokine Secretion Assays repeat wash step.
3. Resuspend cell pellet in **80 µl** of **cold medium**.*
4. Add **20 µl** of **Cytokine Catch Reagent**, mix well and incubate for **5 minutes on ice.***

C Cytokine secretion period

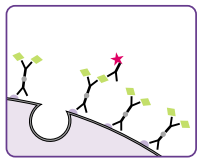


1. Add **warm medium** (37°C) to dilute the cells according to the following table: *

Expected number of 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

2. Incubate cells in 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.

D Labeling cells with Cytokine 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.

   ▲ **Note:** When working with Mouse Cytokine Secretion Assays repeat wash step.
3. Resuspend cell pellet in **80 µl** of **cold buffer**.*
4. Add **20 µl** of **Cytokine Detection Antibody (PE)**.*
5. (Optional) Add additional staining reagents.
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.

* When working with **Mouse IFN-γ Secretion Assay** and expected frequencies of IFN-γ secreting cells ≥ 2%, refer to protocol supplied with the kit.

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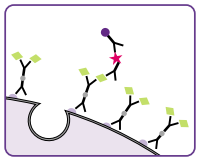
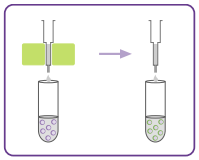
E Magnetic labeling with Anti-PE MicroBeads



1. Resuspend cell pellet in **80 µl** of **cold buffer**.
2. Add **20 µl** of **Anti-PE MicroBeads**, mix well and incubate for **15 minutes** at **4-8°C.**
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**.
5. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.

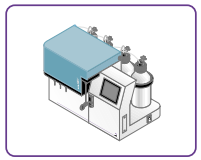
F Magnetic separation using MS Columns



1. Prepare **two MS Columns** per sample by rinsing with **500 µl** of cold buffer and discard effluent.
2. Place the first column into the magnetic field of a suitable MACS Separator.
3. (Optional) Pass the cells through Pre-Separation Filter 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 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 **1 ml** of cold buffer onto the first column. Immediately and firmly flush out fraction with the magnetically labeled cells using 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 successively once the column reservoir is empty.
9. Remove the second column from separator, place the column on a suitable collection tube.
10. Pipette **500 µl** 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.
11. Proceed to flow cytometric analysis, cell culture or other subsequent experiment.

▲ **Note:** For flow cytometric analysis add propidium iodide or 7-AAD to a final concentration of 0.5 µg/ml just prior to acquisition.

G Magnetic separation using the autoMACS



1. Prepare and prime the autoMACS.
2. (Optional) Pass cells through Pre-Separation Filter to remove clumps.
3. Place tube containing magnetically labeled cells in the autoMACS. Choose separation program “Posseld”. Collect the enriched fraction from outlet port “pos2”.
4. Proceed to flow cytometric analysis, cell culture or other subsequent experiment.

▲ **Note:** For flow cytometric analysis add propidium iodide or 7-AAD to a final concentration of 0.5 µg/ml just prior to acquisition.

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