



## Special protocol for Cytokine Secretion Assays

# Two-color Cytokine Secretion Assays

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## 1. Description

### 1.1 Two-color cytokine analysis

Cytokine-secreting cells can be analyzed simultaneously for the production of two different cytokines by the combination of two different Cytokine Secretion Assay - Detection Kits.

### 1.2 Reagent and instrument requirements

- **Two different Cytokine Secretion Assays**, for example:
  - IFN- $\gamma$  Secretion Assay - Detection Kit (PE) (#130-054-202)
  - IFN- $\gamma$  Secretion Assay - Detection Kit (FITC) (#130-090-433)
  - IFN- $\gamma$  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-10 Secretion Assay - Detection Kit (PE) (#130-090-434)
  - IL-10 Secretion Assay - Detection Kit (APC) (#130-090-761)
  - TNF- $\alpha$  Secretion Assay - Detection Kit (PE) (#130-091-268)
  - TNF- $\alpha$  Secretion Assay - Detection Kit (APC) (#130-091-267)
- or
  - Mouse IFN- $\gamma$  Secretion Assay - Detect. Kit (PE) (#130-090-516)
  - Mouse IFN- $\gamma$  Secretion Assay - Detect. Kit (APC) (#130-090-984)
  - Mouse IL-2 Secretion Assay - Detect. Kit (PE) (#130-090-491)
  - Mouse IL-2 Secretion Assay - Detect. Kit (APC) (#130-090-987)
  - Mouse IL-10 Secretion Assay - Detect. Kit (PE) (#130-090-489)
  - Mouse IL-10 Secretion Assay - Detect. Kit (APC) (#130-090-939)
- **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<sub>2</sub>O. Add 146.2 g ethylenediamine-tetraacetic acid (EDTA), adjust pH to 7.5, fill up to 1000 mL with dd H<sub>2</sub>O.

- Culture medium, e.g. RPMI 1640 (#130-091-440) containing 5% human serum, e.g. 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
- Additional staining reagents, e.g. CD4-FITC (#130-080-501), or CD8-FITC (#130-080-601)
- Refrigerated centrifuge (4–8 °C)
- Rotation device for tubes:  
MACSmix™ tube rotator (#130-090-753)

## 2. Experimental setup

### 2.1 Controls

#### Negative control

For accurate detection of cytokine-secreting antigen-specific cells, a negative control sample should always be included. This will provide information about cytokine 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  $\mu$ g/mL for 3–16 hours, may be included in the experiment.

▲ **Note:** For IFN- $\gamma$ , IL-2, and TNF- $\alpha$  Secretion Assays mitogens like PHA or PMA/Ionomycin are not recommended for stimulation of a positive control, as the resulting high frequencies of cytokine-secreting cells do not allow conclusions on the performance (e.g. sensitivity) of the Cytokine Secretion Assays.

### 2.2 Kinetics of restimulation and proposed time schedule

#### Peptides

Upon stimulation with peptide, the cells can be analyzed for cytokine secretion 3–6 hours later.

It is possible to prepare the cells first and take them into culture overnight, but without addition of antigen (see 3.2 step 2.). Peptide is then added the next morning for 3 hours of stimulation, directly followed by the Cytokine Secretion Assay.

#### Proteins

Upon stimulation with protein, the cells can be analyzed for cytokine secretion 6–16 hours later.

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

#### Co-stimulation

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.

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### 2.3 Counterstaining of cytokine-secreting cells

To identify cells of interest, counterstaining of T cells with e.g. CD4 or CD8 is important.

▲ 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 (human) or B cells (mouse) with antibodies conjugated to PerCP™, e.g. CD14-PerCP™ or CD45R/B220-PerCP™, respectively. These cells can then be excluded together with PI stained dead cells by gating.

## 3. Protocol

### 3.1 Cell preparation

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

▲ **Note:** PBMCs may be stored overnight. The cells should be resuspended and incubated in culture medium as described in 3.2 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.

### 3.2 (Antigen-specific) *in vitro* stimulation

▲ Always include a **negative control** in the experiment, a **positive control** may also be included (see 2.1).

▲ When working with human cells do **not use** media containing any **non-human** proteins, like BSA or FCS because of non-specific stimulation. When working with murine cells do **not use** media containing any **non-murine** proteins.



#### Protocol for *in vitro* stimulation

- Resuspend  $10^6$  cells per sample in 100  $\mu$ L of culture medium, e.g. RPMI 1640 (# 130-091-440), containing 5% human or mouse serum. Transfer cells to one well of a 96-well plate per sample.
- Add antigen or control reagent:
  - peptide: 3–6 hours at 37°C, 5–7% CO<sub>2</sub>, e.g. 1–10  $\mu$ g/mL
  - protein: 6–16 hours at 37°C, 5–7% CO<sub>2</sub>, e.g. 10  $\mu$ g/mL
  - SEB: 3–16 hours at 37°C, 5–7% CO<sub>2</sub>, e.g. 1  $\mu$ g/mL

▲ **Note:** For comparison of different experiments the stimulation time should always be the same.
- Collect cells carefully by pipetting up and down. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary, rinse the dish again.

### 3.3 Two-color Cytokine Secretion Assay

#### General considerations

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

▲ For each test with  $10^6$  total cells, prepare:

10 mL of **cold buffer** (4–8 °C)

100  $\mu$ L of **cold medium** (4–8 °C)

1 mL (or 10 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  $10^6$  total cells. When working with fewer than  $10^6$  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  $2 \times 10^6$  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 completely.



#### Labeling cells with Cytokine Catch Reagent

- Use  $10^6$  total cells in a 2 mL closable tube per sample.

▲ **Note:** For larger cell numbers, scale up all volumes accordingly. For fewer than  $10^6$  cells, use same volumes.

- Wash cells by adding 1–2 mL of **cold buffer**, centrifuge at 300xg for 10 minutes at 4–8 °C, pipette off supernatant completely.
- Resuspend the cells in 80  $\mu$ L of **cold medium** per  $10^6$  total cells.
- Pre-mix same volumes** of the two different **Catch Reagents**. Add 20  $\mu$ L of this **cocktail** per  $10^6$  total cells, mix well and incubate for 5 minutes **on ice**.

▲ **Note:** It is important to pre-mix the Catch Reagents prior to adding to the cells. This will provide sufficient binding of both cytokines.



#### Cytokine secretion period

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

Expected number of cytokine-secreting cells	Dilution	Amount of medium to add per $10^6$ total cells
< 5 %	$10^6$ cells/mL	1 mL
$\geq 5$ %	$10^5$ cells/mL	10 mL

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

- Incubate cells in a closed tube for 45 minutes at 37 °C under slow continuous rotation by using the MACSmix™ tube rotator (# 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 Antibodies

- Put the tube on ice.
- Wash the cells by filling up the tube with **cold buffer**, 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.
- Resuspend the cells in 80 µL of **cold buffer** per 10<sup>6</sup> total cells.
- Add 10 µL of **each Cytokine Detection Antibody** per 10<sup>6</sup> total cells, respectively.
- (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™.
- Mix well and incubate for 10 minutes **on ice**.
- Wash cells by adding 2 mL of **cold buffer**, centrifuge at 300xg for 10 minutes at **4–8 °C**, pipette off supernatant.
- Resuspend cells in 500 µL of **cold buffer** and proceed to flow-cytometric acquisition and analysis (see 4.).

## 4. Analysis of cytokine co-expressing 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<sup>5</sup> viable cells from each sample.

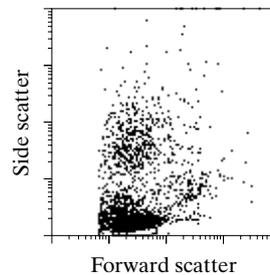
### 4.1 Example: Co-expression of IFN-γ and IL-2 by CD4<sup>+</sup> T cells

To illustrate the analysis, we describe the detection of IFN-γ and IL-2 secreting CD4<sup>+</sup> T cells by using the IFN-γ Secretion Assay (APC) (# 130-090-762) in combination with the IL-2 Secretion Assay (PE) (# 130-090-487). This description, including how to set gates, should serve as a model for the analysis of your own sample.

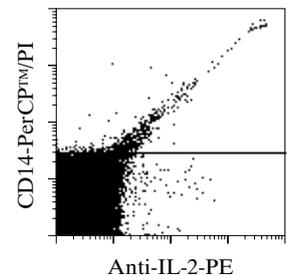
- 10<sup>6</sup> human PBMCs of a CMV<sup>+</sup> donor have been restimulated for 16 hours with, and for the control sample, without CMV lysate.
- The two color Cytokine Secretion Assay was performed on the stimulated and the control sample.
- Counterstaining of T cells** was performed by using CD4-FITC.
- Monocytes** were stained with CD14-PerCP™.
- Dead cells** were stained with PI, which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/mL.
- 200,000 viable cells were acquired by flow cytometry, from the stimulated as well as from the control sample.
- A **lymphocyte gate** based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude monocytes and debris (plot 1.).

- Dead cells and monocytes were excluded according to PI and CD14-PerCP™ staining in a fluorescence 2 (PE) versus fluorescence 3 (PerCP™) plot (plot 2.).
  - The **dead cell exclusion** is crucial for the analysis of rare antigen-specific T cells, as antibodies may bind non-specifically to dead cells. This could lead to false positive events.
  - The sensitivity of the detection is further enhanced by exclusion of undesired non-T cells, like monocytes which may cause non-specific background staining.
- CD4<sup>+</sup> T cells were gated in a fluorescence 1 versus fluorescence 2 plot (not shown).
- For analysis secreted IFN-γ (APC) versus secreted IL-2 (PE) of viable CD4<sup>+</sup> T cells is displayed (plot 3.).

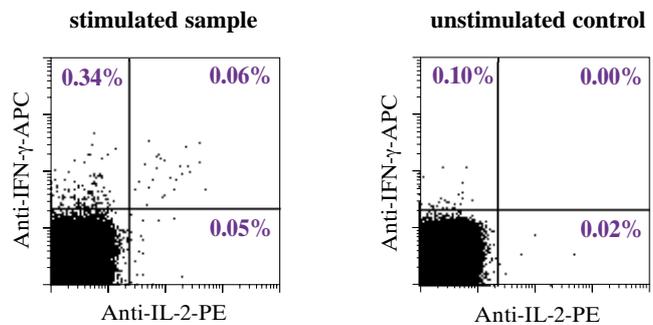
### 1. Lymphocyte gate using FSC versus SSC



### 2. Dead cell and monocyte exclusion



### 3. IFN-γ and IL-2 secreting CD4<sup>+</sup> T cells after stimulation with CMV-lysate



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