Special protocol for Cytokine Secretion Assays

Combined staining of cytokine-secreting cells with peptide-MHC tetramers

human

Index

1. Description

1.1 Combination of Cytokine Secretion Assay with peptide-MHC tetramer staining

The Cytokine Secretion Assay can be performed in conjunction with peptide-MHC tetramer labeling. This provides information on functionality and specificity of human antigen-specific T cells on a single-cell level.

Since stimulation with antigen can strongly down-regulate TCR expression on the specific T cells, tetramer labeling should be performed prior to *in vitro* stimulation and the Cytokine Secretion Assay.

1.2 Reagent and instrument requirements

- **Cytokine Secretion Assay, human, for example:**
  IFN-γ Secretion Assay - Detection Kit (PE) (# 130-054-202)
  IFN-γ Secretion Assay - Detection Kit (FITC) (# 130-090-433)
  IFN-γ 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)
- **Peptide-MHC tetramers** conjugated to R-Phycoerythrin (PE) or allophycocyanin (APC)
- **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 (# 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. 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 Antigen sample

The cells are labeled with the specific peptide-MHC tetramers first (see 1.1), followed by *in vitro* stimulation for 2 hours with specific peptide (see 3.2 and 3.3). Then the Cytokine Secretion Assay is performed according to the standard protocol (see 3.4).

2.2 Negative controls

For accurate detection of cytokine-secreting antigen-specific cells, a negative control sample incubated without peptide or a control peptide should **always** be included in the experiment. This will provide information about the activation status of the cells, e.g. due to ongoing *in vivo* immune reactions.

Depending on the peptide-MHC tetramer, tetramer labeling of the cells (a) may, or (b) may not induce cytokine secretion.
If the stimulatory effect of the peptide-MHC tetramer is unknown, both types of negative controls should be performed initially.

(a) Cytokine secretion is induced:
For the negative control sample the cells should be cultured for 2 hours without addition of the specific peptide or peptide-MHC tetramers. Then the Cytokine Secretion Assay is performed followed by peptide-MHC tetramer labeling.
In contrast, the antigen samples are labeled with peptide-MHC tetramers prior to the Cytokine Secretion Assay (see 2.1).

(b) No cytokine secretion is induced:
Cells from all samples, from the antigen sample as well as from the negative control sample, are first labeled with peptide-MHC tetramers. Subsequently, cells from all samples are incubated for 2 hours with or without (negative control) addition of antigenic peptide followed by performing the Cytokine Secretion Assay.

2.3 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 (SEB) 1 µg/mL for 3–16 hours, may be included in the experiment.
Peptide-MHC tetramer staining should be performed prior to stimulation of the cells with SEB.

▲ **Note:** For IFN-γ, IL-2, and TNF-α 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.

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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 3.3 step 1., but without addition of antigen. The procedure is then started 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 Labeling with peptide-MHC tetramers

1. Incubate 10^6 PBMCs with peptide-MHC tetramers according to the manufacturer's protocol, e.g. 10 µL peptide-MHC tetramers per 100 µL of cell suspension for 1 h at 4–8 °C.
2. Wash cells by adding 1–2 mL of **cold medium**, centrifuge at 300xg for 10 minutes at **4–8 °C**. Pipette off supernatant .

3.3 (Antigen-specific) *in vitro* stimulation

▲ Always include a **negative control** in the experiment (see 2.2). A **positive control** may also be included (see 2.3).

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

Protocol for *in vitro* stimulation

1. Resuspend 10^6 cells per sample in 100 µL of culture medium, e.g. RPMI 1640 (# 130-091-440), containing 5% human serum. Transfer cells to one well of a 96-well plate per sample.
2. Add antigen or control reagent:
   peptide: 1–10 µg/mL
   SEB: 1 µg/mL
   Incubate cells for 2 hours at 37 °C, 5–7% CO_2.
3. 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.

3.4 Cytokine Secretion Assay

General considerations

▲ The assay 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 (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:

50 mL of **cold buffer** (4–8 °C)
100 µL 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 $2x10^6$ 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

1. 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.
2. Wash cells by adding 1–2 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 90 µL of **cold medium** per 10^6 total cells.
4. Add 10 µL of **Cytokine Catch Reagent** per 10^6 total cells, mix well and incubate for 5 minutes **on ice**.

Cytokine secretion period

1. 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
≥ 5 %	10^5 cells/mL	10 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 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 Antibody

1. Put the tube on ice.
2. 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.
3. Resuspend cell pellet in 90 µL of **cold buffer** per 10^6 total cells.
4. Add 10 µL of **Cytokine Detection Antibody** per 10^6 total cells.
5. Add additional staining reagents, e.g. 10 µL of CD8-FITC.

6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 2 mL of **cold buffer**, centrifuge at 300xg for 10 minutes at **4–8 °C**, pipette off supernatant.
8. Resuspend cells in 500 µL of **cold buffer** and proceed to flow-cytometric acquisition and analysis (see 4.).

4. Analysis of cytokine-secreting peptide-MHC tetramer stained 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 each sample.

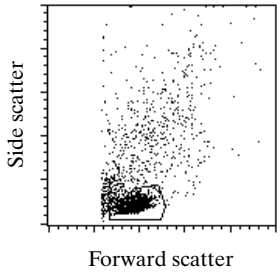
4.1 Example: IFN-γ secretion of CMV-pp65$_{495-503}$-HLA-A2 tetramer labeled cells

To illustrate the analysis, we describe the detection of CMV-pp65-specific, IFN-γ-secreting CD8$^+$ T cells using the IFN-γ Secretion Assay (APC) in combination with CMV-pp65$_{495-503}$-HLA-A2 tetramers conjugated to PE. This description, including how to set gates, should serve as a model for the analysis of your own sample.

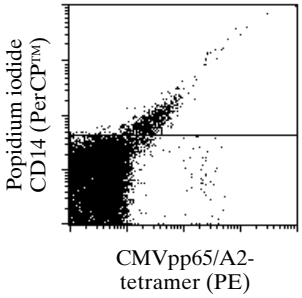
1. 10^6 PBMCs were incubated for 1 h with CMV-pp65$_{495-503}$-HLA-A2 tetramers conjugated to PE at 4–8 °C.
2. The pp65-HLA-A2 tetramer labeled cells were stimulated with pp65-peptide for 2 h at 37 °C (plot 4.A).

   As control samples (see 2.2) pp65-HLA-A2 tetramer labeled cells were incubated without peptide for 2 h at 37 °C (plot 4.B) and cells without peptide-MHC tetramer staining have been stimulated with pp65-peptide (plot 4.C).
3. The samples were stained for IFN-γ secretion using the IFN-γ Secretion Assay - Detection Kit (APC).
4. **Counterstaining of T cells** was performed by using CD8-FITC.
5. **Dead cells** were stained with PI.
6. 200,000 viable cells were acquired by flow cytometry, from the stimulated as well as from the control sample.
7. 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).
8. Dead cells were excluded according to PI staining in a fluorescence 2 (PE) versus fluorescence 3 (PI) 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.
9. CD8$^+$ T cells were gated in a fluorescence 1 versus fluorescence 2 plot (plot 3.).
10. For analysis, secreted IFN-γ (APC) versus pp65-HLA-A2 tetramer (PE) of gated, viable CD8$^+$ T cells are displayed.

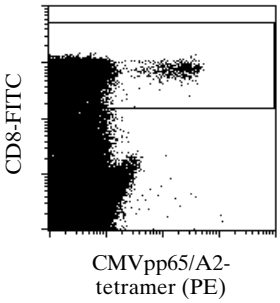
1. Lymphocyte gate using FSC versus SSC



2. Dead cell and monocyte exclusion

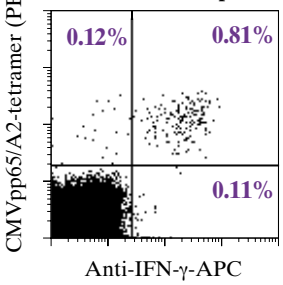


3. CD8$^+$ T cell gate

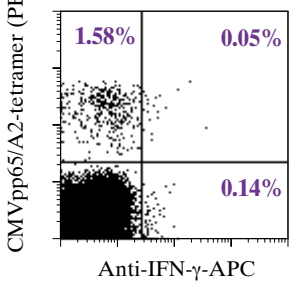


4. Secretion of IFN-γ by CMV peptide-specific CD8$^+$ T cells

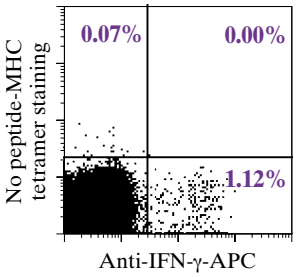
A stimulated sample



B unstimulated control



C stimulated sample without peptide-MHC tetramer staining



5. References

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2. Pittet, MJ; Zippelius, A; Speiser, DE; Assenmacher, M; Guillaume, P; Valmori, D; Lienard, D; Lejeune, F; Cerottini, JC; Romero, P. (2001). Ex vivo IFN-γ secretion by circulating CD8 T lymphocytes: Implications of a novel approach for T cell monitoring in infectious malignant diseases. J. Immunol.; 166: 7634–7640. [1037]