

# Human IFN- $\gamma$ /IL-5 FluoroSpot kit

Product Code: FS-0108-2

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## CONTENTS:

- ▶ Monoclonal antibody 1-D1K (300  $\mu$ l) green top  
Concentration: 1 mg/ml
- ▶ Monoclonal antibody TRFK5 (300  $\mu$ l) green top  
Concentration: 1 mg/ml
- ▶ Monoclonal antibody 7-B6-1-FS-FITC (120  $\mu$ l)
- ▶ Biotinylated monoclonal antibody 5A10 (50  $\mu$ l) yellow top  
Concentration: 1 mg/ml
- ▶ Monoclonal antibody anti-FITC-490 (120  $\mu$ l)
- ▶ SA-550 (120  $\mu$ l)
- ▶ Co-stimulator anti-CD28 mAb CD28-A (100  $\mu$ l)  
Concentration: 0.1 mg/ml
- ▶ Positive control anti-CD3 mAb CD3-2 (100  $\mu$ l)
- ▶ Fluorospot plates (2 IPFL plates)
- ▶ Fluorescence enhancer (25 ml)

1-D1K, TRFK5 and 5A10 are supplied in sterile filtered (0.2  $\mu$ m) PBS with 0.02% sodium azide. Anti-CD28 and anti-CD3 mAbs are supplied in sterile filtered (0.2  $\mu$ m) PBS. 7-B6-1-FS-FITC, SA-550, anti-FITC-490 and Fluorescence enhancer contain 0.002% Kathon CG.

## STORAGE:

Shipped at ambient temperature. On arrival all reagents should be stored refrigerated at 4-8°C.

# Guidelines for Human IFN- $\gamma$ /IL-5 FluoroSpot

The protocol describes double staining for the detection of human IFN- $\gamma$  and IL-5. The protocol may also be adjusted to single staining. IPFL plates are coated with a mixture of mAbs: 1-D1K for IFN- $\gamma$  and TRFK5 for IL-5. Cells +/- stimuli are added and secreted IFN- $\gamma$  and IL-5 will be captured by the specific mAbs. After cell removal, spots are detected in two steps. First, a mixture of 7-B6-1-FS-FITC (IFN- $\gamma$ ) and 5A10-biotin (IL-5) is added, then a mixture of anti-FITC-490 (IFN- $\gamma$ ) and SA-550 (IL-5).

## **A Preparation of plate (sterile conditions)**

1. In the same tube, dilute the coating antibodies 1-D1K to 15  $\mu\text{g/ml}$  and TRFK5 to 15  $\mu\text{g/ml}$  in sterile PBS, pH 7.4. Filter the antibody solution using a 0.2  $\mu\text{m}$ , low protein binding filter.
2. Pre-wet the plate membrane by treatment with 35% ethanol, 15  $\mu\text{l/well}$ , for maximum 1 minute.
3. Wash plate 5 times with sterile water, 200  $\mu\text{l/well}$ .
4. Add 100  $\mu\text{l/well}$  of the antibody solution and incubate overnight at 4-8°C.

## **B Incubation of cells in plate (sterile conditions)**

1. Remove excess antibody and wash plate 5 times with sterile PBS, 200  $\mu\text{l/well}$ .
2. Add 200  $\mu\text{l/well}$  of sterile medium containing 10% of the same serum as used for the cell suspensions. Incubate for at least 30 minutes at room temperature to block/condition the membrane.
3. Remove the medium and add the stimuli followed by the cell suspension. Alternatively, cells and stimuli can be mixed before addition to the plate. The mAb CD28-A can be included (at 0.1  $\mu\text{g/ml}$ ) to enhance antigen-specific stimulation. As a positive control for cytokine production, the mAb CD3-2 is recommended in a dilution of 1:1000.
4. Put the plate in a 37°C humidified incubator with 5% CO<sub>2</sub> and incubate 12-48 hrs. Do not move the plate during this time and take measures to avoid evaporation (e.g. by wrapping the plate in aluminium foil).

## **C Detection of spots**

1. Remove the cells by emptying the plate and wash 5 times with PBS, 200  $\mu\text{l/well}$ .
2. In the same tube, dilute the detection antibodies 7-B6-1-FS-FITC 1:200 and 5A10-biotin to 2  $\mu\text{g/ml}$  in PBS containing 0.1% bovine serum albumin (PBS-0.1% BSA). Filter the antibody solution using a 0.2  $\mu\text{m}$ , low protein binding filter. Add 100  $\mu\text{l/well}$  and incubate for 2 hours at room temperature.
3. Wash as above (step C1).
4. In the same tube, dilute the anti-FITC-490 1:200 and SA-550 1:200 in PBS-0.1% BSA. Filter the solution using a 0.2  $\mu\text{m}$ , low protein binding filter and add 100  $\mu\text{l/well}$ . Incubate for 1 hour at room temperature. From this step on, cover the plate to limit light exposure.
5. Wash as above (step C1).
6. Empty the plate and add 50  $\mu\text{l/well}$  of Fluorescence enhancer and leave the plate for 15 minutes at room temperature.
7. Empty the plate and remove residual Fluorescence enhancer by firmly tapping the plate against clean paper towels.
8. Remove the underdrain (the soft plastic under the plate). Leave the plate in the dark to dry; plate should be completely dry before analysis. Inspect and count spots in a FluoroSpot reader. Store plate in the dark at room temperature.

# Hints and comments

These suggestions are based on the detection of antigen-specific immune responses using PBMC. If using T-cell clones, mixtures of separated cell fractions etc., other protocols may have to be considered.

## Co-stimulation with anti-CD28

Anti-CD28 mAb provides a co-stimulatory signal to antigen-specific responses by binding to CD28 on T cells. Addition of anti-CD28 mAb to the cell culture can be used to enhance antigen-specific responses. Further optimization may be necessary, depending on which cells and stimuli are used. Too high concentration of anti-CD28 mAb may result in an elevation of non-specific cytokine secretion. The co-stimulatory effects of anti-CD28 mAb, as well as a possible impact on non-specific spots, can be assessed by comparing cells cultured with or without anti-CD28 mAb.

## Plates

The IPFL plates included in the kit have a low fluorescent PVDF-based membrane. To obtain maximal antibody binding capacity the plates need to first be activated by a brief treatment with ethanol. It is essential that the membrane is not allowed to dry after the treatment. If this occurs the treatment step (A2-3) needs to be repeated before adding the coating antibodies. The underdrain can be left on the plate all along, but then plates require a longer drying time before spots can be counted (step C8).

## Plate washing

Washing of plates can be done using a multi-channel micropipette. In washing steps not requiring sterile conditions (C1-C5), a regular ELISA plate washer can also be used, provided that the washing head is adapted to the ELISpot/FluoroSpot plates.

## Serum

The serum should be selected to support cell culture and give low background staining. We recommend the use of fetal calf serum. Alternatively serum-free medium evaluated for cell culture can be used.

## Cells

Both freshly prepared and cryopreserved cells may be used in the assay. However it is recommended that the latter are rested for at least one hour to allow removal of cell debris before addition to the plate. Triplicates or duplicates of 250,000 cells per well are often used to assess antigen-specific responses. For polyclonal activators, the cell number may have to be reduced to avoid confluent spot formation. Protocols with other incubation times have to be established by the user.

## Assay controls

The number of cells responding to antigen stimulation is often compared to the number of cells spontaneously secreting cytokine which is determined by incubating the same number of cells in the absence of stimuli. A polyclonal activator such as anti-CD3 mAb (included in the kit) or phytohemagglutinin (1-10 µg/ml) is often included as a control for cell viability and functionality of the assay.

## Buffers

PBS for washing and dilution should be filtered (0.2 µm) for optimal results. We do not recommend the inclusion of Tween or other detergents in the washing and incubation buffers.

## Detection reagents

To reduce unspecific background it is recommended to filter the working dilution of detection reagents. Use a 0.2 µm, low protein binding filter.

## Analysis

We recommend the use of an automated FluoroSpot reader equipped with filters for FITC (excitation 490 nm/emission 510 nm) and Cy3 (excitation 550 nm/emission 570 nm). Filters should have high specificity to avoid bleed-through artifacts. Spots identified with FITC filter represent IFN-γ producing cells and spots identified with Cy3 filter represent IL-5 producing cells. Double producing cells are preferentially identified by a computerised overlay of IL-5 and IFN-γ spots. Fluorescent spots may fade due to excessive exposure to light and it is recommended to analyse the plate within one week of development.

**NOTE; for research use only.**

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MABTECH AB  
Box 1233  
SE-131 28 Nacka Strand  
Sweden  
Tel: +46 8 716 27 00  
Fax: +46 8 716 27 01  
E-mail: [mabtech@mabtech.com](mailto:mabtech@mabtech.com)  
[www.mabtech.com](http://www.mabtech.com)

MABTECH Inc  
M.E.B. 220  
3814 West Street  
Cincinnati, OH 45227  
USA  
Tel: +1 513 871 4500  
Fax: +1 513 871 7353  
E-mail: [mabtech.usa@mabtech.com](mailto:mabtech.usa@mabtech.com)

MABTECH AB Büro Deutschland  
Germany  
Tel: +49 40 4135 7935  
Fax: +49 40 4135 7945  
E-mail: [mabtech.de@mabtech.com](mailto:mabtech.de@mabtech.com)

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MABTECH AUSTRALIA Pty Ltd  
resolvingIMAGES  
Unit 22, 196 Settlement Road  
Thomastown Victoria 3074  
Australia  
Tel: +61 3 9466 4007  
Fax: +61 3 9466 4003  
E-mail: [mabtech.au@mabtech.com](mailto:mabtech.au@mabtech.com)

MABTECH AB Bureau de liaison France  
BP 255, 1300 route des Crêtes  
06905 Sophia Antipolis  
France  
Tel: +33 (0)4 92 38 80 70  
Fax: +33 (0)4 92 38 80 71  
E-mail: [mabtech.fr@mabtech.com](mailto:mabtech.fr@mabtech.com)