FluoroSpot kit for Mouse IFN-γ/IL-2

Product Code: FS-4142-2

CONTENTS:

- Monoclonal antibody AN18 (300 µl) green top
  Concentration: 1 mg/ml
- Monoclonal antibody 1A12 (300 µl) red top
  Concentration: 1 mg/ml
- Monoclonal antibody R4-6A2-BAM (120 µl) yellow top
- Biotinylated monoclonal antibody 5H4 (50 µl) yellow top
  Concentration: 1 mg/ml
- Monoclonal antibody anti-BAM-Green (120 µl)
- Streptavidin-Red (120 µl)
- Monoclonal antibody to CD28 (100 µl)
  Concentration: 0.2 mg/ml
- Fluorospot plates (2 IPFL plates)
- Fluorescence enhancer-II (25 ml)

STORAGE:

Shipped at ambient temperature. On arrival all reagents should be stored refrigerated at 4-8°C. AN18, 1A12 and 5H4-biotin are supplied in sterile filtered (0.2 µm) PBS with 0.02% sodium azide. Anti-CD28 mAb is supplied in sterile filtered (0.2 µm) PBS. R4-6A2-BAM, Streptavidin-Red, anti-BAM-Green and Fluorescence enhancer-II contain 0.15% Kathon CG.

Guidelines for Mouse IFN-γ/IL-2 FluoroSpot

The protocol describes double staining for the detection of mouse IFN-γ and IL-2. The protocol may also be adjusted to single staining. IPFL plates are coated with a mixture of mAbs: AN18 for IFN-γ and 1A12 for IL-2. Cells +/- stimuli are added and secreted IFN-γ and IL-2 will be captured by the specific mAbs. After cell removal, spots are detected in two steps. First, a mixture of R4-6A2-BAM (IFN-γ) and 5H4-biotin (IL-2) is added then a mixture of anti-BAM-Green fluorophore (IFN-γ) and SA-Red fluorophore (IL-2).

A Preparation of plate (sterile conditions)

1. In the same tube, dilute the coating antibodies AN18 to 15 µg/ml and 1A12 to 15 µg/ml in sterile PBS, pH 7.4.
2. Pre-wet the plate membrane by treatment with 35% ethanol, 15 µl/well, for maximum 1 minute.
3. Wash plate 5 times with sterile water, 200 µl/well.
4. Add 100 µl/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 µl/well.
2. Add 200 µl/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 including anti-CD28 mAb followed by the cell suspension. Alternatively, cells and stimuli can be mixed before addition to the plate.
4. Put the plate in a 37°C humidified incubator with 5% CO_2 and incubate over night. 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 µl/well.
2. In the same tube, dilute the detection antibodies R4-6A2-BAM 1:200 and 5H4-biotin to 2 µg/ml in PBS containing 0.1% bovine serum albumin (PBS-0.1% BSA). Add 100 µl/well and incubate for 2 hours at room temperature.
3. Wash as above (step C1).
4. In the same tube, dilute the anti-BAM-Green 1:200 and SA-Red 1:200 in PBS-0.1% BSA and add 100 µl/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 µl/well of Fluorescence enhancer-II and incubate 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. 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 spleen cells. 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 producing cytokine which is determined by incubating the same number of cells in the absence of stimuli. A polyclonal activator such as concavalin A (1-10 μg/ml) is often included as a control for cell viability and functionality of the test system.

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 antibody
To reduce unspecific background it is recommended to filter (0.2 μm) the working dilution of detection mAb.

Analysis
Plates should be completely dry before analysis. Single colour analysis can be made in a microscope equipped with filters for FITC and Cy-3 but we recommend the use of an automated FluoroSpot reader with these filters. Green spots represent IFN-γ producing cells and red spots represent IL-2 producing cells. Double producing cells are preferentially identified by a computerized overlay of IL-2 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

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