Mouse IgG Subclass FluoroSpot kit

Product Code: FS-53515756-2

CONTENTS:

- Capture mAbs: anti-mouse Ig mAbs MT24/JC5-1 (1600 µl)
  Concentration: 0.5 mg/ml
- Detection mAbs
  anti-mouse IgG1-490 mAb MG1 (50 µl)
  anti-mouse IgG2b-380 mAb MG2b (50 µl)
  anti-mouse IgG2a+IgG2c-550 mAbs MG2a/MTG2c (50 µl)
  anti-mouse IgG3-640 mAb MG3 (50 µl)
- Polyclonal activator: R848 (100 µl)
  concentration: 1 mg/ml
- Lyophilized recombinant mouse IL-2 (0.5 µg)
- Fluorescence enhancer-II (25 ml)
  Fluorospot plates (2 IPFL plates)

STORAGE:

Shipped at ambient temperature. On arrival antibodies and Fluorescence enhancer-II should be stored refrigerated at 4-8°C. R848 and IL-2 should be stored at -20°C. Plates should be kept at room temperature.

Guidelines for Mouse IgG Subclass FluoroSpot

This kit contains reagents for the simultaneous detection of mouse IgG subclass (IgG1, IgG2a+IgG2c, IgG2b and IgG3) secreting B cells. The IgG2a allele is expressed in e.g. Balb/c and the IgG2c allele is expressed in e.g., C57BL/6, C57BL/10, SJL and NOD. B cells secreting antigen-specific antibodies as well as B cells secreting antibodies irrespective of antigen specificity can be measured with this kit. For analysis of memory B cells, cells may need to be pre-activated (see Hints and comments).

Antigen-specific IgG subclss FluoroSpot:
Antigen is coated on the FluoroSpot plate and antigen-specific IgG1, IgG2a or IgG2c, IgG2b and IgG3 secreted by B cells bind to the immobilized antigen. Spots are visualized after addition of fluorophore labeled subclass specific antibodies.
Total IgG subclass FluoroSpot
IgG1, IgG2a+IgG2c, IgG2b and IgG3 from secreting B cells are captured by anti-mouse Ig kappa and lambda chain specific mAbs MT24/JC5-1. Spots are visualized using the same detection reagents as for antigen-specific B cells.

A Preparation of plate (sterile conditions)

1. **Antigen-specific IgG subclass FluoroSpot:**
   Dilute the antigen to suitable concentration (e.g. 1-50 µg/ml) in sterile PBS, pH 7.4.
   Total IgG subclass FluoroSpot:
   Dilute capture mAbs MT24/JC5-1 to 40 µg/ml.
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 antigen or 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 cell suspension to the plate. Memory B cells may need pre-activation in tubes prior to this; please see Hints and comments section.
4. Put the plate in a 37°C humidified incubator with 5% CO_2 and incubate 16-24 hours. 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 1:500 (anti-mouse IgG2b-380, anti-mouse IgG1-490, anti-mouse IgG2a+IgG2c-550 and anti-mouse IgG3-640). Add 100 µl/well and incubate for 2 hours at room temperature. From this step on, cover the plate to limit light exposure.
3. Wash as above (step C1).
4. Empty the plate and add 50 µl/well of Fluorescence enhancer-II and incubate the plate for 15 minutes at room temperature.
5. Wash as above (step C1).
6. 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 B-cell clones, mixtures of separated cell fractions etc., other protocols may have to be considered.

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. Do not use mouse serum.

Cells

Both fresh and cryopreserved cells may be used with good results. 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. B cells that have been activated in vivo, for instance as a result of immunization, may be analysed directly in the FluoroSpot wells without prior stimulation. Memory B cells may require polyclonal stimulation before secreting detectable amounts of antibody. Immunoglobulin secretion can be induced by pre-activation of cells with R848 and IL-2. Stimulate cells with a mixture of R848 (1 μg/ml) and IL-2* (10 ng/ml) in tubes for 3-5 days. After pre-activation, wash the cells extensively to remove secreted antibodies. Triplicates with 50.000-250.000 cells/well are recommended but the cell number may need to be modified depending on the expected spot frequencies. Other incubation times have to be established by the user. * Reconstitute IL-2 with 1 ml PBS to obtain 0.5 μg/ml. Leave for 15 min and then vortex. Use directly or store in aliquots at -20°C.

Assay controls

The number of cells responding to stimulation is often compared to the number of cells spontaneously producing the different IgG subclasses, determined by incubating the same number of cells in the absence of stimuli. Polyclonal activators, such as R848 and IL-2, can be used as control stimuli for assessment of cell viability and functionality of the assay. See above.

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 LED380, LED490, LED550 and LED640. IgG2b spots are analyzed with LED380 filter, IgG1 spots with LED490, IgG2a and IgG2c with LED550 filter and IgG3 using a LED640 filter. Fluorescent spots may fade due to excessive exposure to light and it is recommended to analyze the plate within one week of development.

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