Human IgG/IgM FluoroSpot kit

Product Code: FS-05R17G-2

CONTENTS:

- Capture mAbs (MT91/145) 600 µl, yellow top
  concentration: 0.5 mg/ml
- Capture mAbs (MT11/12) 600 µl, red top
  concentration: 0.5 mg/ml
- Detection mAbs anti-human IgG-550 (MT78/145) 50 µl
- Detection mAb anti-human IgM-490 (MT22), 50 µl
- Polyclonal activator: R848, 100 µl
  concentration: 1 mg/ml
- Lyophilized recombinant human IL-2, 1 µg
- Fluorospot plates, 2 IPFL plates
- Fluorescence enhancer-II, 25 ml

Capture antibodies are supplied in sterile filtered (0.2 µm) PBS with 0.02% sodium azide. R848 is supplied in sterile filtered (0.2 µm) PBS with 2% DMSO. Fluorophore-conjugated detection antibodies and Fluorescence enhancer contain 0.002% Kathon CG.

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 Human IgG/IgM FluoroSpot

This kit contains reagents for the simultaneous detection of IgG and IgM secreting B cells. B cells secreting antigen-specific antibodies as well as B cells secreting antibodies irrespective of antigen specificity (total IgG/IgM) can be measured. For analysis of memory B cells, cells may need to be pre-activated (see Hints and comments).

Detection of antigen-specific B cells
Antigen is coated on the FluoroSpot plate and antigen-specific IgG and IgM secreted by B cells bind to the immobilized antigen. Spots are visualized after addition of fluorophore labeled anti-human IgG-550 and anti-human IgM-490.

Detection of total IgG/IgM-secreting B cells
IgG and IgM from secreting B cells are captured by mAbs MT91/145 (for IgG) and mAbs MT11/12 (for IgM). Spots are visualized using the same detection reagents as for antigen-specific B cells.

A Preparation of plate (sterile conditions)

1. **Antigen-specific IgG/IgM:**
   Dilute the antigen to suitable concentration (e.g. 1-50 µg/ml) in sterile PBS, pH 7.4.
   Total IgG/IgM:
   In the same tube, dilute each of the capture mAbs MT91/145 and MT11/12 to 15 µg/ml. Filter the antibody solution using a 0.2 µm, low protein binding filter.
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 capture 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 anti-human IgG-550 and anti-human IgM-490 1:500 in PBS containing 0.5% fetal calf serum. Filter the antibody solution using a 0.2 µm, low protein binding filter. 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. Empty the plate and remove residual Fluorescence enhancer by firmly tapping the plate against clean paper towels.
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 PBMC. 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.

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. Triplicates or duplicates of 100,000-500,000 cells per well are often used to assess antigen-specific responses. For detection of total IgG/IgM, less cells (e.g. 25,000-50,000 cells per well) may be used to avoid confluent spot formation.

B cells that have been activated in vivo, for instance as a result of vaccination, may be analysed directly in the FluoroSpot wells without prior stimulation. Typically, cells secreting antigen-specific antibodies can be detected in the circulation 6 to 9 days after vaccination.

Memory B cells may require polyclonal stimulation before secreting detectable amounts of antibody. Pre-stimulation can be made with a mixture of R848 (1 µg/ml) and rhIL-2 (10 ng/ml) (both included in the kit) in separate tubes/plates for 48-72 hours. After pre-stimulation, wash the cells to ensure removal of any secreted antibodies. Resuspend the cells in medium and add the cell suspension to the FluoroSpot plate.

Reconstitution of rhIL-2: Add 1 ml PBS to obtain 1 µg/ml. Leave at room temperature for 15 minutes and then vortex. Use immediately or store in aliquots at -20°C.

Assay controls

Determination of the number of cells secreting IgG and IgM independent of antigen specificity is recommended as a postive control for the assay. It is also valuable for establishing the proportion of antigen-specific B cells.

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 Cy3 (excitation 550 nm/ emission 570 nm) and FITC (excitation 490 nm/ emission 510 nm). Filters should have high specificity to avoid bleed-through artifacts. IgG spots are analyzed with Cy3 filter and IgM spots with FITC 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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