Human IFN-γ/Granzyme B FluoroSpot kit, precoated

FSP-0110-2
FSP-0110-10
INTRODUCTION

FluoroSpot – General description

This dual FluoroSpot kit enables convenient enumeration of cells secreting either one or both analytes.

FluoroSpot combines the sensitivity of ELISpot with the capacity to analyze secretion of several analytes simultaneously. This highly sensitive cellular assay is robust, easy to perform, and suitable both for testing single samples and for large-scale screening.

FluoroSpot assay principle. A sandwich assay principle is applied in FluoroSpot in which a mixture of monoclonal antibodies (mAbs) with different specificities is coated onto a PVDF membrane in a 96-well plate. FluoroSpot kits supplied with pre-coated plates feature plates that already have the capture antibodies bound to the membrane of the wells. In FluoroSpot, detection of cells secreting multiple analytes is made possible by utilizing biotin- and tag-labeled detection antibodies followed by secondary detection reagents coupled to different fluorophores. Spots from cells secreting multiple analytes are identified by coordinates, i.e. based on spot position.
CONTENTS AND STORAGE

Human IFN-γ/Granzyme B FluoroSpot kit, pre-coated

The kit is shipped at ambient temperature. All reagents should be stored at 4-8°C upon arrival. Plates should be stored at room temperature.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>2-plate kit</th>
<th>10-plate kit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detection antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-IFN-γ mAb 7-B6-1-BAM</td>
<td>120 μl</td>
<td>600 μl</td>
<td></td>
</tr>
<tr>
<td>anti-Granzyme B mAb GB11,</td>
<td>1 mg/ml</td>
<td>50 μl</td>
<td>250 μl</td>
</tr>
<tr>
<td>biotinylated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fluorophore conjugates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-BAM-490</td>
<td>120 μl</td>
<td>600 μl</td>
<td></td>
</tr>
<tr>
<td>SA-550</td>
<td>120 μl</td>
<td>600 μl</td>
<td></td>
</tr>
<tr>
<td><strong>Co-stimulator</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-CD28 mAb CD28A</td>
<td>0.1 mg/ml</td>
<td>100 μl</td>
<td>500 μl</td>
</tr>
<tr>
<td><strong>Stimulus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-CD3 mAb CD3-2</td>
<td>0.1 mg/ml</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td><strong>Fluorescence enhancer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ready-to-use Fluorescence</td>
<td>25 ml</td>
<td>60 ml</td>
<td></td>
</tr>
<tr>
<td>enhancer-II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pre-coated plates</strong></td>
<td>Plates pre-coated with:</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>mAbs 1-D1K and GB10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Anti-CD28 and anti-CD3 mAbs are supplied in sterile filtered (0.2 μm) PBS. The detection antibodies, SA-550, and Fluorescence enhancer contain 0.002 % Kathon CG. Vials have been overfilled to ensure recovery of stated volume.
ASSAY PROTOCOL

Please consult the table below for recommendations on dilutions and cell incubation time. For additional information, read the Tips For A Successful Assay section. The reagents should not be diluted in advance.

<table>
<thead>
<tr>
<th>Component</th>
<th>Dilution</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-IFN-γ mAb 7-B6-1-BAM</td>
<td>1:200</td>
<td>C2</td>
</tr>
<tr>
<td>anti-Granzyme B mAb GB11, biotinylated</td>
<td>1:500</td>
<td>C2</td>
</tr>
<tr>
<td>Fluorophore conjugates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-BAM-490</td>
<td>1:200</td>
<td>C4</td>
</tr>
<tr>
<td>SA-550</td>
<td>1:200</td>
<td>C4</td>
</tr>
<tr>
<td>Co-stimulator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-CD28 mAb CD28A</td>
<td>1:1000</td>
<td>B1</td>
</tr>
<tr>
<td>Stimulus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-CD3 mAb CD3-2</td>
<td>1:1000</td>
<td>B1</td>
</tr>
<tr>
<td>Cell incubation time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24–72 hours</td>
<td></td>
<td>B2</td>
</tr>
</tbody>
</table>

A. Plate preparation (sterile conditions)

1. Remove the plate from the sealed package and wash the plate three times with sterile PBS (200 µl/well).

2. Block/condition the plate by adding cell incubation medium containing 10% fetal calf serum (200 µl/well). Incubate for at least 30 minutes at room temperature.

B. Cell incubation (sterile conditions)

1. Remove the medium and add the stimuli followed by the cell suspension. Cells and stimuli can also be mixed before addition to the plate.

2. Incubate the cells in the plate at 37°C in a humidified incubator with 5% CO₂. Approximate incubation time is indicated above and can be further optimized by the user. Do not move the plate during incubation and avoid evaporation, for example by wrapping the plate in aluminum foil.
C. Detection

1. Remove the cells by emptying and washing the plate five times with PBS (200 µl/well).

2. Dilute all the detection antibodies, in the same tube, in PBS containing 0.1% BSA (PBS-0.1% BSA) to the dilutions described above. Add 100 µl/well and incubate for 2 hours at room temperature.

3. Wash the plate five times with PBS (200 µl/well).

4. Dilute the fluorophore-conjugated reagents, in the same tube, in PBS-0.1% BSA to the concentrations shown above. Add 100 µl/well and incubate for 1 hour at room temperature.

5. Wash the plate five times with PBS (200 µl/well).

6. Empty the plate, add Fluorescence enhancer (50 µl/well) and leave for 15 minutes at room temperature.

7. Empty the plate and remove any residual Fluorescence enhancer by firmly tapping the plate against clean paper towels.

8. Remove the underdrain (the soft plastic drain under the plate), for example using pliers. Dry the plate protected from light. The plate should be completely dry before analysis. Store plate in the dark at room temperature.

9. Spot analysis is performed with an automated FluoroSpot reader equipped with filters for the fluorophores used. Filters should have high specificity to avoid bleed-through artifacts.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Required filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>Excitation 490 nm/emission 510 nm (FITC)</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>Excitation 550 nm/emission 570 nm (Cy3)</td>
</tr>
</tbody>
</table>
**TIPS FOR A SUCCESSFUL ASSAY**

**Buffers**
PBS for washing and dilution should be filtered (0.2 μm) to remove any particles. We do not recommend the inclusion of Tween or other detergents in the washing and incubation buffers.

**Washing**
Plates can be washed using a multi-channel micropipette. A regular plate washer can be used in washing steps when sterile conditions are not required, provided that the washing head is adapted to ELISpot/FluoroSpot plates.

**Serum**
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 FluoroSpot. We recommend that cryopreserved cells are rested for at least 1 hour after thawing to allow removal of cell debris before addition to the plate. Triplicates or duplicates of 250,000 cells/well are usually recommended to assess antigen-specific responses. For polyclonal activators, the cell number should be reduced in order to avoid confluent spot formation.

**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.

**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 an anti-CD28 mAb together with antigen (step B1) can be used to enhance antigen-specific responses. However, if the concentration of anti-CD28 mAb is too high, non-specific cytokine secretion may be elevated. Anti-CD28 can be used to circumvent capture effects, which can occur when different capture antibodies are coated in the same well, where absorption of one cytokine may negatively affect the secretion of another cytokine. See www.mabtech.com for more information about capture effects.

**Analysis**
An automated FluoroSpot reader with separate filters is required for analysis. The reader should be equipped with filters for the fluorophores used. Filters should be selective for the specific wavelengths to avoid bleed-through artifacts. Spots from cells secreting multiple analytes are identified by coordinates, i.e. based on spot position. In order to obtain accurate measurements of cells secreting multiple analytes, the automated reader must be able to create an overlay analysis from the filters. To avoid fading, the plates should be kept dark and should preferably be analyzed within 1 week of development.
**PROTOCOL SUMMARY**

**Start**
- Block/condition plate for 30 min.
- Add stimuli and cells
  - Incubate at 37°C
- Remove cells
  - Wash x5

**Cell incubation**

**Detection**
- Add detection antibody mix
  - Incubate 2h at RT
- Add conjugate mix
  - Incubate 1h at RT
- Add Fluorescence enhancer
  - Incubate 15 min. at RT
- Empty plate
- Remove underdrain
  - and dry plate

**Analysis**

**Sterile conditions**
ABOUT MABTECH

Mabtech AB is a privately owned Swedish biotech company founded in 1986. We develop, manufacture, and market high quality monoclonal antibodies and kits suitable for ELISA, ELISpot, and FluoroSpot. For many years Mabtech has been a world leader in the field of ELISpot as a result of our strong research focus and continued effort to optimize this technique. Close international collaborations with both academia and industry are leading the way for future developments that help the research community achieve optimal results.

Developed and manufactured by MABTECH AB, Sweden, whose quality management system complies with the following standards:

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