# ELISpot Path: CMV Human IFN-γ

## Product Code: 3420-2AST-P2-1

**CONTENTS:**

**1 pre-coated strip plate (mAb 1-D1K) and empty plate frame.**

**7-B6-1-ALP conjugate (150 μl)**

**BCIP/NBT-plus substrate (25 ml)**

**CMV peptide pool for human CD4 and CD8 T cells (1 vial)**

**Positive control anti-CD3 mAb CD3-2 (100 μl)**

**STORAGE:**
Shipped at ambient temperature. On arrival all reagents should be stored refrigerated at 4-8 °C except the peptide pools that should be stored frozen at -20°C or below. Plates may be kept at room temperature. The expiry date indicates how long unopened products, stored according to instructions, are recommended for use. Conjugate is supplied in 0.1 M Tris buffer with 0.002% Kathon CG. Anti-CD3 mAb is supplied in sterile filtered (0.2 μm) PBS. Vials have been overfilled to ensure recovery of stated quantity.

# Guidelines for ELISpot Path: CMV Human IFN-γ
**Please read through before starting the assay**

## A Preparation and blocking of plate

1. Assemble the required number of strips in the extra plate frame and wash 4 times with sterile PBS (200μl/well). Seal the bag with the remaining strips and store at room temperature.
2. Condition the plate with medium containing 10% of the same serum as used for the cell suspensions (200 μl/well). Incubate for at least 30 minutes at room temperature.

## B Incubation of cells in plate

1. Dissolve the peptide pool by addition of 40 μl DMSO, then add 85 μl PBS. The concentration of the stock solution is 200 μg/ml of each peptide. Aliquot and store at -20°C or below. Empty the plate and add 50 μl/well of peptides or controls, followed by 50 μl cell suspension/well. Alternatively, mix cells and stimuli before addition of 100 μl/well. The final peptide concentration should be 2 μg/ml of each peptide. Anti-CD3 is recommended as a positive control at a dilution of 1:1000.
2. Put the plate in a 37°C humidified incubator with 5% $CO_2$ and incubate for 12-48 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. Dilute the 7-B6-ALP 1:200 in filtered PBS containing 0.5% fetal calf serum. Add 100 μl to each well and incubate for 2 hours at room temperature.
3. Wash the plate 5 times with PBS, 200 μl/well.
4. Filter the ready-to-use substrate solution (BCIP/NBT-plus) through a 0.45 μm filter and add 100 μl of substrate per well. Develop until distinct spots emerge. Stop the colour development by washing extensively in tap water. Remove the plate frame from the plastic tray and rinse the underside of the membrane.
5. Leave the plate to dry. Inspect and count spots in an ELISpot reader or in a dissection microscope.
6. 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.

**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 plates.

**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.

**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. Human serum is not recommended as it may contain heterophilic antibodies or intrinsic analyte which may interfere with the assay.

**Conjugate**
To reduce unspecific background it is recommended to filter the detection mAb-enzyme conjugate (0.2 µm).

**Assay controls**
The number of cells responding to stimulation is often compared to the number of cells spontaneously producing the cytokine, which is determined by incubating the same number of cells in the absence of stimuli. A polyclonal activator such as the included anti-CD3 mAb, CD3-2 or phytohemagglutinin (1-10 µg/ml) is often used 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. Avoid the inclusion of Tween or other detergents in the washing and incubation buffers.

**Substrate development**
Develop until distinct spots are visible in positive wells (usually 10-30 minutes). A general darkening of the membrane may occur but disappears after drying.

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