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IFNG
Mouse Interferon gamma ELISPOT Kit

Catalog No: CKM038S **Size:** 5 x 96 plates

Intended use

The cytokine ELISPOT (Enzyme-Linked ImmunoSPOT) assay is designed to enumerate cytokine-secreting cells in single cell suspensions of lymphoid tissue, central nerve system (CNS) tissue, bone marrow or preparations of peripheral blood mononuclear cells (PBMCs). The assay has the advantage of detecting only activated/memory T-cells and has the ability to detect cytokine release in response to antigen by a single cell thereby permitting direct calculation of responder T-cell frequencies. The high sensitivity and easy performance, allowing the determination of peptide-reactive T-cells without prior *in vitro* expansion, makes the ELISPOT assay eminently well suited to monitor T-cell responses. The higher sensitivity of ELISPOT in comparison to that of ELISA or intracellular staining is due to the plate-bound antibodies directly capturing the cytokine released by the cell before it is diluted in the supernatant, trapped by high-affinity receptors or degraded by proteases. The sensitivity of the assay lends itself to measurement of very low frequencies of cytokine-secreting cells (1/300,000).

Brief Description of the ELISPOT assay

Cells are incubated in the wells of the ELISPOT plate precoated with a high-affinity monoclonal antibody to which the cytokine, produced during incubation, will bind. Subsequently, cells are washed away. Areas in which the cytokines have been bound are detected with a combination of biotinylated anti-cytokine detection antibodies and Streptavidin horseradish peroxidase (Streptavidin-HRP). The last step in the assay is the addition of AEC (3-amino-9-ethylcarbazole) yielding a colored zone ('spot'). This zone reveals the site of cytokine secretion.

Reagents and materials supplied with the kit:

Items	Quantity (5 plates)	Storage conditions
Coating antibodies	1 vial	2-4°C
Biotinylated detection antibodies	1 vial	2-4°C
Streptavidin-HRP	1 vial	≤-20°C
AEC coloring system:		
I. AEC stock solution	1 vial (4 ml)	≤-20°C
II. Substrate buffer capsules	5 capsules	room temperature
Blocking stock solution R (10x)	1 vial (10 ml)	2-4°C
Dilution buffer R (10x)	1 vial (10 ml)	2-4°C
Tween-20	1 vial (5 ml)	room temperature
PVDF plates	5	room temperature

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Hazard Information:

AEC (3-Amino-9-ethylcarbazole) stock solution is classified as harmful according to Directive 1999/45/EC and its amendments.
Risk phrases: R22/R36 Safety phrases: S26/S39/S45
The AEC stock solution should be handled only in a chemical fume hood. In case of contact with skin, wash with soap and water and remove contaminated clothing and shoes. Upon ingestion or contact with eyes, rinse mouth (if person is conscious) or eyes with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids. Seek medical advice immediately.
Other kit components are not classified as dangerous according to Directive 67/548/EC or 1999/45/EC and their amendments.

Storage of Kit Reagents

- The vials with lyophilized coating antibodies, and biotinylated detection antibodies can be safely stored at 2-4°C until the expiration date (indicated on the vials). After reconstitution, the reagents are stable for at least 6 months at 2-4°C when kept sterile. However, it is strongly recommended to divide the reconstituted antibody preparations into small aliquots for single use. These aliquots should be stored at ≤-20°C. Under these conditions, the reagents are stable for at least one year.
- The vials with lyophilized Streptavidin-HRP conjugate should be stored at ≤-20°C. After reconstitution, the reagents are stable for at least 2 months at 2-4°C when kept sterile. However, it is strongly recommended to divide the reconstituted antibody preparations into small aliquots for single use. These aliquots should be stored at ≤-20°C. Under these conditions, the conjugate is stable for at least one year. The reconstituted Streptavidin-HRP rapidly loses activity when kept at room temperature.
- The AEC stock solution should be protected from light and is stable for at least 1 year at ≤-20°C. It is recommended to divide the solution into small aliquots in polypropylene vials for single use. These aliquots should be stored at ≤ -20°C protected from light.
- The Substrate buffer capsules are stable for an indefinite length of time when stored in a moisture-free environment at room temperature.
- Blocking stock solution R (10x) and Dilution buffer R (10x) should be stored at 2-4°C until the expiration date (indicated on the vials). After opening, these solutions are stable for at least 6 months when kept sterile.
- Tween-20 can best be stored at room temperature until the expiration date (indicated on the vials).

Materials/reagents required but not provided:

- Sterile distilled water
- 70% ethanol
- 30% ethanol
- Phosphate-buffered saline (PBS): home-made, filter-sterilize or autoclave. For washing purposes only.
- Wash buffer: PBS containing 0.05% Tween-20.

- Sterile and pyrogen free PBS (PBS-I): Invitrogen Cat. No. 10010-015 is recommended.
- Culture medium: see Addendum**.
- Cell stimuli: see Addendum**.
- Pipetting devices
- Squirt (wash or squeeze) bottle with wide spout for washing, see Addendum**.
- CO2-incubator (37°C, 100% humidity, 5% CO2)
- Tissue culture plates for prestimulation (optional)
- A dissecting microscope or an immunospot image analyzer for spot counting

** Addendum to this ELISPOT manual contains guidelines and troubleshooting.

Preparation of Kit Reagents

Prepare reagents under aseptic conditions (e.g. Laminar Flow Hood).

1. Coating antibodies: Reconstitute the lyophilized contents of the vial by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently and allow it to stand for 2 minutes at room temperature. For one ELISPOT plate, thoroughly mix 50 µl with 5 ml PBS-I.
2. Blocking buffer R (1x): Dilute Blocking stock solution R (10x) in PBS-I. For one ELISPOT plate, thoroughly mix 2 ml with 18 ml PBS-I.
3. Dilution buffer R (1x): Dilute Dilution buffer R (10x) in PBS-I. For one ELISPOT plate, thoroughly mix 2 ml with 18 ml PBS-I.
4. Biotinylated detection antibodies: Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently and allow it to stand for 2 minutes at room temperature. For one ELISPOT plate, thoroughly mix 100 µl with 10 ml Dilution buffer R (1x).
5. Streptavidin-HRP conjugate: Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently and allow it to stand for 2 minutes at room temperature. For one ELISPOT plate, thoroughly mix 100 µl with 10 ml Dilution buffer R (1x).
6. AEC coloring system: The AEC coloring system consists of two items: a concentrated AEC stock solution and a substrate buffer capsule. For preparing the AEC substrate solution, dissolve the contents of one substrate buffer capsule in 57 ml water. After complete dissolution, 43 ml 70% ethanol is added to reach a final concentration of 30% ethanol. 10 ml of this solution is thoroughly mixed with 330 µl AEC stock solution (toxic, use a fume hood). After mixing, the solution should be clear. This amount is sufficient for one ELISPOT plate and should be used within 30 minutes after preparation.

ELISPOT method

Be sure to read the 'Preparation of Kit Reagents' and 'Directions for washing' before carrying out the assay.

Use ELISPOT plates and reagents under aseptic conditions (e.g. Laminar Flow Hood) for steps 1 to 6.

1. Prewet the PVDF membranes by adding 25 µl of 70% ethanol to each well. Incubate for 1 minute at room temperature.
2. Aspirate or firmly shake-out the ethanol, then immediately rinse wells 2x with PBS-I. Empty the plate and tap on tissue paper.
3. Add 50 µl of properly diluted coating antibodies into each well. Cover the plate with a lid and incubate overnight at 2-4°C.
4. Decant solution from wells. Wash 3x with 200 µl PBS-I/well. Add 200 µl Blocking buffer R (1x) to each well. Cover the plate with a lid and incubate for 1 hour at 37°C.
5. Decant solution from wells (do not wash the wells). Dilute the cells in Culture medium containing an appropriate stimulus (polyclonal stimulus or antigen). Place cells in the wells of the ELISPOT plate. Add 100 µl/well. Triplicates of $3x10^6$ cells/ml are often used to assess antigen-specific responses. For polyclonal stimuli, the cell number may have to be reduced by $\pm10^4$ cells/ml. No more than $3x10^5$ cells/well should be suspended in the ELISPOT plate. See Addendum.
6. Cover ELISPOT plate with lid and incubate at 37°C, 5% CO_2, and 100% humidity. The incubation time can vary from 5 to 24 hours. Specific activation conditions will vary, depending on cell type, cytokine of interest, kinetics of cytokine release and whether a preincubation step was included in the procedure. See Addendum.
7. Remove the bulk of cells with a firm shake-out action and wash 2x with room temperature PBS (200 µl/well). Wash the wells 5x with 250 µl Wash buffer/well (see Addendum).
8. Discard wash buffer and add 100 µl of properly diluted biotinylated detection antibodies to each well. Seal the plate with an adhesive cover slip and incubate 1 hour at 37°C or overnight at 2-4°C.
9. Decant solution from wells. Remove the underdrain from the bottom of the plate and wash both sides of the PVDF membrane 5x with 250 µl Wash buffer/well. Place 100 µl of properly diluted Streptavidin-HRP solution into each well. Seal the plate with an adhesive cover slip and incubate 1 hour at 37°C.
10. Decant solution from wells. Wash both sides of the PVDF membrane 5x with 250 µl Wash buffer/well.
11. Add 100 µl AEC substrate solution to each well. Cover plate with lid and incubate for 25 minutes at room temperature in the dark.
12. Stop color development by thoroughly rinsing both sides of the PVDF membrane with demineralized water.

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13. Air dry the plate at room temperature and count spots by use of a dissecting microscope or an immunospot image analyzer. To prevent bleaching of spots store the plate at a dry place in the dark.

Typical Data

IFN-gamma specific spots produced by 2.5 x 10^4 mouse (C57bl/6j) splenocytes. Stimulation: PMA/ionomycin

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Addendum to Cell Sciences ELISPOT manual

Guidelines and Troubleshooting

Assay Performance:
There are two major approaches: either the assay is performed *directly* in the ELISPOT well or *indirectly* by introducing a preincubation step in the procedure. The rationale of preincubation is that exogenous proteins must be internalized, processed and presented by antigen presenting cells (APC) via MHC class I/II molecules to $CD8^+$/$CD4^+$ T-cells. Using Tetanus toxoid and PPD as model antigens it has been shown that a preincubation step in a tube prior to analyzing the cells in the ELISPOT assay is required for optimal antigen presentation.

Isolation and handling of human and non-human primate blood cells:
Venous or arterial blood should be collected from humans or non-human primates, fasted for at least 6 hours, using heparin as anti-coagulant. After being drawn, blood should be kept at room temperature for no longer than 16 hours. Peripheral blood mononuclear cells (PBMCs) are isolated from venous blood by density gradient centrifugation and washed twice in medium (RPMI 1640 + L-glutamin + antibiotics). This involves two centrifugation/resuspension steps (8 min, 200 x g, room temperature).
It is recommended that specimen collection from humans and non-human primates is carried out in accordance with NCCLS document M29-T2. No known test method can offer complete assurance that human- or non-human primate-derived blood or tissue samples will not transmit infection. Therefore, all human and non-human primate specimens should be considered potentially infectious.

Directions for cell culture of human or non-human primate PBMCs
Optimal conditions for the generation of cells releasing cytokines or other effector molecules in heterogeneous cell populations should be determined empirically. However it should be realized that different cell types, producing the same effector molecules, require different conditions for stimulation. For instance, the optimal conditions for the detection of IFN-γ secreting $CD8^+$ T-cells in PBMC preparations differ considerably from those for the detection of IFN-γ secreting $CD4^+$ T-cells. Moreover, the production of cytokines, such as TNF-α, IL6 and IL10, is not restricted to T-cells and a high frequency of these cytokine secreting cells can also be attributable to activated monocytes/macrophages. Adherence of these cells to the surface of an ELISPOT well may already be sufficient to trigger IL6 and TNF-α release.

When following the *direct* ELISPOT procedure, it should be realized that cell contact is critical for an optimal immune response. For that reason, 1-3x10^6 PBMCs/ml should be brought into the well (100 µl/well). Polyclonal stimulation of this high number of PBMCs usually leads to more than 500 spot forming cells (SFC)/well interfering with the formation of individual spots. Therefore for polyclonal stimuli, the cell number in the ELISPOT plate should be reduced to less than 5x10^5 cells/ml (100 µl/well). On the other hand, antigen-specific stimulation generally yields less than 100 SFC/10^5 PBMCs making this direct approach highly suited for antigen-specific responses. However, it should be realized that a preincubation step has been recommended for optimal antigen presentation by APC.

In the *direct* procedure, cells are suspended in culture medium with an appropriate stimulus and brought into the well of the ELISPOT plate and incubated for 16 to 24 hours to allow spot formation. For optimal ELISPOT responses with human PBMCs, serum-free medium (AIM-V) has proven to be the best choice. However, RPMI 1640 medium supplemented with 2 mM L-glutamine, antibiotics and 10% FCS (fetal calf serum) is a good medium for various other cell types. No more than 3x10^5 cells/well should be suspended in the ELISPOT plate.

When following the *indirect* procedure, consistent results are obtained if the cells are preincubated in culture medium with an appropriate stimulus for 16 to 42 hours at 4x10^6 cells/ml in a tissue culture plate (100% humidity, 37°C and 5-7% CO_2). It is important that during preincubation cell density is high. Therefore a minimum of 1 ml medium containing 4x10^6 cells is brought in a well of a 24-well plate, 0.5 ml in a well of a 48-well plate or 100 µl in a well of a 96-well plate.
After preincubation, the non-adherent cells are collected and washed twice with culture medium to avoid the carryover of cytokines produced during the preincubation step. This involves two centrifugation/resuspension steps (8 min, 200 x g, room temperature). Then the cells are suspended in culture medium with an appropriate stimulus at 1-3x10^6 cells/ml

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(antigen-specific responses). 100 µl of this cell suspension is transferred to the well of the ELISPOT plate and incubated for 5 to 24 hours to allow spot formation. For polyclonal stimuli, the cell number needs to be reduced to ≤ $5x10^5$ cells/ml. As culture medium RPMI 1640 medium supplemented with 2 mM L-glutamine, antibiotics and 10% FCS is recommended.

Directions for the isolation and cell culture of rodent cells

For rodent cells, the same culturing conditions can be applied as described for human and non-human primate cells. However, most data so far were obtained with rodent spleen cells and only the collection, preparation and culture conditions of mouse or rat spleen cells are described in this section.

Spleens are aseptically removed from rodents and collected in RPMI 1640 medium. A single cell suspension is prepared by gently teasing the spleen tissue through a sterile stainless steel or nylon screens into RPMI 1640 medium and then washed twice with RPMI 1640 medium. This involves two centrifugation/resuspension steps (8 min, 200 x g, room temperature). Cells are cultured in culture medium (RPMI 1640 medium + 2 mM L-glutamine + antibiotics + 10% FCS) supplemented with an appropriate stimulus for triggering cytokine production. Consistent results are obtained if the splenocytes are prestimulated for 24 hours at $2x10^6$ cells in a volume of 0.5 ml in the wells of a 48-well tissue culture plate in a humidified atmosphere at 37°C with 5-7% CO_2. Subsequently, the non-adherent cells are collected by two gentle washing steps using prewarmed (37°C) culture medium. Cells are centrifuged (200 x g) for 5 minutes at room temperature and resuspended in 500 µl culture medium with the same supplements as present during stimulation (including antigen/mitogen). Thereafter varying concentrations of cells (starting at $3x10^6$ cells/ml in triplicate with 1:3 serial dilutions down to ± 10^3 cells/ml, final volume: 100 µl/well) are transferred to the ELISPOT plate for a further incubation of 5-16 hours to allow spot formation.

As with primate PBMCs, the *direct* procedure has shown to be less effective than the *indirect* one, although only spleen cells have been evaluated in both procedures.

Stimuli and their concentrations

For antigen-specific stimulation, the optimal antigen concentration should be determined experimentally but generally varies between 1 and 10 µg/ml of protein or peptide. As antigen-specific positive control in the human IFN-γ, IL2, IL4, IL5, IL10, IL13 and granzyme B ELISPOT assay the ICE peptide pool (1 µg of each peptide/ml) can be used. This pool consists of 23 peptides of Influenza A virus (flu), Cytomegalovirus (CMV) and Epstein Barr virus (EBV) epitopes which are recognized by $CD8^+$ T-cells and presented by 11 class I HLA-A and HLA-B alleles prevalent among Caucasian individuals. As polyclonal stimuli for human and non-human primate PBMCs, concanavalin A (conA; 6-10 µg/ml), a combination of PMA (50 ng/ml) plus ionomycin (1 µg/ml), PHA (10 µg/ml) or anti-CD3/CD28 antibodies can be used. Whereas the first three stimuli can be used in all the different cytokine ELISPOT assays, anti-CD3/CD28 is effective only for the IFN-γ, IL4, IL10 and granzyme B ELISPOT assays. For rodent splenocytes, conA (4 µg/ml) or a combination of PMA (50 ng/ml) plus ionomycin (1 µg/ml) have shown to be effective polyclonal stimuli.

Directions for washing of polystyrene-bottomed plates

- All washing must be performed with Wash buffer (PBS containing 0.05% Tween-20).
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into each well. Take care not to scratch the bottom of the well. After aspiration, fill the wells with at least 250 µl of wash buffer and then aspirate the liquid. After washing, empty the wells of the plate by a firm shake-out action.
- Alternatively, the wash buffer may be put into a squirt bottle (use a squirt bottle with a wide spout). If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After washing, empty the wells of the plate by a firm shake-out action.
- If using an automated washing device, the operating instructions should be carefully followed.

Directions for washing of PVDF membrane-bottomed plates

- All washing must be performed with Wash buffer (PBS containing 0.05% Tween-20).
- For effective washing of PVDF membranes, a squirt bottle with a wide spout produces the best results. The bottle should be used to thoroughly flush all wells of the plate with Wash buffer. While flushing, completely fill the wells with Wash buffer and then remove the Wash buffer by a firm 'shake-out' action. After washing, empty the plate by tapping both sides on absorbent tissue.

- Additional washing of the underside of the PVDF membrane is needed after the incubation steps with detection antibody and conjugates to further reduce background staining. To do so, remove the plastic underdrain of the plate and use the squirt bottle to flood the underside of the membrane with Wash buffer. After washing, remove the Wash buffer by a gentle 'shake-out' action.

Recommended reagents

- Ficoll-Paque: GE Healthcare Cat. No. 17-1440-02 (for isolation of PBMCs by density gradient centrifugation)
- RPMI 1640 medium: Invitrogen Cat. No. 52400-025
- L-glutamine: Invitrogen Cat. No. 35030-024
- Penicillin/Streptomycin: Invitrogen Cat. No. 15140-122
- AIM-V medium: Invitrogen Cat. No. 31035-025
- ICE peptide pool: Cell Sciences Cat. No. CK113
- Concanavalin A (ConA): GE Healthcare Cat. No. 17-0450-01
- Phorbol 12-myrisate 13-acetate (PMA): Sigma Aldrich Cat. No. P8139
- Ionomycin: Sigma Aldrich Cat. No. I0634
- Phytohemagglutinin (PHA): Sigma Aldrich Cat. No. L8902

Troubleshooting

No or low frequency of spots

- Polyclonal stimulation of cells sometimes leads to a low frequency of spot forming cells as a consequence of apoptotic/necrotic cell death. If the culture medium turns yellow during stimulation, cell death is likely to occur. No such phenomenon occurs with antigenically stimulated cells.
- Clumping of cells during preincubation (particularly prominent with a polyclonal stimulus) may lead to underestimation of spot forming cells and inconsistent results. It is therefore critical that before the cells are transferred to the ELISPOT plate, they are thoroughly resuspended to obtain a single cell suspension (indirect procedure only).
- PBMCs isolated from blood kept for more than 16 hours at room temperature may produce a low frequency of spot forming cells.

Weakly stained spots

- The filler in the PBS tablets interferes with the coating process and should not be used as diluent for the coating antibody.
- The AEC stock solution can lose activity when it is exposed to light or prolonged storage at temperatures ≥ 0°C (enzymatic staining only).
- For optimal coloring, the AEC substrate solution can be best applied to the wells at temperatures of 25-30°C (enzymatic staining only).
- The Activator I and II solutions can lose activity when they are exposed to air and/or light, are not properly stored or have been cross-contaminated (silver-staining only).

Artifactual spots and/or high background staining

- Just prior to spot counting, it is important to clean the underside of the polystyrene-bottomed wells with 70% ethanol and to remove dust particles by blowing 4-5 bar compressed air into the wells (dust particles are a source of artifactual spots).
- The reconstituted antibody solutions should not be used if there is an indication of bacterial growth or if the solutions have become turbid.
- Bacterial or fungal infections in PBMC preparations or culture medium can produce spot like structures in the well.
- Inadequate post-coating of the ELISPOT well or insufficient washing between the different incubation steps may be the cause of artifactual spots or high background staining.
- When the indirect ELISPOT procedure step is followed, wash the cells thoroughly before they are transferred to the ELISPOT plate to avoid the carryover of cytokines released in the preincubation medium.
- Complete drying of the PVDF membranes (overnight at room temperature and in the dark) after the completion of the assay, is important for obtaining optimal spot intensity and low background staining.

- PBMCs from HTLV-1-infected humans and STLV-infected monkeys may contain a high frequency of spontaneously secreting IFN-γ producing cells.

Other

- Do not stack the plates during incubation.
- Do not puncture the PVDF membrane by pipetting/washing procedures. The membrane is fragile and may easily be damaged.
- To identify the optimal cell concentration for spot formation, include a wide range of cell concentrations in the first experiment.
- Spots may become irregular and ambiguous when the ELISPOT plate is moved during incubation. Even minor vibrations caused by closing the door of the incubator can affect spot formation.
- Granulocytes have a negative impact on spot formation.
- During incubation with blocking solution, membrane-leakage occasionally occur. This phenomenon, however, does not negatively affect assay results (PVDF membrane-bottomed plates only).

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