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Human IL-21 ELISPOT Kit with Sterile PVDF Plates

Catalog No: CKR060A
CKR060S

Size: 2-plate format
5-plate format

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.

Contents of the kit:

Catalog #	Item	Storage Temp.	Quantity (2-plate format)	Quantity (5-plate format)
CKR060-A	Coating antibody (lyophilized)	4°C	1 vial	1 vial
CKR060-B	Biotinylated detection antibody (lyophilized)	4°C	1 vial	1 vial
CKR060-C	Streptavidin-HRP conjugate (lyophilized)	≤-20°C	1 vial	1 vial
	AEC coloring system:			
CKR060-D	I. AEC stock solution	≤-20°C (in dark)	1 vial (4 ml)	1 vial (4 ml)
CKR060-E	II. Substrate buffer capsule	RT	1 vial (2.5 ml)	1 vial (5 ml)
CKR060-F	Blocking stock solution (10x)	4°C	1 vial (4 ml)	1 vial (10 ml)
CKR060-G	Dilution buffer R (10x)	4°C	1 vial (4 ml)	1 vial (10 ml)
CKR060-H	Tween-20	RT	1 vial (5 ml)	1 vial (5 ml)
CKR060-P	Sterile 96-well PVDF plate (Millipore MSIPS4510)	RT	2	5
CKR060-S	Adhesive cover slip	RT	5	10

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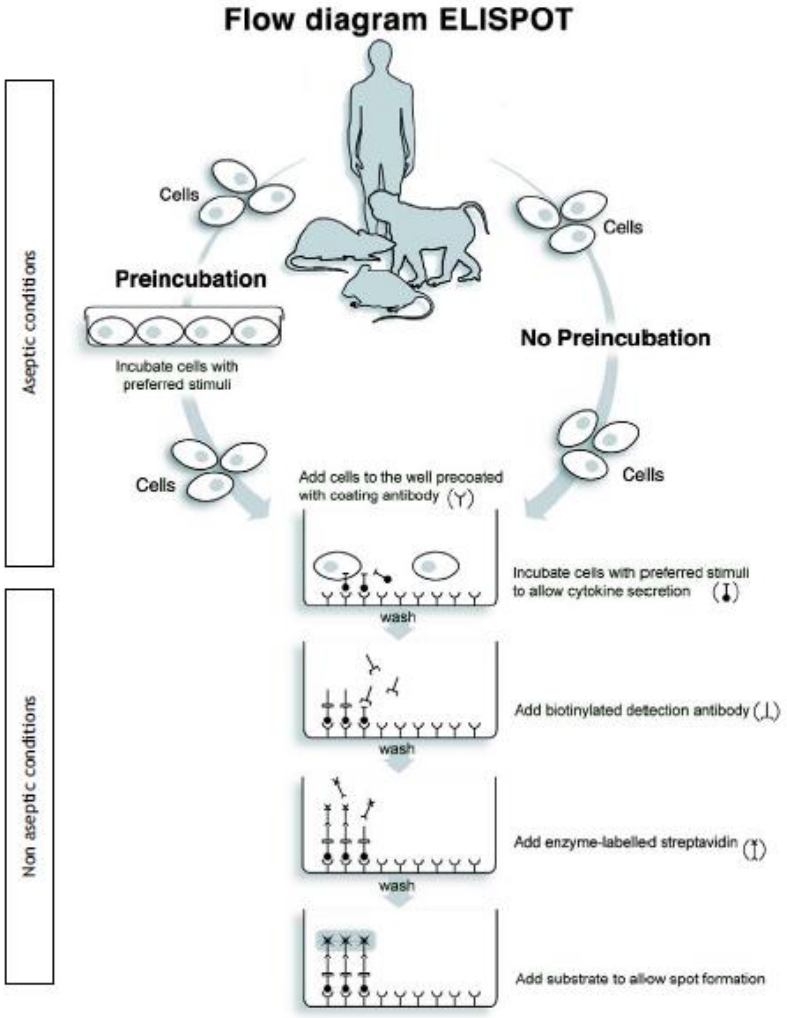

Hazard Information

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.

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Additional reagents/materials required for assay performance:

- Sterile distilled water
- 70% ethanol
- Sterile and pyrogen-free PBS, pH 7.4 (PBS-I)
  Filter-sterilize or autoclave. For washing purposes only.
- Wash buffer: PBS, 0.05% Tween-20 (0.5ml Tween-20 mixed with 1L PBS is sufficient for one ELISPOT plate)
- Sterile and pyrogen free PBS, pH 7.4 (PBS-I).
- Culture medium: RPMI 1640 with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% FCS.
- Cell stimuli: see "Cell sample preparation" and Addendum**..
- Pipetting devices
- Squirt (wash or squeeze) bottle with wide spout for washing, see Addendum**.
- CO_2-incubator (37°C, 100% humidity, 5% CO_2)
- Tissue culture plates for preincubation (optional)
- Tubes and containers to prepare solutions
- A reflected light microscope or an immunospot image analyzer for spot counting
  ** Addendum to this ELISPOT manual contains guidelines and troubleshooting.

Storage of Reagents

- The vials with lyophilized **coating antibody (CKR060S-A)** and **biotinylated detection antibody (CKR060S-B)** can be safely stored at 2-4°C until the expiration date. After reconstitution, the reagents are stable for at least 12 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 vial with lyophilized **Streptavidin-HRP conjugate (CKR060S-C)** should be stored at ≤ -20°C until the expiration date. After reconstitution, the reagent is stable for at least 2 months at 2-4°C when kept sterile. However, it is strongly recommended to divide the reconstituted conjugate into small aliquots for single use. These aliquots should be stored in the dark at ≤ -20°C. Under these conditions the reagent is stable for at least one year. The reconstituted Streptavidin-HRP rapidly loses activity when kept at room temperature.

- The **AEC stock solution (CKR060S-D)** should be protected from light, stored at ≤ -20°C until the expiration date. It is recommended to divide the solution into small aliquots for single use in polypropylene vials. These aliquots should be stored at ≤ -20°C protected from light.

- The **Substrate buffer capsules (CKR060S-E)** are stable until the expiration date when stored at room temperature, in a moisture-free environment.

- **Blocking stock solution 10x (CKR060S-F)** and **Dilution buffer R 10x (CKR060S-G)** should be stored at 2-4°C until the expiration date. After opening these solutions are stable for at least 6 months when kept sterile.

- **Tween-20 (CKR060S-H)** is best stored at room temperature until the expiration date.

Preparation of reagents

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

1. **Coating antibodies (CKR060S-A):** Reconstitute the lyophilized contents of the vial by injecting **250 µl** of sterile distilled water. Mix gently and allow it to stand for 5 minutes at room temperature. For one ELISPOT plate, thoroughly mix 50 µl with 5 ml PBS-I.
2. **Blocking stock solution (CKR060S-F):** Dilute Blocking stock solution (10x) in PBS-I. For one ELISPOT plate, thoroughly mix 2 ml with 18 ml PBS-I.
3. **Dilution buffer R (CKR060S-G):** 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 (CKR060S-B):** Reconstitute the lyophilized contents by injecting **500 µl** of sterile distilled water. Mix gently for approximately 15 seconds and allow it to stand for 5 minutes at room temperature. For one ELISPOT plate, thoroughly mix 100 µl with 10 ml Dilution buffer R (1x).
5. **Streptavidin-HRP conjugate (CKR060S-C):** Reconstitute the lyophilized contents by injecting **500 µl** of sterile distilled water. Mix gently for approximately 15 seconds and allow it to stand for 5 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 (CKR060S-D) and a substrate buffer capsule (CKR060S-E). For preparing the AEC substrate solution, the contents of one substrate buffer capsule is dissolved 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 660 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.

Cell sample preparation

Both fresh and cryopreserved cells can be used for ELISPOT analysis. Guidelines for specimen collection and handling are described in the ‘‘Addendum T cell ELISPOT assay’’.

Optimal conditions for the generation of cells releasing cytokines or other effector molecules in heterogeneous cell populations should be determined in advance. This is advisable because different cell types can produce the same effector molecules but 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, IL-6 and IL-10, is not restricted to T cells and many spot forming cells can also be attributable to activated monocytes/macrophages. Adherence of this last cell type to the surface of an ELISPOT well may already be sufficient to trigger TNF-α and IL-6 release.

Assay controls

Before starting an ELISPOT experiment, proper assay controls need to be chosen, which is mainly dependent on the selected analyte, targeted cell type and experimental set-up.

Positive controls

As positive controls both antigen-specific and polyclonal stimuli can be used to demonstrate that the cells are functional and the assay works well. Well-defined reagents such as the ICE peptide pool (a pool of synthetic peptides of common viral epitopes) and monoclonal antibodies (e.g. anti-CD3/CD28), are often preferred since these reagents are proven stimuli. In addition, also vaccine proteins (e.g. tetanus toxoid, Hepatitis B proteins) can be used, depending on whether all study subjects have been vaccinated.

Polyclonal stimuli such as, ConA, PHA, PMA/ionomycin, can be used for many different cell types of various species. An overview of ELISPOT stimuli and the recommended concentration ranges can be found in our “Addendum T cell ELISPOT assay”. In general, the optimal antigen concentration for antigen-specific stimulations varies between 0.5 and 10 µg of protein or peptide/ml but should be determined experimentally.

Negative controls

To reveal any false positive signals, or spontaneously secreting cells, cells are also incubated without stimuli at the same cell concentration as the cells incubated with the specific antigen of interest. In addition, a limited number of wells may be used including all reagents, but without the addition of cells, to exclude the possibility of false positivity due to the reagents or media.

Cell culture conditions preincubation

A 24-48 hours preincubation step at high cell density (> 10^6) may be required when full-length proteins or long peptides are used for in vitro re-stimulation. These long antigens must first be internalized, processed and presented by APCs via MHC class I/II molecules before they can stimulate cytokine (or other effector molecule) release by T cells. The high number of cells enhances the probability of contact between stimulating and responding cells. Omitting this step leads in most cases to a significant lower frequency of spot forming cells.

For preincubation, suspend cells in culture medium with an appropriate stimulus at 4 x 10^6 cells/ml in a tissue culture plate and incubate 24-48 hours (37°C with 5% CO2 in a humidified atmosphere). Use a minimum of 1 ml/well in a 24-well plate, 0.5 ml/well in a 48-well plate or 100 µl/well in a 96-well plate.
After preincubation, the non-adherent cells are collected and washed twice with fresh culture medium without stimuli or fetal calf serum. This will avoid the carryover of cytokines or other effector molecules produced during the preincubation step (two centrifugation/resuspension steps; 8 min, 200x g, RT). Thereafter cells are counted and suspended in culture medium with the same stimulus as used during preincubation at 1-3 x 10^5 cells/well (antigen-specific responses). For polyclonal stimulation, the recommended cell concentration per well should be reduced to 2 x 10^2 - 10^5 cells per well.

Stimulation with small peptides (8-12 amino acids)

Small peptides can directly be presented by APCs to T cells. Consequently, such peptides can be used in the ELISPOT assay without a preincubation step. For antigen-specific stimulation, 1-3x105 cells per well is recommended. For polyclonal stimulation, the recommended cell concentration per well should be reduced to 2 x 10^2 - 10^5 cells per well.

NOTES:

- It is recommended to test the samples in triplicate and in serial dilutions in the ELISPOT procedure.
- No more than 3 x 10^5 cells/well should be suspended in the ELISPOT plate. At higher concentrations the cells will be stacked upon each other, resulting in poor spot formation.

ELISPOT METHOD

Use ELISPOT plates and reagents under aseptic conditions (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 paper towels.
3. Add 50 µl of diluted coating antibodies into each well. Cover the plate with a lid and incubate overnight at 4°C.
4. Decant solution from wells. Wash 3x with 200 µl PBS-I/well. Add 200 µl Blocking buffer (1x) to each well. Cover the plate with a lid and incubate for at least 1 hour at RT. During this incubation, start the cell sample preparations with the appropriate stimulus.
5. Decant the Blocking solution from wells (do not wash the wells). Add prepared cells to the wells of the ELISPOT plate, 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 modified by $\pm10^4$ cells/ml. No more than $3x10^5$ cells/well should be suspended in the ELISPOT plate. See attached Addendum.)
6. Cover ELISPOT plate with lid and incubate at 37°C, 5% CO_2, and 100% humidity. The incubation time can vary from 24 to 72 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 attached Addendum.
7. Remove the bulk of cells with a firm shake-out action and wash 2x with room temperature PBS-I (200 µl/well). Wash the wells 5x with 250 µl Wash buffer/well (see attached Addendum).
8. Decant last wash buffer from wells and add 100 µl of properly diluted biotinylated detection antibodies to each well. Seal the plate with an adhesive cover slip and incubate 2 hours at room temperature 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 Wash buffer. Add 100 µl of properly diluted Streptavidin-HRP solution into each well. Seal the plate with an adhesive cover slip and incubate 1 hour at room temperature.
10. Empty plate and wash both sides of the PVDF membrane 5x with Wash buffer.
11. Add 100 µl freshly prepared AEC substrate solution to each well. Cover plate with lid and incubate for 30 minutes at room temperature in the dark.
12. Stop color development by emptying the plate and thoroughly rinse both sides of the PVDF membrane with demineralized water.
13. Air dry the plate at room temperature (in the dark) and count spots with a reflected light microscope or an immunospot image analyzer. To prevent bleaching of spots, store the plate in a dry place in the dark.

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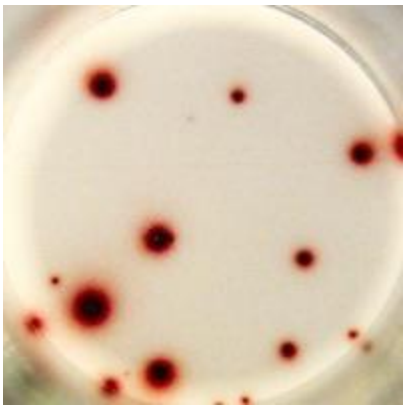
Typical data for CKR060S:

IL-21 specific spots produced by $5x10^4$ human PBMC.
Stimulation: Phytohemagglutinin.



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Addendum to Cell Sciences ELISPOT manual

Guidelines and Troubleshooting

Assay Performance:
There are two ways: 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. 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-glutamine + antibiotics).
Specimen collection from humans and non-human primates should be 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 (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. For the indirect procedure, AIM-V medium is dissuaded.

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Directions for the isolation and cell culture of rodent cells

For rodent cells, the same culturing conditions can be applied as described for human 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.

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 controls 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, the wells of the plate are emptied 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, the wells of the plate are emptied by a firm shake-out action.
- If using an automated washing device, the operating instructions should carefully be 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 has shown to produce the best results. The bottle should be used to thoroughly flush all wells of the plate with Wash buffer. While flushing, the wells are completely filled with Wash buffer and thereafter, the Wash buffer is removed by a firm ‘shake-out’ action. After washing, the plate is emptied 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, the Wash buffer is removed by a gentle ‘shake-out’ action.

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Recommended reagents

- Ficoll-Paque: for isolation of PBMCs by density gradient centrifugation
- RPMI 1640 medium
- L-glutamine
- Penicillin/Streptomycin
- AIM-V medium
- ICE peptide pool: Cell Sciences Cat. No. CK113
- Concanavalin A (ConA)
- Phorbol 12-myrisate 13-acetate (PMA)
- Ionomycin
- Phytohemagglutinin (PHA)
- Anti-CD3/CD28 for human PBMCs: Cell Sciences Cat. No. CSI17148

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.

Faintly 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 stored 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 procedure).

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.

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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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