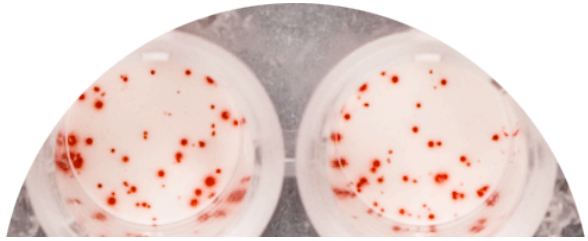
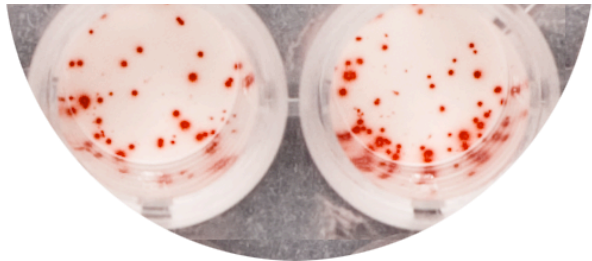


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# Instruction Manual T cell ELISPOT kit



Enzymatic staining procedure  
on PVDF plates



2-plate and 5-plate format

*For research use only.*  
Not for use in diagnostic or therapeutic procedures.

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# Abbreviations

AEC	3-Amino-9-EthylCarbazole
APC	Antigen-Presenting Cell
CTL	Cytotoxic T Lymphocyte
CD	Cluster of Differentiation
CNS	Central Nervous System
ConA	Concanavalin A
ELISA	Enzyme-Linked Immunoassay
ELISPOT	Enzyme-Linked ImmunoSPOT
FCS	Fetal Calf Serum
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HRP	HorseRadish Peroxidase
ICE	Influenza A, Cytomegalovirus, Epstein-Barr virus
IFN	Interferon
IL	Interleukin
LPA	Lymphocyte Proliferation Assay
MHC	Major Histocompatibility Complex
min	minute(s)
PBS	Phosphate Buffered Saline
PBS-I	Sterile and Pyrogen-free PBS
PBMC	Peripheral Blood Mononuclear Cell
PHA	Phytohaemagglutinin
PMA	Phorbol 12-Myristate 13-Acetate
PVDF	Polyvinylidene fluoride
RT	Room Temperature (temperature between 18 °C and 26 °C)
RT-PCR	Reverse Transcription Polymerase Chain Reaction
sec	seconds
SEB	Staphylococcal Enterotoxin B

# Catalogue numbers T cell ELISPOT kits

This manual applies to the following T cell ELISPOT kits

Analyte	Human	Old World Monkey	Mouse	Rat
IFN- $\gamma$	CT230-PR2 (2-plate) CT230-PR5 (5-plate)	CT121-PR2 (2-plate) CT121-PR5 (5-plate) CT126-PR2 (2-plate) CT126-PR5 (5-plate)	CT317-PR2 (2-plate) CT317-PR5 (5-plate)	CT079-PR2 (2-plate) CT079-PR5 (5-plate)
IL-1 $\beta$	CT242-PR2 (2-plate) CT242-PR5 (5-plate)	CT123-PR2 (2-plate) CT123-PR5 (5-plate)		
IL-2	CT231-PR2 (2-plate) CT231-PR5 (5-plate)	CT127-PR2 (2-plate) CT127-PR5 (5-plate)	CT435-PR2 (2-plate) CT435-PR5 (5-plate)	
IL-4	CT232-PR2 (2-plate) CT232-PR5 (5-plate)	CT128-PR2 (2-plate) CT128-PR5 (5-plate)	CT319-PR2 (2-plate) CT319-PR5 (5-plate)	CT081-PR2 (2-plate) CT081-PR5 (5-plate)
IL-5	CT233-PR2 (2-plate) CT233-PR5 (5-plate)	CT129-PR2 (2-plate) CT129-PR5 (5-plate)	CT321-PR2 (2-plate) CT321-PR5 (5-plate)	
IL-6	CT234-PR2 (2-plate) CT234-PR5 (5-plate)	CT130-PR2 (2-plate) CT130-PR5 (5-plate)	CT436-PR2 (2-plate) CT436-PR5 (5-plate)	
IL-10	CT235-PR2 (2-plate) CT235-PR5 (5-plate)	CT131-PR2 (2-plate) CT131-PR5 (5-plate)	CT320-PR2 (2-plate) CT320-PR5 (5-plate)	
IL-12/23p40		CT135-PR2 (2-plate) CT135-PR5 (5-plate)		
IL-12p70	CT240-PR2 (2-plate) CT240-PR5 (5-plate)			
IL-13	CT236-PR2 (2-plate) CT236-PR5 (5-plate)	CT132-PR2 (2-plate) CT132-PR5 (5-plate)		
IL-17A	CT416-PR2 (2-plate) CT416-PR5 (5-plate)	CT401-PR2 (2-plate) CT401-PR5 (5-plate)		
IL-17F	CT418-PR2 (2-plate) CT418-PR5 (5-plate)	CT403-PR2 (2-plate) CT403-PR5 (5-plate)		
IL-21	CT419-PR2 (2-plate) CT419-PR5 (5-plate)			
G-CSF	CT680-PR5 (5-plate)	CT122-PR5 (5-plate)		
GM-CSF	CT241-PR2 (2-plate) CT241-PR5 (5-plate)	CT124-PR2 (2-plate) CT124-PR5 (5-plate)		

Analyte	Human	Old World Monkey	Mouse	Rat
Granzyme B	CT229-PR2 (2-plate) CT229-PR5 (5-plate)			
Perforin	CT681-PR2 (2-plate) CT681-PR5 (5-plate)	CT136-PR2 (2-plate) CT136-PR5 (5-plate)		
TNF- $\alpha$	CT237-PR2 (2-plate) CT237-PR5 (5-plate)	CT133-PR2 (2-plate) CT133-PR5 (5-plate)	CT322-PR2 (2-plate) CT322-PR5 (5-plate)	

NOTE: the accompanying 'Typical data' and 'Addendum T cell ELISPOT assay', which contains guidelines and troubleshooting for ELISPOT analyses are available on our website ([www.ucytech.com/manuals](http://www.ucytech.com/manuals)).

# Introduction

The ELISPOT assay is one of the most sensitive tests to monitor *ex-vivo* cellular immune responses at the single cell level. The assay can accurately detect secreted proteins, such as cytokines, released by T cells in response to an antigen. The cell suspensions, used in the test, can originate from blood (PBMCs), lymphoid, spleen, bone marrow or CNS tissue.

Classical T cell monitoring assays (e.g. LPA and CTL assay), measure CD4<sup>+</sup> or CD8<sup>+</sup> cell mediated immune responses. Both LPA and CTL assays have their drawbacks including the use of radioactivity, low throughput screening, decreased sensitivity in cryopreserved specimens and technical burden. RT-PCR analysis, to measure T cell responses can also be used. However, this assay detects mRNA instead of actually secreted protein.

The ELISPOT assay, not afflicted with these shortcomings, has proven to be more sensitive than an ELISA<sup>1</sup> or intracellular cytokine staining<sup>2,3</sup>. The high sensitivity is due to the plate-bound antibodies that directly capture the secreted proteins released by the cell before they dilute in the culture medium, are taken up by cells via cell-surface receptors or are degraded by proteases. This property enables the detection of very low frequencies of cytokine secreting cells (1/300,000) and also offers the possibility of high throughput screening.

The majority of ELISPOT assays are nowadays performed on PVDF-membrane bottomed plates. The fractal surface and hydrophobic properties of the membrane are ideal for strong binding of ELISPOT capture antibodies to the membrane surface. The subsequent interaction of cell-secreted proteins by these antibodies forms the first step in spot formation. For the visualization of spots, the U-CyTech's ELISPOT assay makes use of the Streptavidin-HRP/AEC coloring system, providing red-colored spots that reveal the site of protein secretion by individual cells. The PVDF membrane's white color provides the ideal background for the visualization of these intense red and well-distinguishable spots.

Nowadays, U-CyTech's ELISPOT procedure with enzymatic staining on PVDF plates is widely used in different fields of biomedical research. For example, researchers use these ELISPOT assays to determine T cell responses in preclinical evaluations of vaccine candidates or to analyze the impact of chronic immunosuppression on T cell reactivity against latent viruses in transplant recipients.<sup>4,5</sup>

## References

1. Tanguay and Killion (1994). Direct comparison of ELISPOT and ELISA-based assays for detection of individual cytokine-secreting cells. *Lymphokine Cytokine Res* 13: 259.
2. Carter and Swain (1997). Single cell analyses of cytokine production. *Curr Opin Immunol* 9: 177.
3. Herold *et al.* (2009). Validity and reproducibility of measurement of islet autoreactivity by T-cell assays in subjects with early type 1 diabetes. *Diabetes* 58: 2588-95.
4. Rollier *et al.* (2016). T- and B-cell responses to multivalent prime-boost DNA and viral vectored vaccine combinations against hepatitis C virus in non-human primates. *Gene Ther* 23: 753-759.
5. Bererhi *et al.* (2012). Clinical and immunological features of very long-term survivors with a single renal transplant. *Transpl Int.* 545:54.

Please find more references of studies using our ELISPOT kits on our website:  
[www.ucytech.com/ELISPOT](http://www.ucytech.com/ELISPOT) or [www.ucytech.com/references](http://www.ucytech.com/references).

## Brief description ELISPOT assay

U-CyTech ELISPOT assays are simple and sensitive immunoassays for the detection of protein secreting cells at the single cell level.

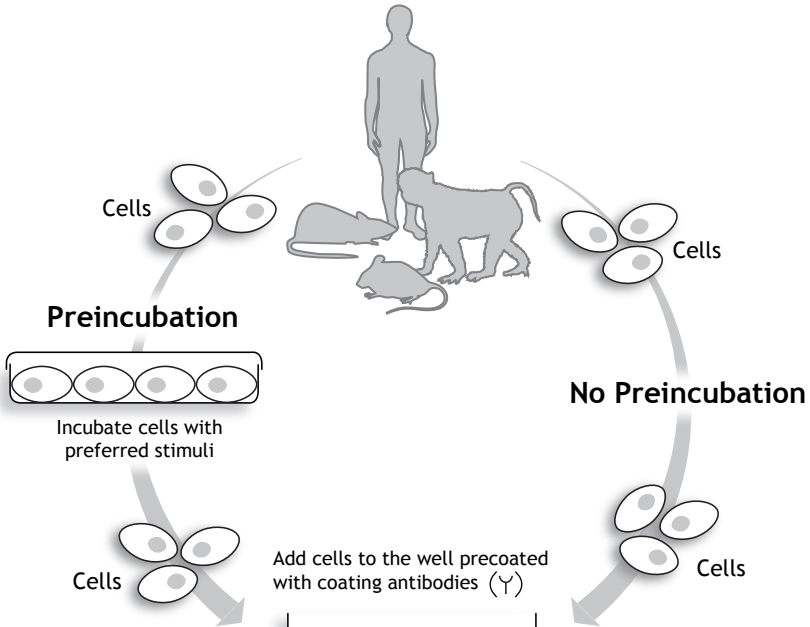
The ELISPOT procedure is illustrated in the “Flow diagram ELISPOT” on page 8. First cells are collected from a donor. Depending on the experimental set-up, the cells are either preincubated (see the next paragraph) or directly added to the wells of the ELISPOT plate that is coated with a high affinity antibody\*. The cell suspension is incubated in the presence of an antigen or polyclonal stimulus to trigger protein release from the cell. Subsequently, the cells are washed away and the antibody-bound proteins are detected with a combination of a biotinylated detection antibody and Streptavidin-HRP conjugate. In the last step, AEC substrate is added which yields a red spot that reveals the protein secretion site (the so-called footprints of individual cells).

Preincubation (24-48 hours) is required when full-length proteins or long peptides are recommended for re-stimulation. These antigens must first be internalized, processed and presented by antigen-presenting cells (APCs) via MHC class I/II molecules before they can stimulate protein (e.g. cytokine) release by T cells. Omitting this step leads to a significant lower frequency of spot forming cells. On the other hand, small peptides (8-12 amino acids) can directly be presented by APC to CD8<sup>+</sup> cells and consequently need no preincubation step. Read “Cell sample preparation” on page 14 for more information.

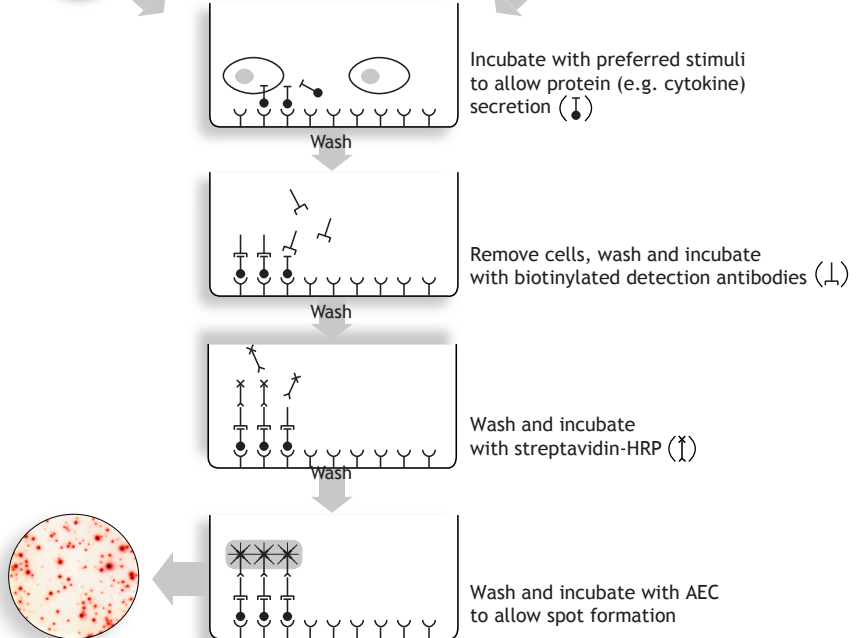
\* specific for a protein of interest

# Flow diagram T cell ELISPOT

Sterile conditions



Non-sterile conditions





# Warnings and precautions

- This kit is designed for research use only, and not for use in diagnostic or therapeutic procedures.
- Please note that human and non-human primate blood components or other biological materials should be considered as potentially infectious and handled with the usual precautions under Bio-Hazard conditions. Follow universal precautions such as established by the US government agencies, Centers for Disease Control and Prevention and Occupational Safety and Health Administration, when handling and disposing of (potentially) infectious waste.

## Hazard Information

Except for the AEC stock solution and Dilution buffer R, the items in this kit are not classified as dangerous according to Regulation (EC) no. 1272/2008 and its amendments.

### Warning:

AEC (3-amino-9-ethylcarbazole) stock solution is classified as dangerous and Dilution buffer R is classified as irritation according to Regulation (EC) no. 1272/2008 and its amendments:

#### AEC stock solution:



Acute toxicity, oral (Category 4), Carcinogenicity (Category 1A), Skin irritation (Category 2), Eye irritation (Category 2), Specific target organ toxicity - single exposure (Category 3).

Hazard statements: Harmful if swallowed (H302), Causes skin irritation (H315), Causes serious eye irritation (H319), May cause respiratory irritation (H335), May cause cancer (H350).

The AEC stock solution should be handled only in a chemical fume hood. Use only non-sparking tools and keep away from open flames and hot surfaces.

#### Dilution buffer R:



Skin irritation (Category 2), Eye irritation (Category 2), Skin sensitization (Category 1), Chronic aquatic toxicity (Category 3).

Hazard statements: Causes skin irritation (H315), May cause an allergic skin reaction (H317), Causes serious eye irritation (H319), Harmful to aquatic life with long lasting effects (H412).

#### AEC stock solution and Dilution buffer R:

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 min. Assure adequate flushing by separating the eyelids. Seek medical advice immediately.

Please find the Material Safety Data Sheet on [www.ucytech.com/manuals](http://www.ucytech.com/manuals).

# Contents of the kit

Items	Quantity (2-plate format)	Quantity (5-plate format)	Storage conditions
Coating antibody*	1 vial	1 vial	4°C
Biotinylated detection antibody*	1 vial	1 vial	4°C
Streptavidin-HRP conjugate*	1 vial	1 vial	-20°C
AEC coloring system:			
I. AEC stock solution	4 ml	4 ml	-20°C in the dark
II. Substrate buffer capsules	2	5	RT
Blocking stock solution (10x)	4 ml	10 ml	4°C
Dilution buffer R (10x)***	4 ml	10 ml	4°C
Tween-20***	5 ml	5 ml	RT
96-well ELISPOT plate** with lid	2	-	RT
Adhesive cover slip	5	-	RT

\* Lyophilized

\*\* PVDF membrane-bottomed Millipore plates (cat. no. MSIP S4510).

\*\*\* Not included in Human IL-21 ELISPOT kit (cat. no. CT419-PR2 and CT419-PR5). This kit contains Dilution buffer Q (10x). The wash buffer in the Human IL-21 assay is PBS only.

# Storage and stability

## **Coating antibody and biotinylated detection antibody**

The vials with lyophilized coating antibody and biotinylated detection antibody can be safely stored at 4°C until the expiry date (indicated on the vials). After reconstitution, the antibodies are stable for at least 12 months at 4°C when kept sterile. However, it is recommended to divide the reconstituted antibody solutions into small aliquots for single use. These aliquots should be stored at  $\leq -20^{\circ}\text{C}$  (stable for at least two years).

## **Streptavidin-HRP conjugate**

The vial with lyophilized Streptavidin-HRP conjugate should be stored at  $\leq -20^{\circ}\text{C}$  and remains stable until the expiry date (indicated on the vial). After reconstitution, the reagent is stable for at least 2 months at 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 at  $\leq -20^{\circ}\text{C}$  in the dark (stable for at least 12 months).

## **AEC stock solution**

The AEC stock solution should be stored at  $\leq -20^{\circ}\text{C}$  and remains stable until the expiry date (indicated on the vial)\*. It is strongly recommended to divide the solutions into small aliquots for single use in polypropylene vials. These aliquots should be stored at  $\leq -20^{\circ}\text{C}$  in the dark (stable for at least 12 months).

\*Avoid exposure to light and air and tightly close the vial after use.

## **Substrate buffer capsules**

The substrate buffer capsules are stable until the expiry date (indicated on the vial) when stored at RT in a moisture-free environment.

## **Blocking stock solution (10x) and Dilution buffer (10x)**

The vials with Blocking stock solution and Dilution buffer can be safely stored at 4°C until the expiry date (indicated on the vials). After opening, these solutions are stable for at least 6 months when kept sterile.

## **Tween-20**

Tween-20 can be safely stored at RT and is stable until the expiry date (indicated on the vial).

## Materials and equipment (required but not provided)

- 96-well PVDF membrane-bottomed plates (for 5-plate kit format): Millipore cat.no. MSIP S4510 is recommended.
- Tubes and containers/plates to prepare the solutions.
- Tissue culture plates for preincubation (optional).
- Sterile distilled water.
- 70% ethanol.
- PBS pH 7.4 (home-made). For washing purposes only.
- Sterile and pyrogen-free PBS pH 7.4 (PBS-I): Thermo Fisher Scientific cat. no. 10010 is recommended (Gibco®).
- Culture medium: RPMI-1640 supplemented with 2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS).
  - RPMI-1640: Thermo Fisher Scientific cat. no. 52400 (Gibco®).
  - L-Glutamine: Thermo Fisher Scientific cat. no. 25030-081 (Gibco®; 200 mM).
  - Penicillin-Streptomycin: Thermo Fisher Scientific cat. no. 15140-122 (Gibco®, 100x).
  - FCS should be selected on low background staining: Thermo Fisher Scientific cat. no. 16000 (Gibco®).

Culture medium AIM V® (Thermo Fisher Scientific cat. no. 31035-025) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin is an alternative culture medium for the ELISPOT procedure without a preincubation step.

- Cell stimuli, see “Cell sample preparation” on page 14 and [www.ucytech.com/ELISPOT-stimuli](http://www.ucytech.com/ELISPOT-stimuli).
- Pipetting devices.
- For washing: squirt (wash or squeeze) bottle with wide sprout, see Addendum\*.
- CO<sub>2</sub> incubator (37°C, 100% humidity, 5% CO<sub>2</sub>).
- A reflected light microscope or an Immunospot image analyzer for spot counting.

## Preparation solutions and reagents

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

### **Blocking buffer (1x)**

Dilute Blocking stock solution (10x) in PBS-I.

For one ELISPOT plate: 2 ml is gently and thoroughly mixed with 18 ml PBS-I.

### **Dilution buffer (1x)**

Dilute Dilution buffer (10x) in PBS-I.

For one ELISPOT plate: 2 ml is gently and thoroughly mixed with 18 ml PBS-I.

### **Coating antibody**

Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix the solution gently for approximately 15 sec and allow the vial to stand for 5 min at RT.

For one ELISPOT plate (2-plate kit): 100 µl is gently and thoroughly mixed with 5 ml PBS-I.

For one ELISPOT plate (5-plate kit): 50 µl is gently and thoroughly mixed with 5 ml PBS-I.

### **Biotinylated detection antibody**

Reconstitute the lyophilized contents of the vial by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix the solution gently for approximately 15 sec and allow it to stand for 5 min at RT.

For one ELISPOT plate: 100 µl is gently and thoroughly mixed with 10 ml Dilution buffer (1x).

### **Streptavidin-HRP conjugate**

Reconstitute the lyophilized contents of the vial by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix the solution gently for approximately 15 sec and allow it to stand for 5 min at RT.

For one ELISPOT plate: 100 µl is gently and thoroughly mixed with 10 ml Dilution buffer (1x).

### **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, 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 min after preparation. The remaining substrate buffer can be discarded.

\* Do not bring AEC stock solution in contact with polystyrene pipettes and vials.

### **PBS (for washing purposes only)**

5.4 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O; 1.3 mM KH<sub>2</sub>PO<sub>4</sub>; 150 mM NaCl; pH 7.4 (sterile).

For one ELISPOT plate: prepare 1 L PBS.

### **Wash buffer**

PBS containing 0.05% Tween-20.

For one ELISPOT plate: 0.5 ml Tween-20 is gently but thoroughly mixed with 1 L PBS.

NOTE: Use PBS only as Wash buffer in the Human IL-21 ELISPOT assay.

# 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- $\gamma$  secreting CD8<sup>+</sup> T cells in PBMC preparations differ considerably from those for the detection of IFN- $\gamma$  secreting CD4<sup>+</sup> T cells<sup>1</sup>.

Moreover, the production of cytokines, such as TNF- $\alpha$ , 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- $\alpha$  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 cat. no. CT387) and monoclonal antibodies (e.g. anti-CD3/CD28 cat. no. CT372), 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” and on page 17. In general, the optimal antigen concentration for antigen-specific stimulations varies between 0.5 and 10  $\mu\text{g}$  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.

## References

1. Schmittel *et al.* (2001). Application of the IFN-gamma ELISPOT assay to quantify T cell responses against proteins. *J Immunol Methods* 247: 17

### **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  $4 \times 10^6$  cells/ml in a tissue culture plate and incubate 24-48 hours ( $37^\circ\text{C}$  with 5%  $\text{CO}_2$  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  $\mu\text{l}$ /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  $1-3 \times 10^5$  cells/well (antigen-specific responses). For polyclonal stimulation, the recommended cell concentration per well should be reduced to  $2 \times 10^2 - 10^5$  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,  $1-3 \times 10^5$  cells per well is recommended. For polyclonal stimulation, the recommended cell concentration per well should be reduced to  $2 \times 10^2 - 10^5$  cells per well.

### **NOTES:**

- It is recommended to test the samples in triplicate and in serial dilutions in the ELISPOT procedure.
- No more than  $3 \times 10^5$  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.

See also 'Guidelines for cell concentrations and cell incubation times' on page 17 for more information on cell concentration/well, incubation times and stimuli.

# ELISPOT procedure

*All solutions should be at RT prior to use. The steps 1 till 11 should be performed under sterile conditions. In addition, estimate the time needed to prepare all cell preparations which have to be ready for step 9 and plan accordingly.*

1. Prewet each well of the PVDF plate with 25  $\mu$ l of 70% ethanol. Incubate for 1 min at RT.
2. Aspirate or firmly shake-out the ethanol. Immediately thereafter wells are rinsed 2x with PBS-I. The plate is subsequently emptied and tapped on tissue paper.
3. Add 50  $\mu$ l of diluted coating antibody solution into each well of the ELISPOT plate.
4. Cover the plate with a lid and incubate overnight at 4 °C.
5. Remove coating antibody solution and rinse each well 3x with 200  $\mu$ l PBS-I. The plate is subsequently emptied.
6. Add 200  $\mu$ l Blocking buffer (1x) into each well.
7. Cover the plate with a lid and incubate for at least 1 hour at RT. During this incubation step start preparing the cell sample preparations (see “Cell sample preparation” on page 14).
8. If the cell preparations are ready, decant the blocking solution from wells (do not wash the wells).
9. Bring the cell preparations into the wells of the ELISPOT plate. Add 100  $\mu$ l/well.
10. Cover ELISPOT plate with lid and incubate at 37°C, 5% CO<sub>2</sub> and 100% humidity. The incubation time can vary from 24 to 72 hours. Specific activation conditions will vary, depending on cell type, protein of interest, kinetics of protein release and whether a preincubation step was included in the procedure. See “Cell sample preparation” on page 14, “Guidelines for cell concentrations and cell incubation times” on page 17 and Typical data.
11. Remove the bulk of cells with a firm shake-out action and rinse each well 2x with 200  $\mu$ l PBS-I. The plate is subsequently emptied.
12. Wash the plate 5x with 250  $\mu$ l Wash buffer/well (see “Addendum T cell ELISPOT assay” for directions on washing).
13. Add 100  $\mu$ l of diluted biotinylated detection antibody into each well.
14. Seal the plate with adhesive cover slip and incubate 2 hours at RT (or overnight at 4°C).
15. Empty plate. Remove and discard the underdrain from the bottom of the plate and wash both sides of the PVDF membrane 5x with Wash buffer.
16. Add 100  $\mu$ l diluted Streptavidin-HRP conjugate into each well.
17. Seal the plate with an adhesive cover slip and incubate 1 hour at RT.

Sterile conditions

Non sterile conditions



18. Empty plate and wash both sides of the PVDF membrane 5x with Wash buffer.
19. Add 100  $\mu$ l freshly prepared AEC solution into each well.
20. Cover plate with lid and incubate for 30 min at RT in the dark.
21. Stop the reaction by emptying the plate and thoroughly rinse both sides of the PVDF membrane with demineralized water.
22. Air-dry the plate at RT (in the dark).
23. Count spots by use of a reflected light microscope or an Immunospot image analyzer.

To prevent bleaching of spots store the plate at a dry place in the dark.

## Guidelines for cell concentrations and cell incubation times

Cell type	Analyte <sup>stimuli</sup>	Incubation time without preincubation	Incubation time after preincubation*	Cell concentration antigen stimulation	Cell concentration polyclonal stimulation	
Human and Old World Monkey PBMCs	IFN- $\gamma$ <sup>1-4,6,7</sup> IL-1 $\beta$ <sup>1,5</sup> IL-2 <sup>1-4,6,7</sup> IL-4 <sup>1-4,6,7</sup> IL-5 <sup>1-4,7</sup> IL-6 <sup>1-3,5</sup> IL-10 <sup>1-5,7</sup> IL-12p70 <sup>**5</sup>	IL-12/23p40 <sup>5</sup> IL-13 <sup>1-4,7</sup> IL-17A <sup>1-4,7</sup> IL-17F <sup>1</sup> IL-21 <sup>1-3</sup> G-CSF <sup>1-3</sup> GM-CSF <sup>1,3,5</sup> TNF- $\alpha$ <sup>1-3</sup>	24-48h	24h	2x10 <sup>5</sup> cells/well	2x10 <sup>3</sup> to 1x10 <sup>5</sup> cells/well
	Granzyme B <sup>1-4,6</sup>	Perforin <sup>1,2,6</sup>	48-72h	24h	2x10 <sup>5</sup> cells/well	2x10 <sup>3</sup> to 1x10 <sup>5</sup> cells/well
Rodent spleen cells	IFN- $\gamma$ <sup>1-3</sup> IL-2 <sup>1-3</sup> IL-4 <sup>1-3</sup> IL-5 <sup>1-3</sup>	IL-6 <sup>1-3</sup> IL-10 <sup>1-3</sup> TNF- $\alpha$ <sup>1-3</sup>	24-48h	24-48h	2x10 <sup>5</sup> cells/well	2x10 <sup>2</sup> to 1x10 <sup>5</sup> cells/well

### General cell stimuli commonly used in ELISPOT

Polyclonal stimuli: 1. PMA & ionomycin (50 ng/ml & 1  $\mu$ g/ml) 2. PHA (10-30  $\mu$ g/ml) 3. ConA (human and Old world monkey: 6-10  $\mu$ g/ml) (rodent: 4  $\mu$ g/ml) 4.  $\alpha$ -CD3 &  $\alpha$ -CD28 (0.05  $\mu$ g/ml & 0.05  $\mu$ g/ml) 5. LPS & IFN- $\gamma$  (100 ng/ml & 10-100 units/ml)

Antigen-specific stimuli (human PBMCs): 6. ICE peptide pool (1  $\mu$ g/ml for each peptide) 7. Tetanus toxoid (0.5 LF/ml)

\*Preincubation time: 24 - 48h (all cell types); cell concentration 4x10<sup>6</sup> cells/ml

\*\*NOTE: Preincubation is not recommended for IL-12p70.

# Notes

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# Technical assistance

If you require assistance, information or have any questions, please contact our company:

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On our website ([www.ucytech.com/manuals](http://www.ucytech.com/manuals)) you can find: Manuals, Typical data, Addendum and MSDS of our ELISPOT kits.