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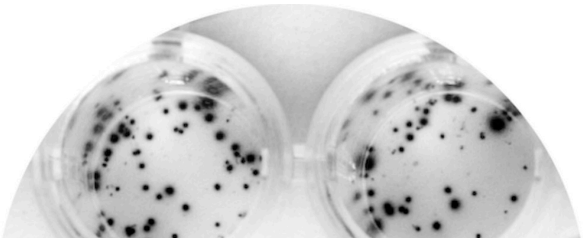
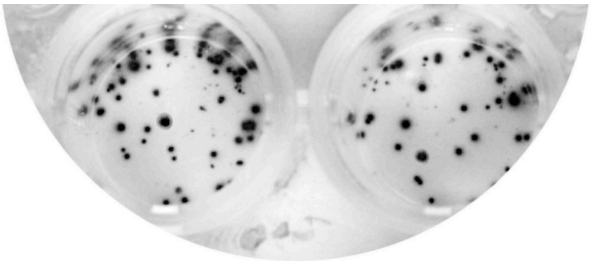
Instruction Manual T cell ELISPOT kit



Silver staining procedure on PVDF plates



2-plate and 5-plate format

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

Contents

Abbreviations

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
GABA	Gold-labeled Anti-Biotin Antibodies
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
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
RT	Room Temperature (temperature between 20 °C and 26 °C)
RT-PCR	Reverse Transcription Polymerase Chain Reaction
sec	seconds
TNF	Tumor Necrosis Factor

Catalogue numbers T cell ELISPOT kits

This manual applies to the following T cell ELISPOT kits

Analyte	Human	Old World Monkey	Mouse	Rat
IFN-γ	CT230-PB2 (2-plate) CT230-PB5 (5-plate)	CT121-PB2 (2-plate) CT121-PB5 (5-plate) CT126-PB2 (2-plate) CT126-PB5 (5-plate)	CT317-PB2 (2-plate) CT317-PB5 (5-plate)	CT079-PB2 (2-plate) CT079-PB5 (5-plate)
IL-1ß	CT242-PB2 (2-plate) CT242-PB5 (5-plate)	CT123-PB2 (2-plate) CT123-PB5 (5-plate)		
IL-2	CT231-PB2 (2-plate) CT231-PB5 (5-plate)	CT127-PB2 (2-plate) CT127-PB5 (5-plate)	CT435-PB2 (2-plate) CT435-PB5 (5-plate)	
IL-4	CT232-PB2 (2-plate) CT232-PB5 (5-plate)	CT128-PB2 (2-plate) CT128-PB5 (5-plate)	CT319-PB2 (2-plate) CT319-PB5 (5-plate)	CT081-PB2 (2-plate) CT081-PB5 (5-plate)
IL-5	CT233-PB2 (2-plate) CT233-PB5 (5-plate)	CT129-PB2 (2-plate) CT129-PB5 (5-plate)	CT321-PB2 (2-plate) CT321-PB5 (5-plate)	
IL-6	CT234-PB2 (2-plate) CT234-PB5 (5-plate)	CT130-PB2 (2-plate) CT130-PB5 (5-plate)	CT436-PB2 (2-plate) CT436-PB5 (5-plate)	
IL-10	CT235-PB2 (2-plate) CT235-PB5 (5-plate)	CT131-PB2 (2-plate) CT131-PB5 (5-plate)	CT320-PB2 (2-plate) CT320-PB5 (5-plate)	
IL-12/23p40		CT135-PB2 (2-plate) CT135-PB5 (5-plate)		
IL-12p70	CT240-PB2 (2-plate) CT240-PB5 (5-plate)			
IL-13	CT236-PB2 (2-plate) CT236-PB5 (5-plate)	CT132-PB2 (2-plate) CT132-PB5 (5-plate)		
IL-17A	CT416-PB2 (2-plate) CT416-PB5 (5-plate)	CT401-PB2 (2-plate) CT401-PB5 (5-plate)		
IL-17F	CT418-PB2 (2-plate) CT418-PB5 (5-plate)	CT403-PB2 (2-plate) CT403-PB5 (5-plate)		
G-CSF	CT680-PB5 (5-plate)	CT122-PB5 (5-plate)		
GM-CSF	CT241-PB2 (2-plate) CT241-PB5 (5-plate)	CT124-PB2 (2-plate) CT124-PB5 (5-plate)		

Analyte	Human	Old World Monkey	Mouse	Rat
Granzyme B	CT229-PB2 (2-plate) CT229-PB5 (5-plate)			
Perforin	CT681-PB2 (2-plate) CT681-PB5 (5-plate)	CT136-PB2 (2-plate) CT136-PB5 (5-plate)		
TNF-α	CT237-PB2 (2-plate) CT237-PB5 (5-plate)	CT133-PB2 (2-plate) CT133-PB5 (5-plate)	CT322-PB2 (2-plate) CT322-PB5 (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).

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 $CD4^+$ or $CD8^+$ 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[1] or intracellular cytokine staining[2,3]. 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. Spot staining is achieved with gold-labeled anti-biotin antibodies (GABA) using silver precipitation for spot visualization, a highly effective and sensitive method. Spots do not fade in time and ELISPOT plates can be reanalyzed several years later after being stored at room temperature.

Nowadays, U-CyTech's ELISPOT procedure with silver staining on PVDF plates is widely used in different fields of biomedical research. For example, researchers use these ELISPOT assays to identify important target antigens in subjects with type 1 diabetes[4], or to characterize T-cell cytokine secretion profiles against specific cancer cells[5].

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. Gottlieb *et al*. (2014). Chromogranin A is a T cell antigen in human type 1 diabetes. J Autoimmun; 50:38-41.
5. Chiriva-Internati *et al*. (2012). Identification of AKAP-4 as a new Cancer/Testis Antigen for detection and immunotherapy of prostate cancer. Prostate 72:12-23.

Please find more references of studies using our ELISPOT kits on our website: www.ucytech.com/ELISPOT or www.ucytech.com/references.

Brief description ELISPOT assay

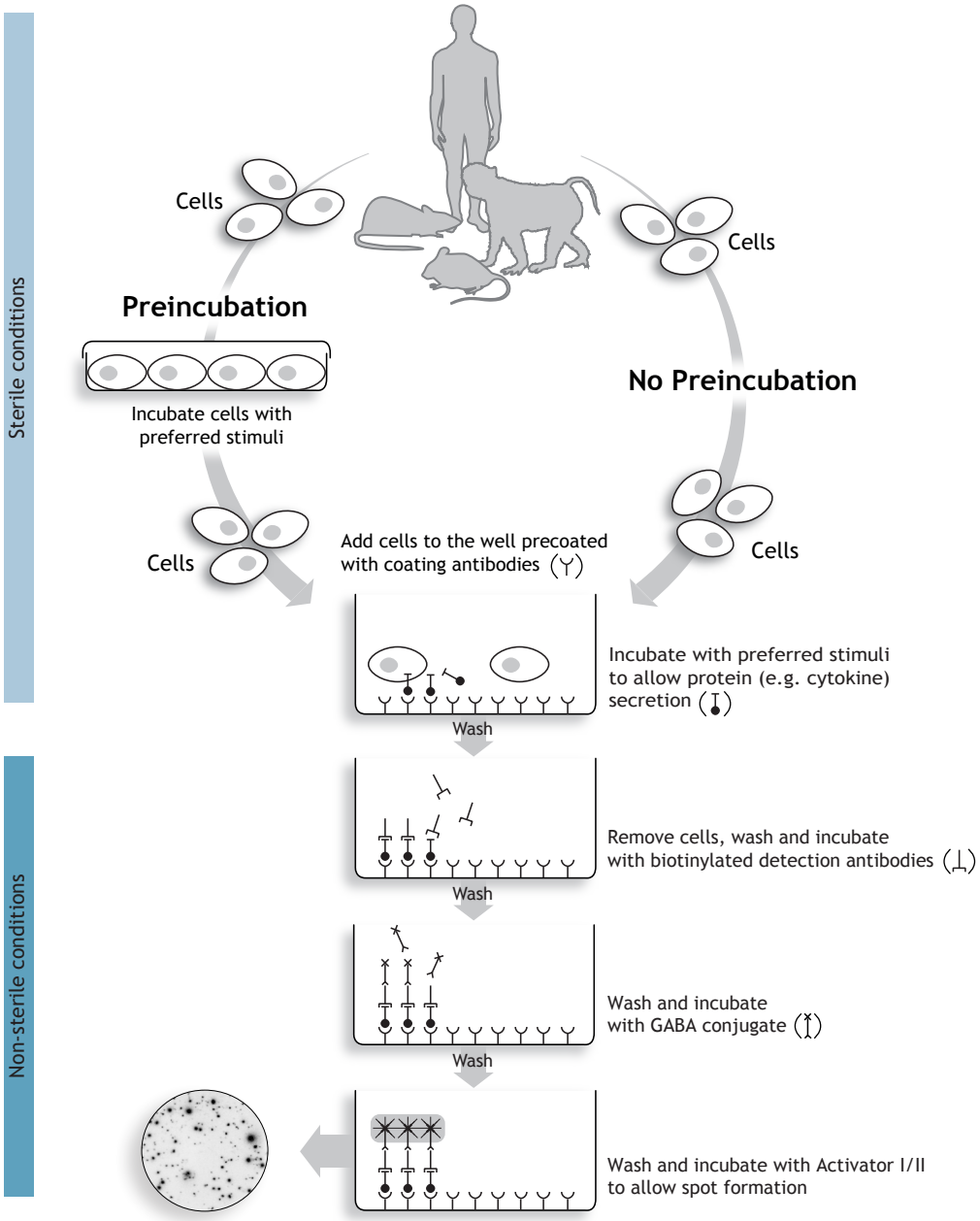
U-CyTech ELISPOT assays are simple and sensitive immunoassays for the detection of protein secreting cells at the single cell level.

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 GABA conjugate. In the last step, a silver salt solution (Activator I/II) is added which allows silver to precipitate on the gold particles producing a spot that reveals the protein secretion site (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 $CD8^+$ 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



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 Activator I+II solutions, the items in this kit are not classified as dangerous according to Regulation (EC) no. 1272/2008 and its amendments.

Activator I + II:

Warning:
Activator I+II solutions are classified as dangerous according to Regulation (EC) no. 1272/2008 and its amendments: Serious eye damage (Category 1), Skin sensitization (Category 1) and Chronic aquatic toxicity (Category 1).

Hazard statement: May cause an allergic skin reaction (H317), Causes serious eye damage (H318), Very toxic to aquatic life with long lasting effects (H410).

The Activator I+II solutions should be kept away from acids and sources of ignition; use only non-sparking tools. Keep away from light, air and heat. In case of contact with skin, wash with soap and water and remove contaminated clothing and shoes. Upon ingestion rinse mouth (if person is conscious) and call physician immediately. Do not induce vomiting. In case of contact with eyes, irrigate 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 and Safety Data Sheet on 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
GABA conjugate*	1 vial	1 vial	4 °C
Activator I	4 ml	9.5 ml	4 °C in the dark
Activator II	4 ml	9.5 ml	4 °C in the dark
Blocking stock solution (10x)	4 ml	10 ml	4 °C
Dilution buffer B (10x)	4 ml	8 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).

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 ≤ -20°C (stable for at least two years).

GABA conjugate
The vial with lyophilized GABA conjugate can be safely stored at 4°C until the expiry date (indicated on the vial). After reconstitution, it is strongly recommended to divide the solution into small aliquots for single use at ≤ -20°C (stable for at least two years).

Activators I and II
The Activators I and II should be stored at 4°C and are stable until the expiry date (indicated on the vials)*. It is strongly recommended to divide the solutions into small aliquots for single use. These aliquots should be stored at ≤ -20°C in the dark (stable for at least two years).

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

Blocking stock solution (10x) and Dilution buffer B (10x)
The vials with blocking stock solution and Dilution buffer B 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 safely be 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.
- PBS-I: Sterile and pyrogen-free PBS pH 7.4; 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.
- Antigen of interest.
- Pipetting devices.
- For washing: squirt (wash or squeeze) bottle with wide sprout, see “Addendum T cell ELISPOT assay”.
- CO_2 incubator (37°C, 100% humidity, 5% CO_2).
- 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 B (1x)
Dilute Dilution buffer B (10x) in PBS-I.
For one ELISPOT plate: 1.5 ml is gently and thoroughly mixed with 13.5 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 B (1x).

GABA 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 5 ml Dilution buffer B (1x).

Activator I and II
Bring the two bottles of Activator to RT prior to use. Shake the bottles gently but intensively before mixing 1.8 ml of Activator I with 1.8 ml Activator II (for one ELISPOT plate). Use immediately thereafter.

PBS (for washing purposes only)
5.4 mM $Na_2HPO_4 \cdot 2H_2O$; 1.3 mM KH_2PO_4; 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 and thoroughly mixed with 1 L PBS.

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[1].

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 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 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.

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 4x10^6 cells/ml in a tissue culture plate and incubate 24-48 hours (37°C with 5% 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 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 1-3x10^5 cells/well (antigen-specific responses). For polyclonal stimulation, the recommended cell concentration per well should be reduced to 2x10^2 - 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, 1-3x10^5 cells per well is recommended. For polyclonal stimulation, the recommended cell concentration per well should be reduced to 2x10^2 - 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 3x10^5 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.

Sterile conditions

1. Prewet each well of the PVDF plate with 25 µl of 70% ethanol. Incubate for 1 min at RT.
2. Aspirate or firmly shake-out the ethanol. Immediatey thereafter wells are rinsed 2x with PBS-I. The plate is subsequently emptied and tapped on tissue paper.
3. Add 50 µl 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 µl PBS-I. The plate is subsequently emptied.
6. Add 200 µl Blocking buffer (1x) into each well.
7. Cover the plate with a lid and incubate for at least 1 hour at 37°C. 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 µl/well.
10. 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, 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 µl PBS-I. The plate is subsequently emptied.

Non sterile conditions

12. Wash the plate 5x with 250 µl Wash buffer/well (see ‘‘Addendum T cell ELISPOT assay’’ for directions on washing).
13. Add 100 µl of diluted biotinylated detection antibody into each well.
14. Seal the plate with adhesive cover slip and incubate 2 hours at 37°C (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 50 µl diluted GABA conjugate into each well.
17. Seal the plate with an adhesive cover slip and incubate 1 hour at 37°C.

18. Empty plate and wash both sides of the PVDF membrane 5x with Wash buffer.
19. Add 35 µl freshly prepared Activator I/II solution into each well. Distribute the Activator I/II uniformly over the bottom of the well.
20. Cover plate with lid and incubate for 30-60 min at RT. Monitor spot development by light microscope.
21. When clear spots have developed, 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.

Visually, spots have a grayish color, microscopically they are black. Silver-stained spots are highly stable and spot quality is preserved indefinitely when the plate is stored at a dry place.

Guidelines for cell concentrations and cell incubation times

Cell type	Analyte[stimuli]		Incubation time without preincubation	Incubation time after preincubation*	Cell concentration antigen stimulation	Cell concentration polyclonal stimulation
Human and Old world Monkey PBMCs	IFN-γ[1-4,6,7] IL-1ß[1,5] IL-2[1-4,6,7] IL-4[1-4,6,7] IL-5[1-4,7] IL-6[1-3,5] IL-10[1-5,7] IL-12p70**[5]	IL-12/23p40[5] IL-13[1-4,7] IL-17A[1-4,7] IL-17F[1] G-CSF[1-3] GM-CSF[1,3,5] TNF-α[1-3]	24-48h	24h	$2x10^5$ cells/ well	$2x10^3$ to $1x10^5$ cells/well
	Granzyme B[1-4,6]	Perforin[1,2,6]	48-72h	24h	$2x10^5$ cells/ well	$2x10^3$ to $1x10^5$ cells/well
Rodent spleen cells	IFN-γ[1-3] IL-2[1-3] IL-4[1-3] IL-5[1-3]	IL-6[1-3] IL-10[1-3] TNF-α[1-3]	24-48h	24-48h	$2x10^5$ cells/ well	$2x10^2$ to $1x10^5$ cells/well

General cell stimuli commonly used in ELISPOT

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

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

**Preincubation time: 24 - 48h (all cell types); cell concentration $4x10^6$ cells/ml*

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

Notes

Notes

Technical assistance

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

U-CyTech biosciences
Phone: +31.30.253 5960
E-mail: info@ucytech.com

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