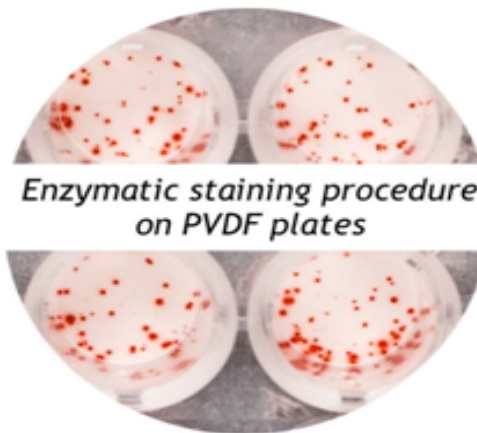


Instruction Manual
ELISPOT kit



*Enzymatic staining procedure
on PVDF plates*

2-plate and 5-plate format



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

This ELISPOT manual applies for the following U-CyTech ELISPOT kits

(please find below the catalogue number of the ELISPOT kit)

Analyte	Species			
	Human	Old World Monkey	Mouse	Rat
IFN-	CT230-PR2 (2-plate)	CT121-PR2 (2-plate)	CT317-PR2 (2-plate)	CT080-PR2 (2-plate)
	CT230-PR2 (5-plate)	CT121-PR5 (5-plate)	CT317-PR5 (5-plate)	CT080-PR5 (5-plate)
		CT126-PR2 (2-plate)		
		CT126-PR5 (5-plate)		
IL-1	CT242-PR2 (2-plate)	CT123-PR2 (2-plate)		
	CT242-PR5 (5-plate)	CT123-PR5 (5-plate)		
IL-2	CT231-PR2 (2-plate)	CT127-PR2 (2-plate)	CT435-PR2 (2-plate)	
	CT231-PR5 (5-plate)	CT127-PR5 (5-plate)	CT435-PR5 (5-plate)	
IL-4	CT232-PR2 (2-plate)	CT128-PR2 (2-plate)	CT319-PR2 (2-plate)	CT081-PR2 (2-plate)
	CT232-PR5 (5-plate)	CT128-PR5 (5-plate)	CT319-PR5 (5-plate)	CT081-PR5 (5-plate)
IL-5	CT233-PR2 (2-plate)	CT129-PR2 (2-plate)	CT321-PR2 (2-plate)	
	CT233-PR5 (5-plate)	CT129-PR5 (5-plate)	CT321-PR5 (5-plate)	
IL-6	CT234-PR2 (2-plate)	CT130-PR2 (2-plate)	CT436-PR2 (2-plate)	
	CT234-PR5 (5-plate)	CT130-PR5 (5-plate)	CT436-PR5 (5-plate)	
IL-10	CT235-PR2 (2-plate)	CT131-PR2 (2-plate)	CT320-PR2 (2-plate)	
	CT235-PR5 (5-plate)	CT131-PR5 (5-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)	CT132-PR2 (2-plate)		
	CT236-PR5 (5-plate)	CT132-PR5 (5-plate)		
IL-17	CT416-PR2 (2-plate)	CT401-PR2 (2-plate)		
	CT416-PR5 (5-plate)	CT401-PR5 (5-plate)		
GM-CSF	CT241-PR2 (2-plate)	CT124-PR2 (2-plate)		
	CT241-PR5 (5-plate)	CT124-PR5 (5-plate)		
Granzyme B	CT229-PR2 (2-plate)			
	CT229-PR5 (5-plate)			
Pertenn	CT681-PR2 (2-plate)	CT136-PR2 (2-plate)		
	CT681-PR5 (5-plate)	CT136-PR5 (5-plate)		
TNF-	CT237-PR2 (2-plate)	CT133-PR2 (2-plate)	CT322-PR2 (2-plate)	
	CT237-PR5 (5-plate)	CT133-PR5 (5-plate)	CT322-PR5 (5-plate)	



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.

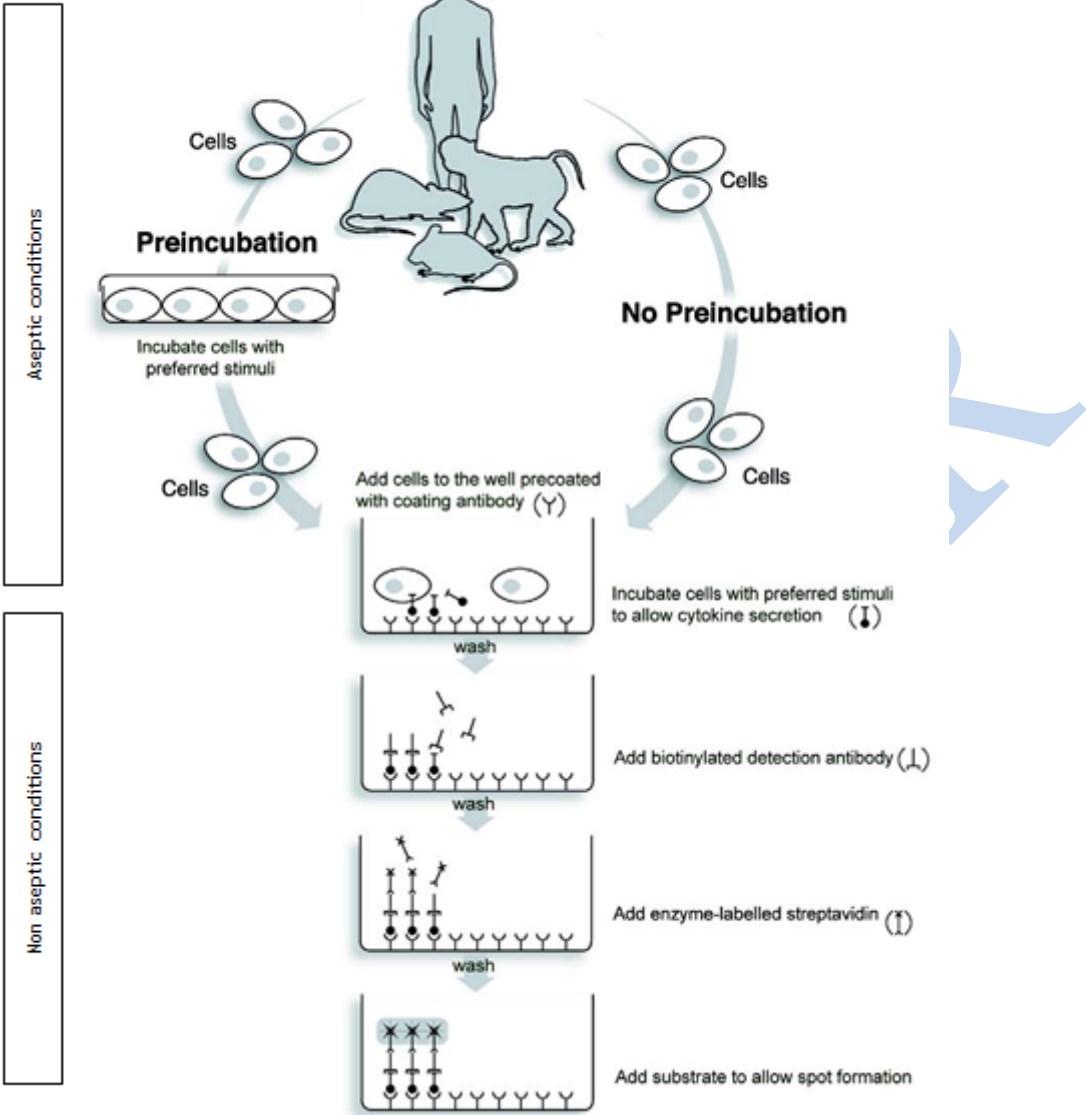
The higher sensitivity of ELISPOT in comparison to that of ELISA¹ or intracellular staining², is due to the plate-bound antibodies directly capturing the cytokine released by the cell before it is diluted in the supernatant, trapped by high-affinity receptors or degraded by proteases. The sensitivity of the assay lends itself to measurement of very low frequencies of cytokine-secreting cells (1/300,000).

1. Tanguay, S. and Killian, J.J. 1994. *Lymphokine Cytokine Res.* 13: 259.
2. Carter, L.L. and Swain, S.L. 1997. *Curr. Opin. Immunol.* 9: 177.

Brief description ELISPOT assay

Cells are incubated in the wells of the ELISPOT plate pre-coated 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. The different steps of the assay are illustrated in the Flow diagram on the next page.


Flow diagram ELISPOT



Contents of kit

Items	Quantity	Quantity
	(2-plate format)	(5-plate format)
Coating antibodies (lyophilized)	1 vial	1 vial
Biotinylated detector antibodies (lyophilized)	1 vial	1 vial
Streptavidin-HRP conjugate (lyophilized)	1 vial	1 vial
AEC coloring system:		
I. AEC stock solution	4 ml	4 ml
II. Substrate buffer capsules	2	5
Blocking stock solution R (10x)	4 ml	10 ml
Dilution buffer R (10x)	4 ml	10 ml
Tween-20	5 ml	5 ml
96-well ELISPOT plate with lid	2*	-
Adhesive cover slip	5	-

* PVDF membrane-bottomed Millipore plates.



Hazard information

Warning:
AEC (3-Amino-9-ethylcarbazole) stock solution is classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EC and its amendments:

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:
H302: Harmful if swallowed. H315: Causes skin irritation.
H319: Causes serious eye irritation. H335: May cause respiratory irritation.
H350: May cause cancer

Precaution statements:
P201: Obtain special instructions before use.
P210: Keep away from heat/sparks/open flames/hot surfaces. - No smoking.
P261: Avoid breathing dust/fume/gas/mist/vapors/sprays.
P280: Wear protective gloves/ protective clothing/eye protection/face protection.
P301 + P312: IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.
P302 + P352: IF ON SKIN: Wash with plenty of soap and water
P305 + P351 + P338: IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.
P308 + P313: IF exposed or concerned: Get medical advice/attention.

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.
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 Regulation (EC) no. 1272/2008 and Directive 67/548/EC or 1999/45/EC and their amendments.

Please find the Material Safety Data Sheet on www.ucytech.com/manuals.

Reagents/materials required but not provided

- PVDF membrane-bottomed plates (for 5-plate kit format): Millipore cat. no. [MSIP_S4510](#).
- Sterile distilled water.
- 70% 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-): [Invitrogen cat. no. 10010-015](#) is recommended.
- [Pipetting devices](#).
- CO₂-incubator (37°C, 100% humidity, 5% CO₂).
- Culture medium: see Addendum ELISPOT assay^{††}.
- Cell stimuli: see Addendum ELISPOT assay^{††}.
- Squirt (wash or squeeze) bottle with wide spout for washing, see Addendum ELISPOT assay^{††}.
- Tissue culture plates for [prestimulation](#) (optional).
- A dissecting microscope or an [immunospot image analyzer](#) for spot counting.

^{††} The accompanying Addendum ELISPOT assay contains guidelines and [troubleshooting for ELISPOT analyses](#). The Addendum ELISPOT assay is also available on our website (www.ucytech.com/manuals) or contact [U-CyTech biosciences \(order@ucytech.com\)](mailto:order@ucytech.com).

Storage reagents

- The vials with lyophilized coating antibodies and [biotinylated](#) detection antibodies can be safely stored at 4°C until the expiry date (indicated on the vials). After reconstitution, the reagents are stable for minimal 6 months at 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 minimal one year.
- The vial with lyophilized [Streptavidin-HRP conjugate](#) should be stored at -20°C until the expiry date (indicated on the vial). After reconstitution, the reagent is stable for minimal 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 -20°C. Under these conditions the reagent is stable for minimal one year.](#)
The reconstituted [Streptavidin-HRP](#) rapidly loses activity when kept at room temperature.
- The AEC stock solution should be protected from light and should be stored at -20°C until the expiry date (indicated on the vial). 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 are stable until the expiry date (indicated on the vial) when stored at room temperature and in a moisture-free environment.
- Blocking stock solution R (10x) and Dilution buffer R (10x) should be stored at 4°C until the expiry date (indicated on the vials). After opening these solutions are stable for minimal 6 months when kept sterile.
- Tween-20 can best be stored at room temperature until the expiry date (indicated on the vials).

Preparation kit reagents

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

1. Coating 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 of the 2-plate format kit, 100 μ l is thoroughly mixed with 5 ml PBS-I.

For one ELISPOT plate of the 5-plate format kit, 50 μ l is thoroughly mixed with 5 ml PBS-I.

2. Blocking buffer R (1x)

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

For one ELISPOT plate, 2 ml is thoroughly mixed with 18 ml PBS-I.

3. Dilution buffer R (1x)

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

For one ELISPOT plate, 2 ml is thoroughly mixed 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, 100 μ l is thoroughly mixed 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, 100 μ l is thoroughly mixed 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, 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 330 μ l 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

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 μ l of 70% ethanol to each well. Incubate for 1 minute at room temperature.
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 μ l of properly 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 μ l PBS-I/well. Subsequently 200 μ l Blocking buffer R (1x) is added to each well. The plate is covered with a lid and incubated for 1 h 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). Bring cells in the wells of the ELISPOT plate. Add 100 μ l/well.

Triplicates of 3×10^5 cells/ml are often used to assess antigen-specific responses. For polyclonal stimuli, the cell number may have to be reduced to $\pm 10^4$ cells/ml. No more than 3×10^5 cells/well should be suspended in the ELISPOT plate. See Addendum ELISPOT assay.

6. Cover ELISPOT plate with lid and incubate at 37°C, 5% CO₂ and 100% humidity. The incubation time can vary from 5 to 24 h. 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 ELISPOT assay.
7. Remove the bulk of cells with a firm shake-out action and wash 2x with PBS of room temperature (200 μ l/well). Thereafter wells are washed 5x with 250 μ l Wash buffer/well (see Addendum ELISPOT assay).
8. Discard wash buffer and add 100 μ l of properly diluted biotinylated detection antibodies to each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C or overnight at 4°C.
9. Decant solution from wells. Remove and discard the underdrain from the bottom of the plate and wash both sides of the PVDF membrane 5x with Wash buffer. Bring 100 μ l of properly diluted Streptavidin-HRP solution into each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C.
10. Decant solution from wells. Wash both sides of the PVDF membrane 5x with Wash buffer.
11. Add 100 μ l 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 demineralised water.
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.

For further information, please visit our website or contact U-CyTech biosciences (info@ucytech.com).

