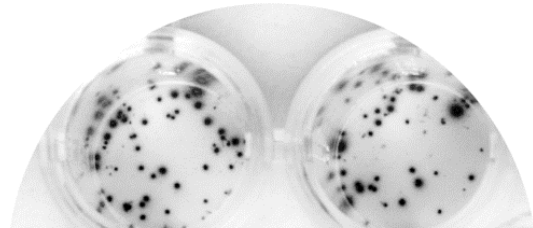


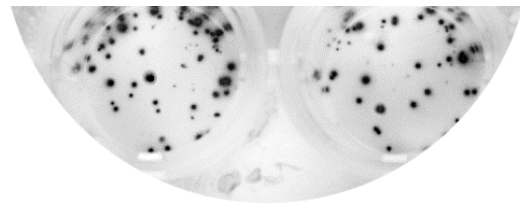


Manufactured By: U-CyTech Biosciences

Instruction Manual 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.



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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- γ	ACT230-PB2 (2-plate)	ACT121-PB2 (2-plate)	ACT317-PB2 (2-plate)	ACT080-PB2 (2-plate)
	ACT230-PB2 (5-plate)	ACT121-PB5 (5-plate)		
IL-1B	ACT242-PB2 (2-plate)	ACT123-PB2 (2-plate)	ACT317-PB5 (5-plate)	ACT080-PB5 (5-plate)
	ACT242-PB5 (5-plate)	ACT123-PB5 (5-plate)		
IL-2	ACT231-PB2 (2-plate)	ACT127-PB2 (2-plate)	ACT435-PB2 (2-plate)	
	ACT231-PB5 (5-plate)	ACT127-PB5 (5-plate)	ACT435-PB5 (5-plate)	
IL-4	ACT232-PB2 (2-plate)	ACT128-PB2 (2-plate)	ACT319-PB2 (2-plate)	
	ACT232-PB5 (5-plate)	ACT128-PB5 (5-plate)	ACT319-PB5 (5-plate)	
IL-5	ACT233-PB2 (2-plate)	ACT129-PB2 (2-plate)	ACT321-PB2 (2-plate)	
	ACT233-PB5 (5-plate)	ACT129-PB5 (5-plate)	ACT321-PB5 (5-plate)	
IL-6	ACT234-PB2 (2-plate)	ACT130-PB2 (2-plate)	ACT436-PB2 (2-plate)	
	ACT234-PB5 (5-plate)	ACT130-PB5 (5-plate)	ACT436-PB5 (5-plate)	
IL-10	ACT235-PB2 (2-plate)	ACT131-PB2 (2-plate)	ACT320-PB2 (2-plate)	
	ACT235-PB5 (5-plate)	ACT131-PB5 (5-plate)	ACT320-PB5 (5-plate)	
IL-12/23p40		ACT135-PB2 (2-plate)		
		ACT135-PB5 (5-plate)		
IL-12p70	ACT240-PB2 (2-plate)			
	ACT240-PB5 (5-plate)			
IL-13	ACT236-PB2 (2-plate)	ACT132-PB2 (2-plate)		
	ACT236-PB5 (5-plate)	ACT132-PB5 (5-plate)		
IL-17	ACT416-PB2 (2-plate)	ACT401-PB2 (2-plate)		
	ACT416-PB5 (5-plate)	ACT401-PB5 (5-plate)		
GM-CSF	ACT241-PB2 (2-plate)	ACT124-PB2 (2-plate)		
	ACT241-PB5 (5-plate)	ACT124-PB5 (5-plate)		
Granzyme B	ACT229-PB2 (2-plate)			
	ACT229-PB5 (5-plate)			
Perforin	ACT681-PB2 (2-plate)	ACT136-PB2 (2-plate)		
	ACT681-PB5 (5-plate)	ACT136-PB5 (5-plate)		
TNF- α	ACT237-PB2 (2-plate)	ACT133-PB2 (2-plate)	ACT322-PB2 (2-plate)	
	ACT237-PB5 (5-plate)	ACT133-PB5 (5-plate)	ACT322-PB5 (5-plate)	

Intended use

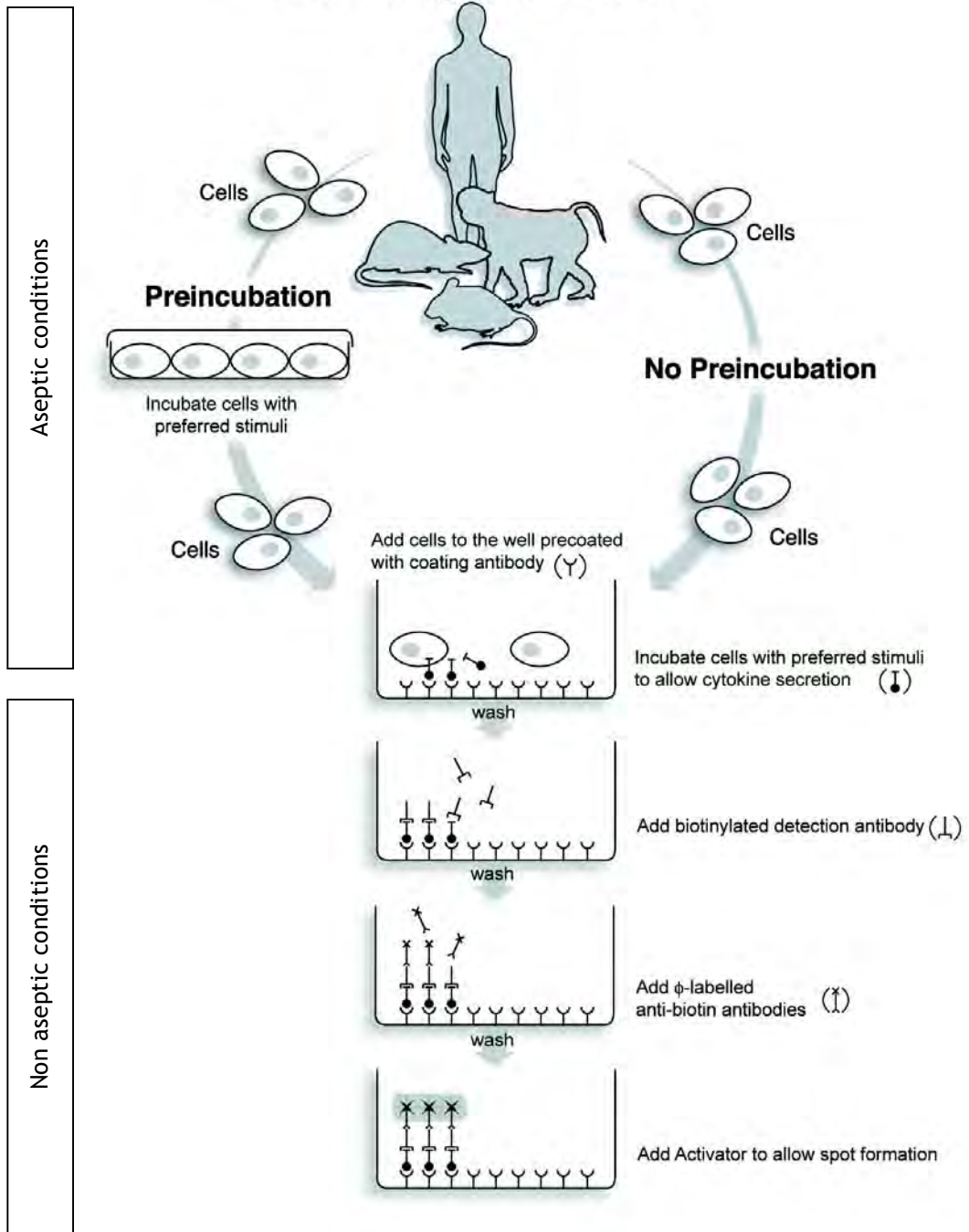
The cytokine ELISPOT (Enzyme-Linked ImmunoSPOT) assay is designed to enumerate cytokine-secreting cells in single cell suspensions of lymphoid tissue, central nerve system (CNS) tissue, bone marrow or preparations of peripheral blood mononuclear cells (PBMCs). The assay has the advantage of detecting 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 Killion, 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 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 ϕ -labeled goat anti-biotin antibodies. The last step in the assay is the addition of a reagent allowing the precipitation of silver on ϕ revealing the site of cytokine secretion (i.e spot formation). The different steps of the assay are illustrated in the Flow diagram on the next page.

Flow diagram ELISPOT



Contents of kit

Items	Quantity (2-plate format)	Quantity (5-plate format)
Coating antibodies (lyophilized)	1 vial	1 vial
Biotinylated detector antibodies (lyophilized)	1 vial	1 vial
φ-labeled anti-biotin antibodies (GABA) (lyophilized)	1 vial	1 vial
Activator I	4 ml	9.5 ml
Activator II	4 ml	9.5 ml
Blocking stock solution B (10x)	4 ml	10 ml
Dilution buffer B (10x)	3.5 ml	8 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:

Activator I+II solutions are classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EC and its amendments:

Serious eye damage (Category 1)

Skin sensitization (Category 1)

Chronic aquatic toxicity (Category 1)

Hazard statements:

H317: May cause an allergic skin reaction. H318: Causes serious eye damage.

H410: Very toxic to aquatic life with long lasting effects.

Precaution statements:

P273: Avoid release to the environment.

P280: Wear protective gloves/protective clothing/eye protection/face protection.

P305 + P351 + P338: IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.

P501: Dispose of contents/container to an approved waste disposal plant.

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

Toxic to aquatic organisms; avoid release in the environment.

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

** 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) or contact U-CyTech biosciences (order@ucytech.com).

Storage reagents

- The vials with lyophilized coating antibodies, biotinylated detection antibodies and GABA 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 Activators I and II should be protected from light and stored at 4°C until the expiry date (indicated on the vials). Since the reagents are susceptible to oxidation by air, it is important that after use, the vials are tightly closed. It is recommended to divide the solutions into small aliquots for single use. These aliquots should be stored at ≤ -20°C in the dark. Frozen samples are stable for at least two years.
- Blocking stock solution B (10x) and Dilution buffer B (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 µl is thoroughly mixed with 5 ml PBS-I.

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

2. Blocking buffer B (1x)

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

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

3. Dilution buffer B (1x)

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

For one ELISPOT plate, 1.5 ml is thoroughly mixed with 13.5 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 µl is thoroughly mixed with 10 ml Dilution buffer B (1x).

5. GABA (φ-labeled anti-biotin 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 µl is thoroughly mixed with 5 ml Dilution buffer B (1x).

6. Activators

For one ELISPOT plate, mix gently but thoroughly 1.8 ml of Activator I with 1.8 ml Activator II. Keep temperature at 4°C during mixing. Use immediately thereafter.

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 µl 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 µl 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 µl PBS-I/well. Subsequently 200 µl Blocking buffer B (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 µl/well.

Triplicates of 3×10^6 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 µl/well). Thereafter wells are washed 5x with 250 µl Wash buffer/well (see Addendum ELISPOT assay).
8. Discard wash buffer and add 100 µl of properly diluted biotinylated detection antibodies to each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C or overnight at 4°C.
9. Decant solution from wells. Remove the underdrain from the bottom of the plate and wash both sides of the PVDF membrane 5x with 250 µl Wash buffer/well. Bring 50 µl of properly diluted GABA solution into each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C (100% humidity).
10. Decant solution from wells. Wash both sides of the PVDF membrane 5x with 250 µl Wash buffer/well.
11. Add 35 µl of freshly prepared Activator I/II solution to each well. Uniformly distribute the Activator I/II solution over the well. Cover plate with lid and incubate at room temperature in the dark.
12. Monitor spot development by light microscopy (from 25 to 30 minutes). When clear spots have developed, stop the reaction by rinsing the wells with demineralised water.
13. Air dry the plate at room temperature and count spots by use of an dissecting 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 for indefinitely when the plate is stored at a dry place.