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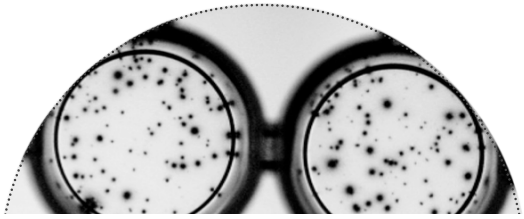
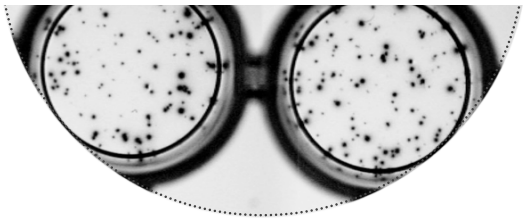
Instruction Manual
Human Granzyme B ELISPOT kit



Silver staining procedure on transparent 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)

	Species			
Analyte	Human	Old World Monkey	Mouse	Rat
Granzyme B	CT229-T2 (2-plate) CT229-T5 (5-plate)			

Intended use

The ELISPOT (Enzyme-linked Immunospot) assay has been designed to identify and enumerate individual cells releasing specific proteins in single cell suspensions of lymphoid tissue, CNS tissue, bone marrow or preparations of peripheral blood mononuclear cells (PBMC). The assay is being used increasingly to detect activated T cells and macrophages secreting cytokines, B cells releasing antibodies and activated cytotoxic T cells (CTLs) and NK cells secreting perforin and/or a family of granule-associated serine proteases such as granzyme A and B. The major advantage of the ELISPOT assay is that it detects only activated cells at the single cell level, allowing direct determination of secretory cell frequencies. The high sensitivity and easy performance, without prior in vitro expansion, makes the ELISPOT assay an attractive tool to enumerate cells producing a particular effector molecule during treatment or pathological conditions. The higher sensitivity of ELISPOT in comparison to that of ELISA[1] or intracellular staining[2] 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 effector molecule, produced during incubation, will bind. Subsequently, cells are washed away. Areas in which the molecule has been trapped are detected with a combination of biotinylated 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 effector molecule secretion (i.e spot formation). The different steps of the assay are illustrated in the Flow diagram on the next page.

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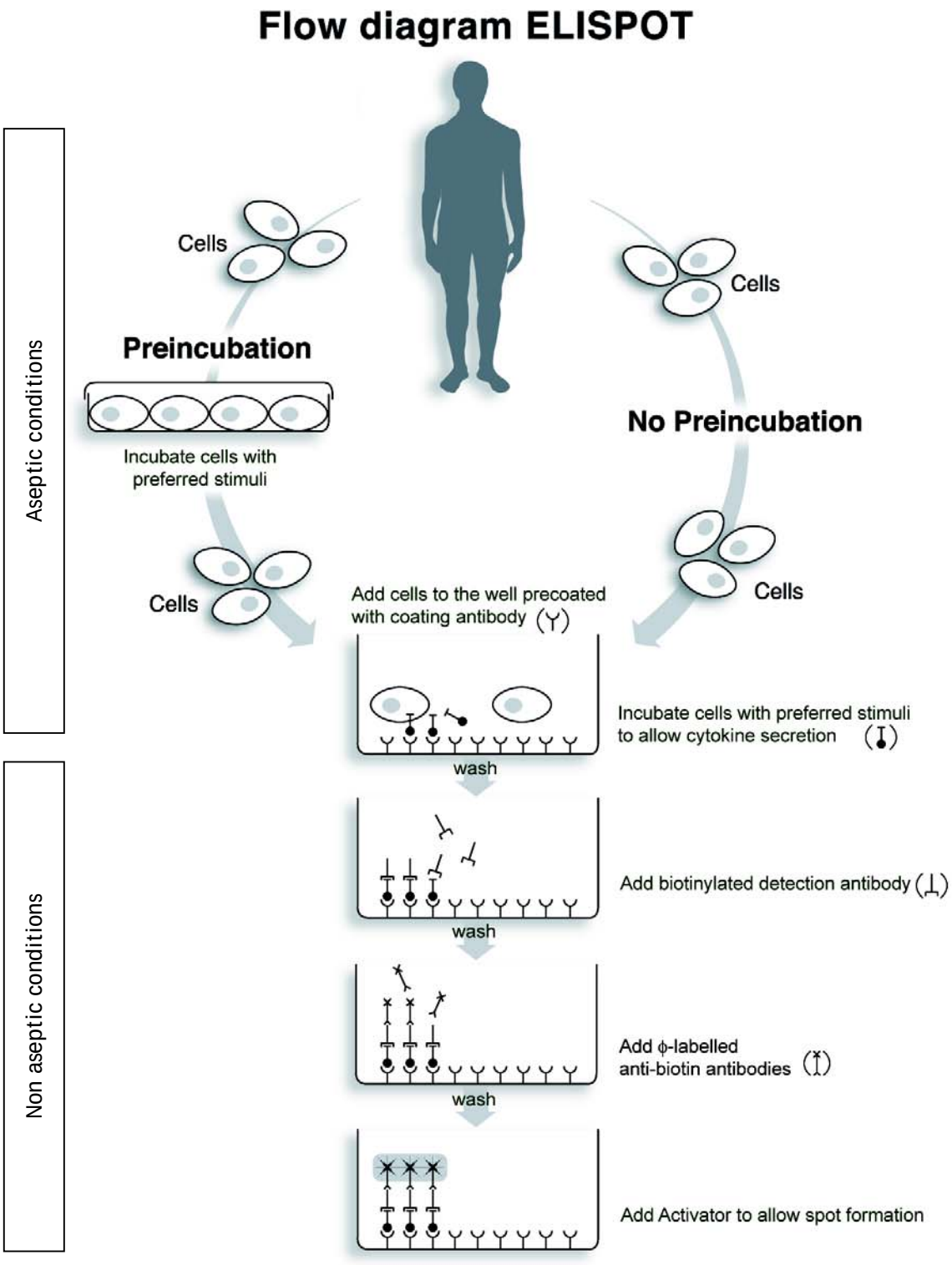
Flow diagram ELISPOT



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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
Coating buffer (10x)	8 ml	8 ml
Blocking stock solution B (10x)	4 ml	10 ml
Dilution buffer T (10x)	3.5 ml	8 ml
Tween-20	5 ml	5 ml
96-well ELISPOT plate* with lid	2	6
Adhesive cover slip	5	10

* Transparent polystyrene-bottomed Nunc MaxiSorp 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.

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Reagents/materials required but not provided

- Sterile distilled water.
- 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.
- CO_2-incubator (37°C, 100% humidity, 5% CO_2).
- Culture medium: see Addendum**.
- Cell stimuli: see Addendum**.
- Plate washer: automated or manual, see Addendum**.
- Tissue culture plates for prestimulation (optional).
- An inverted 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.
- Coating buffer (10x), Blocking stock solution B (10x) and Dilution buffer T (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).

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Preparation kit reagents

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

1. Coating buffer (10x)

   Dilute Coating buffer (10x) in sterile distilled water.

   For one ELISPOT plate, 1 ml is thoroughly mixed with 9 ml sterile distilled water.

2. 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 Coating buffer (1x).

   For one ELISPOT plate of the 5-plate format kit, 50 µl is thoroughly mixed with 5 ml Coating buffer (1x).

3. 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.

4. Dilution buffer T (1x)

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

   For one ELISPOT plate, 1.5 ml is thoroughly mixed with 13.5 ml PBS-I.

5. 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 T (1x).

6. GABA (f-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 T (1x).

7. Activators

   Allow the two bottles to reach room temperature. Gently shake the bottles well, before mixing 1.8 ml of Activator I with 1.8 ml Activator II (for one ELISPOT plate). Use immediately thereafter.

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ELISPOT method

Use ELISPOT plates and reagents under aseptic conditions (e.g. Laminar Flow Hood) for steps 1 to 4.

1. Add 50 µl of properly diluted coating antibodies into each well and fill up to 100 µl/well with Coating buffer (1x). Cover the plate with a lid and incubate overnight at 4°C.
2. 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.
3. 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 $3x10^6$ 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 $3x10^5$ cells/well should be suspended in the ELISPOT plate. See Addendum ELISPOT assay.
4. Cover ELISPOT plate with lid and incubate at 37°C, 5% CO_2, and 100% humidity. The incubation time can vary from 5 to 24 h. Specific activation conditions will vary, depending on cell type, effector molecule of interest, kinetics of molecule release and whether a preincubation step was included in the procedure. See Addendum ELISPOT assay.
5. 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 6x with 250 µl Wash buffer/well (see Addendum ELISPOT assay).
6. 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.
7. Decant solution from wells. Wash wells 6x 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.
8. Decant solution from wells. Wells are washed 6x with 250 µl Wash buffer/well and subsequently emptied by a firm 'shake-out' action (wells should not contain residual Wash buffer).
9. 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.
10. Monitor spot development by light microscopy (from 30 to 60 minutes). When clear spots have developed, stop the reaction by rinsing the wells with demineralised water.
11. Air dry the plate at room temperature and count spots by use of an inverted 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.

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