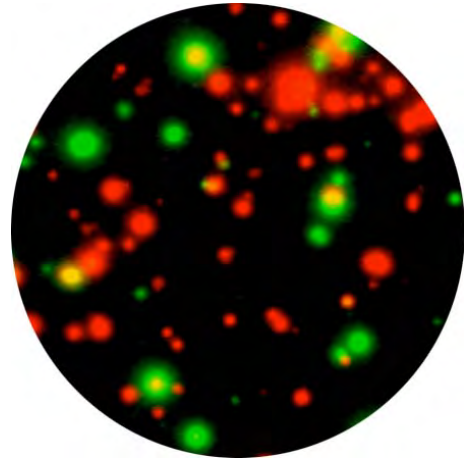




Manufactured By: U-CyTech Biosciences

Instruction Manual FluoroSpot kit



2-plate format



For research use only.

Not for use in diagnostic or therapeutic procedures.



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This FluoroSpot manual applies for the following U-CyTech FluoroSpot kits
(please find below the catalogue number of the FluoroSpot kit)

Analyte	Species	
	Human	Old World Monkey
IFN- γ / IL-2	ACT852-F2	ACT902-F2
IFN- γ / IL-5	ACT853-F2	ACT903-F2
IFN- γ / IL-10	ACT850-F2	ACT900-F2
IFN- γ / IL-13	ACT854-F2	ACT904-F2
IFN- γ / IL-17	ACT855-F2	ACT905-F2
IFN- γ / TNF- α	ACT851-F2	ACT901-F2

Intended use

The dual-color cytokine FluoroSpot assay is a modification of the Enzyme-Linked ImmunoSPOT (ELISPOT) assay and is designed to detect double cytokine-secreting T cells at the single cell level. The assay is based on the use of fluorescent- instead of enzymatic-labeled conjugates. Previous attempts to develop an immunoenzymatic dual-color ELISPOT assay were less successful because of problematic interpretation of mixed color spots. The dual-color FluoroSpot assay, however, provides good discrimination between single and double cytokine-secreting cells and is particularly suited to detect T cell subpopulations with a characteristic cytokine profile. The sensitivity of the assay lends itself to measure very low frequencies of cytokine-secreting T cells (1/300,000).

Brief description dual FluoroSpot assay

Cells are incubated in the wells of the FluoroSpot plate precoated with two high-affinity antibodies specific for different cytokines. After binding of the released cytokines, cells are washed away. Areas in which the cytokines have been captured by the two coating antibodies are detected by incubation with a mixture of two different anti-cytokine detector antibodies followed by an incubation step with two fluorescent-labeled conjugates. The generation of spots that emit light at different wavelengths enables the precise localization of single and double cytokine-producing cells. Green and red fluorescent spots represent cells that produce one cytokine of interest whereas yellow spots originate from cells that release both cytokines of interest.

Contents of kit

Items	Quantity (2-plate format)
Coating antibodies (lyophilized)	2 vials
Detector antibodies (lyophilized)	2 vials
Fluorescent-labeled conjugate (lyophilized)	1 vial
Blocking stock solution R (10x)	4 ml
Dilution buffer R (10x)	4 ml
Tween-20	5 ml
Spot enhancer (4x)	6 ml
96-well FluoroSpot plate with lid	2
Adhesive cover slip	5

Hazard information

The items in this kit are not classified as hazardous according to Regulation (EC) no. 1272/2008 and to Directives 67/548/EC and 1999/45/EC and their amendments.

Please find the Material Safety Data Sheet on www.aniara.com.

Reagents/materials required but not provided

- 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.
- Culture medium: see Addendum FluoroSpot*.
- Cell stimuli: see Addendum FluoroSpot*.
- Pipetting devices.
- Squirt (wash or squeeze) bottle with wide spout for washing, see Addendum FluoroSpot*.
- CO₂-incubator (37°C, 100% humidity, 5% CO₂).
- Tissue culture plates for prestimulation.
- A fluorescent microscope or a fluorescent immunospot image analyzer for spot counting. The devices have to be equipped with a multicolor optical filter set for FITC (Absorption/Emission: 495/519 nm; visualizing green spots) and R-Phycoerythrin (R-PE, Absorption/Emission: 546/578 nm visualizing red spots).

* The accompanying Addendum FluoroSpot contains guidelines and troubleshooting for FluoroSpot analyses. This addendum is also available on our website or contact us (order@ucytech.com).

Storage reagents

- The vials with lyophilized coating antibodies and detector antibodies can be safely stored at 4°C. After reconstitution, the reagents are stable for minimal 6 months at 4°C when kept sterile (protect detector antibodies from exposure to direct light). However, it is strongly recommended to divide the reconstituted reagents into small aliquots for single use. The aliquots with antibodies should be stored at $\leq -20^{\circ}\text{C}$. Under these conditions the reagents are stable for minimal one year.
- The vial with the lyophilized fluorescent-labeled conjugate should be kept at 4°C in the dark. After reconstitution, the reagent is stable for minimal 6 months at 4°C when kept sterile and protected from light. DO NOT FREEZE.
- Blocking stock solution R (10x), Dilution buffer R (10x) and Spot enhancer (4x) should be stored at 4°C. After opening these solutions are stable for minimal 6 months when kept sterile.
- Tween-20 can best be stored at room temperature.

Note : Unopened vials expire at the dates indicated on the labels of the vials.

Preparation kit reagents

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

1. Coating antibodies (2 vials)

Reconstitute the contents of each vial by injecting 200 μl sterile water. Mix gently and allow them to stand for 2 minutes at room temperature.

For one FluoroSpot plate, mix 100 μl from both vials with 5 ml PBS-I.

2. Blocking buffer R (1x)

For one FluoroSpot plate, mix 2 ml of Blocking stock solution R (10x) with 18 ml PBS-I.

3. Dilution buffer R (1x)

For one FluoroSpot plate, mix 2 ml Dilution buffer R (10x) with 18 ml PBS-I.

4. Detector antibodies (2 vials)

Reconstitute the contents of each vial by injecting 200 μl sterile water. Mix gently and allow both vials to stand for 2 minutes at room temperature.

For one FluoroSpot plate, mix 100 μl from both vials with 10 ml Dilution buffer R (1x).

5. Fluorescent-labeled conjugate

Reconstitute the contents of the vial by injecting 200 μl sterile water. Mix gently and allow it to stand for 2 minutes at room temperature.

For one FluoroSpot plate, mix 100 μl with 10 ml Dilution buffer R (1x).

6. Spot enhancer (4x)

For one FluoroSpot plate, 2.5 ml Spot enhancer (10x) is thoroughly mixed with 7.5 ml PBS-I.

FluoroSpot method

Use FluoroSpot plates and reagents under aseptic conditions (e.g. Laminar Flow Hood) for steps 1 to 7.

1. Prestimulate the cells with appropriate stimulus. See Addendum Fluorospot for details.
2. Prewet the plate membrane by adding 25 μ l of 70% ethanol to each well. Incubate for 1 minute at room temperature.
3. Aspirate or firmly shake-out the ethanol. Immediately thereafter wells are rinsed 2x with 200 μ l PBS-I. The plate is subsequently emptied and tapped on tissue paper.
4. Add 50 μ l of the diluted mixture of two coating antibodies into each well. Cover the plate with a lid and incubate overnight at 4°C. Best results are obtained by a 40 hour incubation. Make sure the membrane does not dry out.
5. Decant solution from wells. Wash 3x with 200 μ l PBS-I per well. Subsequently add 200 μ l Blocking buffer R (1x) to each well. The plate is covered with a lid and incubated for 1 hour at 37°C.
6. Decant solution from wells (do not wash the wells). Dilute the prestimulated cells in culture medium containing an appropriate stimulus (polyclonal or antigen). Bring 100 μ l of the cell suspension in the wells of the FluoroSpot plate.

Triplicates of 2×10^6 cells/ml are often used to assess antigen-specific responses. No more than 3×10^6 cells/ml should be suspended in each well. For polyclonal stimuli, the cell number may have to be reduced to approximately 1×10^5 cells/ml. See Addendum FluoroSpot.

7. Cover FluoroSpot plate with lid and incubate at 37°C, 5% CO₂, and 100% humidity. The incubation time is 20-24 hours.
8. Remove the bulk of cells with a firm shake-out action and wash 2x with PBS at room temperature (200 μ l/well). Thereafter wells are washed 5x with Wash buffer with a squirt bottle with a wide spout (see Addendum FluoroSpot).
9. Discard wash buffer and add 100 μ l of diluted mixture of the two detector antibodies into each well. Seal the plate with an adhesive cover slip and incubate 1 hour at 37°C or overnight at 4°C. Incubation should be performed in the dark.
10. Decant solution from wells. Remove and discard the underdrain of the plate and wash both sides of the membrane 5x with Wash buffer (use squirt bottle with wide spout). Tap dry, and add 100 μ l of properly diluted fluorescent-labeled conjugate into each well. Seal the plate with an adhesive cover slip and incubate 1 hour at 37°C in the dark.
11. Decant solution from wells. Wash both sides of the membrane 5x with Wash buffer. Tap dry and add 100 μ l/well of diluted Spot enhancer to each well. Incubate for 15 minutes at room temperature. Discard Spot enhancer solution and remove residual fluid by tapping on tissue paper. Wash underside of the membrane (not inside the wells) 2x with demineralised water and dry plate for 5 minutes at 37°C in the dark.
12. Analyze the spots by use of a fluorescent microscope or a fluorescent immunospot image analyzer.

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