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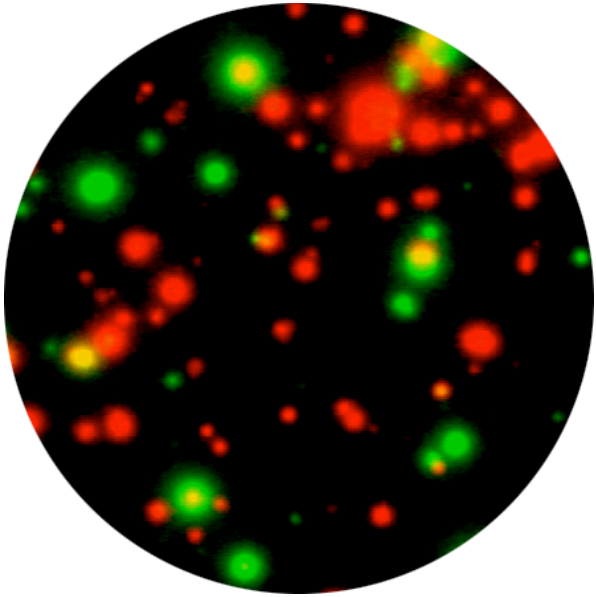
Instruction Manual FluoroSpot kit



2-plate format

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

Contents

Abbreviations

APC	Antigen-Presenting Cell
CD	Cluster of Differentiation
ConA	Concanavalin A
ELISPOT	Enzyme-Linked ImmunoSPOT
FCS	Fetal Calf Serum
ICE	Influenza A, Cytomegalovirus, Epstein-Barr virus
IFN	Interferon
IL	Interleukin
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
PVDF	Polyvinylidene fluoride
RT	Room Temperature (temperature between 20 °C and 26 °C)
sec	seconds

Catalogue numbers FluoroSpot kits

This manual applies to the following FluoroSpot kits

Analytes	Human	Old World Monkey
IFN-γ / Granzyme B	CT849-F2	
IFN-γ / IL-1β	CT856-F2	
IFN-γ / IL-2	CT852-F2	CT902-F2
IFN-γ / IL-4	CT857-F2	
IFN-γ / IL-5	CT853-F2	CT903-F2
IFN-γ / IL-6	CT858-F2	
IFN-γ / IL-10	CT850-F2	CT900-F2
IFN-γ / IL-13	CT854-F2	CT904-F2
IFN-γ / IL-17A	CT855-F2	CT905-F2
IFN-γ / TNF-α	CT851-F2	CT901-F2

Introduction

The 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, which can be visualized by fluorescence microscopy or by a fluorescent immunospot image analyzer equipped with narrow-band fluorophore filters. Fluorescent detection is as sensitive as ELISPOT, or even more sensitive, and also enables the detection of very low frequencies of cytokine secreting cells (1/300,000). With use of different coloring dyes (Alexa 488 [green] and R-PE [red]) single and double-secreting (yellow spots) cells can be discriminated, what makes the assay particularly suited to detect distinct T cell subpopulations with a characteristic cytokine profile. Another advantage is the need for less sample material.

The FluoroSpot Assay is performed on transparent, flat-bottomed, 96-well plates with PVDF-membranes designed for low autofluorescence. The fractal surface and hydrophobic properties of the PVDF-membrane are ideal for strong binding of two different capture antibodies to the membrane surface. The subsequent interaction of cell-secreted proteins with these antibodies forms the first step in spot formation. For the visualization of spots, the U-CyTech's ELISPOT assay makes use of a mixture of detection antibodies conjugated with FITC and biotin. Secondly, spots are detected by adding a mixture of a fluorescent-labeled conjugate containing Alexa 488-labeled anti-FITC antibodies and R-PE-labeled-streptavidin. Spot Enhancer is used to improve the fluorescent signal.

References

- Okamoto *et al.* (1998). Development of a dual color enzyme-linked immunospot assay for simultaneous detection of murine T helper type 1- and T helper type-2 cells. Immunopharmacology 39, 107-116.
- Boulet *et al.* (2007). A dual color ELISPOT method for the simultaneous detection of IL-2 and IFN-gamma HIV-specific immune responses. J Immunol Method. 320: 18-29.
- Gazagne *et al.* (2003). A FluoroSpot assay to detect single T lymphocytes simultaneously producing multiple cytokines. J Immunol Method. 283: 91-98.

Brief description FluoroSpot assay

The dual-color cytokine FluoroSpot assay is a modification of the ELISPOT assay and is designed to detect double protein-secreting T cells at the single cell level.

The FluoroSpot assay procedure is illustrated in the "Flow diagram FluoroSpot" on page 6. First, cells are collected from a donor. The cells are preincubated (24-48 hours) before they are added to the wells of the FluoroSpot plate that is coated with two high affinity antibodies*. 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 mixture of two different detection 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 protein-producing cells. Green and red fluorescent spots represents cells that produce one protein of interest whereas yellow spots originate from cells that release both proteins of interest.

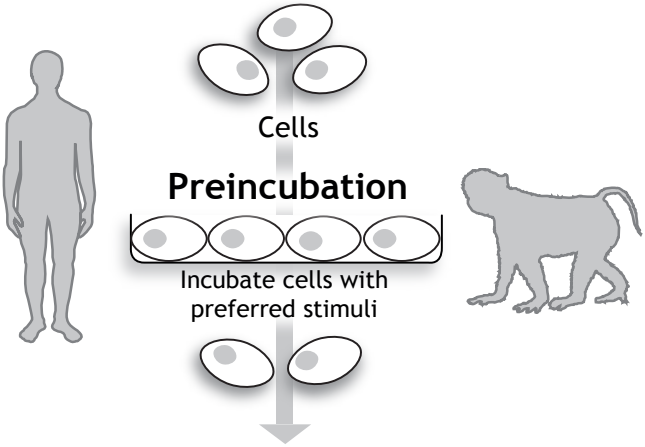
A preincubation step (24-48 hours) is required for optimal results. Antigens must first be internalized, processed and presented by antigen-presenting cells (APCs) via MHC class I/II molecules before they can stimulate protein (e.g. cytokine) release by T cells. Omitting this step leads to a significant lower frequency of spot forming cells and/or small sized spots. Read "Cell sample preparation" on page 12 for more information.

* specific for two different proteins of interest

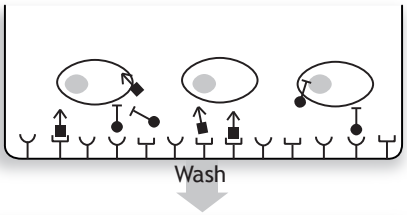
Flow diagram FluoroSpot

Sterile conditions

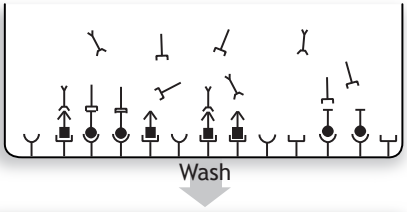
Non-sterile conditions



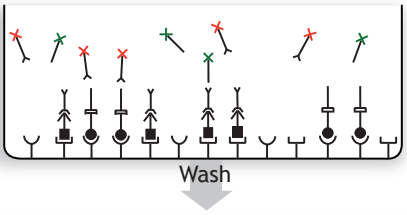
Add cells to the well precoated with coating antibodies



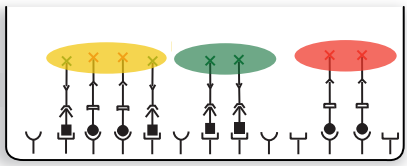
Incubate with preferred stimuli to allow protein (e.g. cytokine) secretion



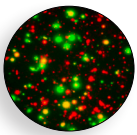
Remove cells, wash and incubate with detection antibodies



Wash and incubate with fluorescent-labeled conjugate



Wash and incubate with spot enhancer



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 Dilution buffer R, the items in this kit are not classified as dangerous according to Regulation (EC) no. 1272/2008 and its amendments.

Dilution buffer R: 

Warning:
Dilution buffer R is classified as irritating according to Regulation (EC) no. 1272/2008 and its amendments: Skin irritation (Category 2), Eye irritation (Category 2), Skin sensitization (Category 1), Chronic aquatic toxicity (Category 3).

Hazard statements: Causes skin irritation (H315), May cause an allergic skin reaction (H317), Causes serious eye irritation (H319), Harmful to aquatic life with long lasting effects (H412).

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

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

Contents of the kit

Items	Quantity (2-plate format)	Storage conditions
Coating antibodies*	2 vials	4°C
Detection antibodies*	2 vials	4°C in the dark
Fluorescent-labeled conjugate*	1 vial	4°C in the dark
Blocking stock solution (10x)	4 ml	4°C
Dilution buffer R (10x)	4 ml	4°C
Tween-20	5 ml	RT
Spot enhancer (4x)	6 ml	4°C
96-well FluoroSpot plate** with lid	2	RT
Adhesive cover slip	5	RT

* Lyophilized

** IPFL PVDF membrane-bottomed Millipore plates (cat. no. S5EJ104107).

Storage and stability

Coating antibodies and detection antibodies
The vials with lyophilized coating and detection antibodies can be safely stored at 4°C in the dark 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 in the dark (stable for at least two years).

Fluorescent-labeled conjugate
The vial with lyophilized fluorescent-labeled conjugate should be stored at 4 °C in the dark until the expiry date (indicated on the vial). After reconstitution, the reagent is stable for at least 6 months at 4°C when kept sterile and protected from light. Do NOT FREEZE.

Blocking stock solution (10x), Dilution buffer R (10x) and Spot enhancer
The vials with Blocking stock solution (10x) and Dilution buffer R (10x) and Spot enhancer (4x) 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 be safely stored at RT and is stable until the expiry date (indicated on the vial).

Materials and equipment (required but not provided)

- 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®).
- Cell stimuli, see “Cell sample preparation” on page 12 and www.ucytech.com/ELISPOT-stimuli.
- Pipetting devices.
- For washing: squirt (wash or squeeze) bottle with wide sprout, see Addendum*.
- CO_2 incubator (37°C, 100% humidity, 5% CO_2).
- 37°C incubator.
- 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 Assay” contains guidelines and troubleshooting for FluoroSpot analyses

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 FluoroSpot plate: 2 ml is gently but thoroughly mixed with 18 ml PBS-I.

Dilution buffer R (1x)
Dilute Dilution buffer R (10x) in PBS-I.
For one FluoroSpot plate: 2 ml is gently but thoroughly mixed with 18 ml PBS-I.

Coating antibodies (2 vials)
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 FluoroSpot plate (2-plate kit): 100 µl from both vials is gently but thoroughly mixed with 5 ml PBS-I.

Detection antibodies (2 vials)
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 FluoroSpot plate: 100 µl is gently but thoroughly mixed with 10 ml Dilution buffer R (1x).

Fluorescent-labeled 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 FluoroSpot plate: 100 µl is gently but thoroughly mixed with 10 ml Dilution buffer R (1x).

Spot enhancer (1x)
Dilute Spot enhancer (4x) in PBS-I.
For one FluoroSpot plate: 2.5 ml Spot enhancer is thoroughly mixed with 7.5 ml PBS-I.

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 FluoroSpot plate: prepare 1 L PBS.

Wash buffer
PBS containing 0.05% Tween-20.
For one FluoroSpot plate: 0.5 ml Tween-20 is gently but thoroughly mixed with 1 L PBS.

Cell sample preparation

Both fresh and cryopreserved cells can be used for FluoroSpot analysis. Guidelines for specimen collection and handling are described in the "Addendum FluoroSpot 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.

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 a Fluorospot well may already be sufficient to trigger TNF-α and IL-6 release.

Assay controls
Before starting an FluoroSpot experiment, proper assay controls need to be chosen, which is mainly dependent on the selected analytes, targeted cell types 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 FluoroSpot stimuli and the recommended concentration ranges can be found in our "Addendum FluoroSpot Assay" and on page 15. 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.

Cell culture conditions preincubation

A 24-42 hours preincubation step at high cell density (> 10^6) is required for full-length proteins or peptides. 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 24-42 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 $1\text{-}3x10^5$ cells/well (antigen-specific responses). For polyclonal stimulation, the recommended cell concentration per well should be reduced to $2x10^3$ - 10^5 cells per well.

NOTES:

- It is recommended to test the samples in triplicate and in serial dilutions in the FluoroSpot procedure.
- No more than $3x10^5$ cells/well should be suspended in the FluoroSpot 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 15 for more information.

FluoroSpot procedure

All solutions should be at RT prior to use. 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 (200 µl/well). The plate is subsequently emptied and tapped on tissue paper.
3. Add 50 µl of the diluted mixture of two coating antibodies into each well of the FluoroSpot 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 1 hour at RT. During this incubation step start preparing the cell sample preparations (see “Cell sample preparation” on page 12).
8. If the cell preparations are ready, decant the blocking buffer from wells (do not wash the wells).
9. Bring the cell preparations into the wells of the FluoroSpot plate. Add 100 µl/well.
10. Cover FluoroSpot plate with lid and incubate at 37°C, 5% CO_2 and 100% humidity. The incubation time is approximately 24 hours.
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 Fluorospot assay’’ for directions on washing).
13. Add 100 µl of diluted mixture of the two detection antibodies into each well.
14. Seal the plate with adhesive cover slip and incubate 2 hours at RT in the dark (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 100 µl diluted fluorescent-labeled conjugate into each well.
17. Seal the plate with an adhesive cover slip and incubate 1 hour at RT in the dark.
18. Empty plate and wash both sides of the PVDF membrane 5x with Wash buffer.

19. Add 100 µl of diluted Spot enhancer to each well.

20. Cover plate with lid and incubate for 15 min at RT in the dark.

21. Empty plate and remove residual fluid by tapping on tissue paper. Wash underside of the PVDF membrane (not inside the wells) 2x with demineralized water.

22. Dry the plate for 5 min at 37°C (in the dark).

23. Count spots by use of a fluorescent microscope or a fluorescent immunospot image analyzer.

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

Guidelines for cell concentrations and incubation times

Cell type	Analytes[stimuli]	Preincubation time	Incubation time after preincubation	Cell concentration antigen stimulation	Cell concentration polyclonal stimulation
Human and Old world Monkey PBMCs	IFN-γ / Granzyme B [1-4,6] IFN-γ / IL-4 [1-4,6,7] IFN-γ / IL-5 [1-4,7] IFN-γ / IL-10 [1-4,7]	48h	24h	$2x10^5$ cells/ well	$2x10^3$ to $1x10^5$ cells/well
	IFN-γ / IL-1ß [1] IFN-γ / IL-2 [1-4,6,7] IFN-γ / IL-6 [1-3] IFN-γ / IL-13 [1-4,7] IFN-γ / IL-17A [1-4,7] IFN-γ / TNF-α [1-3]	24-48h	24h	$2x10^5$ cells/ well	$2x10^3$ to $1x10^5$ cells/well

General cell stimuli commonly used in FluoroSpot

Polyclonal stimuli: 1. PMA & ionomycin (50 ng/ml & 1 µg/ml) 2. PHA (10-30 µg/ml) 3. ConA: 6-10 µg/ml) 4. α-CD3 & α-CD28 (0.05 µg/ml & 0.05 µg/ml)

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

Technical assistance

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

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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 FluoroSpot kits.