U-CyTech biosciences

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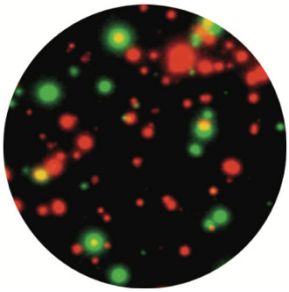
Manual FluoroSpot kit



The dual-color FluoroSpot assay is a modification of the T cell ELISPOT assay, one of the most sensitive assays for monitoring *ex vivo* cellular immune responses at the single cell level. The FluoroSpot assay was developed for the simultaneous detection of two secreted proteins, such as cytokines, which are released by e.g. T cells in response to an antigen. The cell suspensions, used in the assay are usually derived from blood (PBMC).

The corresponding 'Data sheet' (specific to each kit) and 'SDS' can be found at www.ucytech.com. Recommendations for performing of the FluoroSpot assay can be found at www.ucytech.com/frequently-asked-questions-fluorospot.

Contents of the kit

Items	Quantity (2-plate format)	Storage conditions
Coating antibodies*	2 vials	2 to 8 °C
Detection antibodies*	2 vials	2 to 8 °C**
Fluorescent-labeled conjugate*	1 vial	2 to 8 °C**
Blocking stock solution (10x)	1 vial (4 ml)	2 to 8 °C
Dilution buffer (10x)	1 vial (4 ml)	2 to 8 °C
Tween-20	1 vial (5 ml)	RT**
Spot enhancer (4x)	1 vial (6 ml)	2 to 8 °C
96-well FluoroSpot plate*** with lid	2 plates	RT
Adhesive plate covers	5 covers	RT

RT Room temperature (temperature between 20 °C and 26 °C)
* Lyophilized
** Store protected from light
*** PVDF membrane-bottomed plates with low background fluorescence

Warnings

This kit is designed for *research use only* and not for use in diagnostic or therapeutic procedures.

Hazard information

The items in this kit are for professional use only. Follow general safety rules for laboratories.

Except for the Dilution buffer, the items in this kit are not classified as hazardous substances/mixtures according to Regulation (EC) no. 1272/2008 and its amendments.

Classification of hazardous items according to Regulation (EC) no. 1272/2008 and its amendments:

Dilution buffer:



Skin sensitization (Category 1): H317: May cause an allergic skin reaction.

Chronic aquatic toxicity (Category 3): H412: Harmful to aquatic life with long lasting effects.

EUH208: Contains CMIT/MIT. May produce an allergic reaction. EUH210: SDS available on request.

Product contains 0.0273% (w/w) ProClin™ 300.
Avoid exposure to Dilution buffer. Do not let product enter drains.

General first aid measures when handling all kit items:

In case of contact with skin, remove contaminated clothing/shoes and wash contaminated area with water (or shower). Upon swallowing or contact with eyes, rinse mouth (if person is conscious) or eyes with plenty of water for several minutes. Assure adequate flushing by separating the eyelids. After inhalation, provide fresh air.

In case of skin or eye irritation, if breathing becomes difficult or feeling unwell or concerned, consult physician and show SDS of this kit to the doctor in attendance.

Upon swallowing Dilution buffer: Do NOT induce vomiting. Consult physician immediately.

Please find the Safety Data Sheet (SDS) at www.ucytech.com.

Storage and stability

Coating antibodies

The vials with lyophilized coating antibody can be safely stored at 2 to 8 °C until the expiration date (indicated on the vials). After reconstitution, the antibodies are stable at 2 to 8 °C for at least 12 months when kept sterile. However, it is recommended that the reconstituted antibody solutions be divided into small aliquots for single use. These aliquots should be stored at -32 to -18 °C (stable for at least two years).

Detection antibodies

The vials with lyophilized detection antibody are stable until the expiration date (indicated on the vials) when stored protected from light at 2 to 8 °C. After reconstitution, the antibodies are stable at 2 to 8 °C for at least 12 months when kept sterile and protected from light. However, it is recommended that the reconstituted antibody solutions be divided into small aliquots for single use. These aliquots should be stored protected from light at -32 to -18 °C (stable for at least two years).

Conjugate

The vial with lyophilized fluorescent-labeled conjugate is stable until the expiration date (indicated on the vial) when stored at 2 to 8 °C protected from light. After reconstitution, the reagent is stable at 2 to 8 °C for at least 6 months when kept sterile and protected from light. DO NOT FREEZE.

Blocking stock solution, Dilution buffer and Spot enhancer

The vials with Blocking stock solution, Dilution buffer and Spot enhancer can be safely stored at 2 to 8 °C until the expiry date (indicated on the vial). After opening, these solutions are stable for at least 6 months when kept sterile.

Tween-20

Tween-20 can safely be stored at RT (protected from light) and is stable until the expiration date (indicated on the vial).

Materials and equipment (required but not provided)

- Tubes and containers/plates to prepare solutions
- Sterile distilled water and demineralized water
- 70% ethanol
- PBS pH 7.4 (home-made). For washing purposes only. Ingredients: $Na_2HPO_4 \cdot 2H_2O$, KH_2PO_4, NaCl and distilled water.
- Sterile and pyrogen-free liquid PBS pH 7.4 (PBS-I): Thermo Fisher Scientific cat. no. 10010 is recommended. (Do not use PBS tablets. The filler in the tablets interferes with the coating process).
- Cell culture medium: RPMI-1640 supplemented with 2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum.
- Cell stimuli: antigen of interest and positive control.
- Laminar flow hood (for sterile conditions)
- Pipetting devices
- For washing: squirt (wash or squeeze) bottle without sprout.
- CO_2 incubator (37 °C, 100% humidity, 5% CO_2)
- A fluorescent microscope or an automated ELISPOT reader with fluorescent applications for spot counting.

  The device must be equipped with a multicolor optical filter set for FITC (excitation/emission: 495/519 nm; visualizing green spots) and R-Phycoerythrin (R-PE, excitation/emission: 546/578 nm; visualizing red spots).

Preparation solutions and reagents

Note: Prepare reagents under sterile conditions (e.g. laminar flow hood).

PBS (for wash buffer)

5.4 mM $Na_2HPO_4 \cdot 2H_2O$; 1.3 mM KH_2PO_4; 150 mM NaCl in distilled water (adjust to pH 7.4 and filter sterilize [0.2 µm] or autoclave).
For 1 FluoroSpot plate: prepare 1 liter PBS.

Wash buffer

PBS containing 0.05% Tween-20.

For 1 FluoroSpot plate: add 0.5 ml of Tween-20 to 1 liter PBS and mix gently but thoroughly.

Blocking buffer (1x)

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

For 1 FluoroSpot plate: mix 2 ml Blocking stock solution (10x) gently but thoroughly with 18 ml PBS-I.

Dilution buffer (1x)

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

For 1 FluoroSpot plate: mix 2 ml Dilution buffer (10x) gently but thoroughly with 18 ml PBS-I.

Coating antibodies

Reconstitute the lyophilized antibodies by injecting an appropriate volume (indicated on the vial) of sterile distilled water into the vials. Mix the solution gently for approximately 15 sec and allow it to stand at RT for 5 min. Avoid vigorous shaking.

For 1 FluoroSpot plate: 100 µl from both vials is mixed gently but thoroughly with 5 ml PBS-I.

Detection antibodies

Reconstitute the lyophilized antibodies by injecting an appropriate volume (indicated on the vial) of sterile distilled water into the vials. Mix the solution gently for approximately 15 sec and allow it to stand at RT for 5 min. Avoid vigorous shaking.

For 1 FluoroSpot plate: 100 µl from both vials is mixed gently but thoroughly with 10 ml dilution buffer (1x).

Conjugate

Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on the vial) of sterile distilled water into the vial. Mix the solution gently for approximately 15 sec and allow it to stand at RT for 5 min. Avoid vigorous shaking.

For 1 FluoroSpot plate: 100 µl is mixed gently but thoroughly with 10 ml dilution buffer (1x).

Spot enhancer

Dilute Spot enhancer (4x) in PBS-I.

For 1 FluoroSpot plate: 2.5 ml is mixed gently but thoroughly with 7.5 ml PBS-I.

Assay controls

Before starting an FluoroSpot experiment, proper assay controls need to be chosen, which is mainly dependent on the selected analyte, cell type and experimental set-up.

Assay controls	Test	Reveals
Positive control	Cells incubated with stimuli (a proven antigen-specific or polyclonal stimulus).	Functionality of the cells and whether the assay works well.
Negative control	Cells incubated without stimulus (at the same cell concentration as the experimental antigen of interest).	The number of spontaneously secreting cells and false positive results.
Background	No cells but all other reagents.	False positive results due to reagents or cell culture media.

Notes:

- A positive and negative control should be tested for each sample on the FluoroSpot plate.
- All assay controls should follow the same procedure and incubation times as the antigen-specific stimulation of the experimental antigen of interest. The only difference between the positive controls and the antigen-specific stimulation/negative control might be a lower final cell concentration per well on the FluoroSpot plate to avoid confluent or poorly defined spots.
- It is recommended to test the samples in triplicate and in serial dilutions in the FluoroSpot procedure. (Since a certain cell number is needed for sufficient stimulation, the assay does not always show linearity in serial dilutions.)
- No more than $3x10^5$ cells/well should be added in the FluoroSpot plate. Higher concentration of cells will cause multiple cell layers, resulting in poor spot formation. For polyclonal stimulation, the recommended cell concentration per well should be reduced to $2x10^2$ – $1x10^5$ cells/well. The volume of the cell preparations in the 96-well FluoroSpot plate is 100 µl/well.

FluoroSpot assay procedure

Note: All solutions should be at RT (20-26 °C) prior to use. Steps 1 till 11 should be performed under sterile conditions. In addition, estimate the time needed to prepare all cell suspensions, which should be ready for step 9, and plan accordingly.

Sterile conditions

1. Prewet the PVDF membrane of each well of the FluoroSpot plate with 25 µl of 70% ethanol. Incubate for 1 min at RT.
2. Aspirate or firmly shake-out the ethanol. Immediately thereafter wells are rinsed twice with 200 µl PBS-I/well. The plate is subsequently emptied and tapped on tissue paper.
3. Add 50 µl of diluted mixture of the two coating antibodies into each well of the FluoroSpot plate.
4. Cover the plate with a lid and incubate overnight at 2 to 8 °C.

Sterile conditions

5. Remove coating antibody solution and rinse each well 3x with 200 µl PBS-I. The plate is subsequently emptied with a firm shake-out action.
6. Add 200 µl blocking buffer (1x) into each well.
7. Cover the plate with a lid and incubate for at least 1 h at RT. During this incubation step start preparing the cell sample suspensions.
8. If the cell suspensions are ready, remove the blocking buffer from the wells with a firm shake-out action (do not wash the wells).
9. Bring the cell suspensions 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 can vary from 24 to 72 h. Specific activation conditions will vary, depending on cell type, proteins of interest, kinetics of protein release and whether a preincubation step was included in the procedure.
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

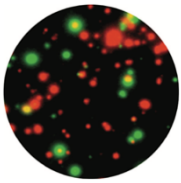
12. Wash the plate 5x with 250 µl wash buffer/well.
13. Add 100 µl of diluted mixture of the two detection antibodies into each well.
14. Seal the plate with an adhesive plate cover and incubate 2 h at RT protected from light (or overnight at 2 to 8 °C).
15. Empty plate. Remove and discard the underdrain from the bottom of the plate and wash both sides of the PVDF membrane 5x with wash buffer.
16. Add 100 µl diluted conjugate into each well.
17. Seal the plate with an adhesive cover slip and incubate 1 h at RT protected from light.
18. Empty plate and wash both sides of the PVDF membrane 5x with wash buffer.
19. Add 100 µl of diluted spot enhancer into each well.
20. Cover plate with lid and incubate for 15 min at RT protected from light.
21. Empty plate and remove residual fluid by tapping on tissue paper. Wash underside of the PVDF membrane (not inside the wells) twice with demineralized water.
22. Dry the plate 5 min at 37 °C (protected from light).
23. Count spots by using a fluorescent microscope or a ELISPOT reader with fluorescent applications within a week of completing the assay.

Note:

Store the plate at RT at a dry place protected from light to prevent photobleaching of spots.

This manual is applicable to following U-CyTech's FluoroSpot kits

Analyte	Human	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-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



Analyte	Spot color	Label
IFN-γ	green	FITC (excitation/emission: 495/519 nm)
Other cytokine	red	R-PE (excitation/emission: 546/578 nm)
Both cytokines	yellow	FITC + R-PE

If you require assistance, information or have any questions, please contact our Customer Service at cs@ucytech.com.