U-CyTech biosciences

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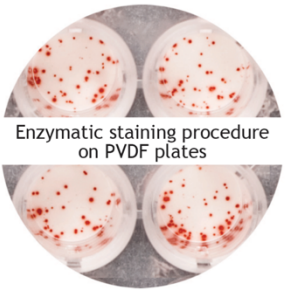
Manual
T cell ELISPOT kit


Enzymatic staining procedure on PVDF plates

The T cell ELISPOT assay is one of the most sensitive tests to monitor *ex vivo* cellular immune responses at single cell level. The assay can accurately detect secreted proteins, such as cytokines, released by e.g. T cells in response to antigen. The cell suspensions, used in the assay, can originate from blood (PBMC), lymphoid, spleen, bone marrow or CNS tissue.

The corresponding 'Data sheet' (specific to each kit) and 'SDS' can be found at www.ucytech.com/manuals. References of studies using our T cell ELISPOT kits, guidelines and recommendations for performing the T cell ELISPOT assay can be found at www.ucytech.com/t-cell-elispot-assay-guidelines.

Contents of the kit

Items	Quantity (2-plate format)	Quantity (5-plate format)	Storage conditions
Coating antibody*	1 vial	1 vial	4 °C
Biotinylated detection antibody*	1 vial	1 vial	4 °C
Streptavidin-HRP conjugate*	1 vial	1 vial	≤-20 °C***
AEC coloring system:			
I. AEC stock solution	1 vial (4 ml)	1 vial (4 ml)	≤-20 °C***
II. Substrate buffer (10x)	1 vial (2.5 ml)	1 vial (5 ml)	4 °C
Blocking stock solution (10x)	1 vial (4 ml)	1 vial (10 ml)	4 °C
Dilution buffer R (10x)****	1 vial (4 ml)	1 vial (10 ml)	4 °C
Tween-20****	1 vial (5 ml)	1 vial (5 ml)	RT***
96-well ELISPOT plate** with lid	2 plates	-	RT
Adhesive cover slip	5 slips	-	RT

RT Room temperature (temperature between 20 °C and 26 °C)
* Lyophilized
** PVDF membrane-bottomed Millipore plates (cat. no. MSIP S4510)
*** Store protected from light
**** Not included in Human IL-21 T cell ELISPOT kits (cat. no. CT419-PR2 and CT419-PR5). These kits contain Dilution buffer Q (10x; storage conditions: 4 °C). The wash buffer in the Human IL-21 T cell ELISPOT assay is PBS only.

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 AEC stock solution and Dilution buffer R, 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:

AEC stock solution: 

Flammable liquid (Category 3): H226: Flammable liquid and vapor.

Acute toxicity, oral (Category 4): H302: Harmful if swallowed.

Carcinogenicity (Category 1B): H350: May cause cancer.

Skin irritation (Category 2): H315: Causes skin irritation.

Eye irritation (Category 2): H319: Causes serious eye irritation.

Specific target organ toxicity - single exposure (Category 3): H335: May cause respiratory irritation.

Product consists of 1% (w/v) 3-amino-9-ethylcarbazole in N,N-diethylformamide.
The AEC stock solution should be handled in a chemical fume hood. Use only non-sparking tools and keep away from open flames, hot surfaces and electrostatic discharges. Avoid contact with skin, eyes and clothing. Avoid inhalation of vapor or mist. Do not let product enter drains.

Dilution buffer R: 

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 R. 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 AEC stock solution or Dilution buffer R: Do NOT induce vomiting. Consult physician immediately.

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

Storage and stability

Coating and detection antibodies

The vials with lyophilized coating and biotinylated detection antibody can be safely stored at 4 °C until the expiry date (indicated on the vials). After reconstitution, the antibodies are stable at 4 °C for at least 12 months at 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 ≤-20 °C (stable for at least two years).

Conjugate

The vial with lyophilized streptavidin-HRP conjugate is stable until the expiry date (indicated on the vial) when stored protected from light at ≤-20 °C. After reconstitution, the reagent is stable at 4°C for at least 2 months when kept sterile and protected from light. However, it is strongly recommended that the solution be divided into small aliquots for single use. These aliquots should be stored protected from light at ≤-20 °C (stable for at least one year).

AEC

The AEC stock solution should be stored at ≤-20 °C and is stable until the expiry date (indicated on the vial)*. Tightly close the vial after use. It is recommended that the solution be divided into small aliquots for single use in polypropylene vials. These aliquots should be stored at ≤-20 °C protected from light (stable for at least one year).

* Avoid exposure to light and air: tightly close the vial after use. Avoid contact with polystyrene pipettes and vials.

Substrate buffer

The substrate buffer is stable until the expiry date (indicated on the vial) when stored at 4 °C. Tightly close the vial after use.

Blocking and Dilution buffer

The vials with Blocking stock solution and Dilution buffer can be safely stored at 4 °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 expiry date (indicated on the vial).

Materials and equipment (required but not provided)

- 96-well PVDF membrane-bottomed plates (for 5-plate kit format): Millipore cat. no. MSIP S4510 is recommended.
- Adhesive cover slips (for 5-plate kit format).
- 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. More information at www.ucytech.com/cell-sample-preparation.
- Cell stimuli: antigen of interest and positive control. More information on positive controls at www.ucytech.com/guidelines-stimuli.
- Laminar flow hood (for sterile conditions), fume hood (for AEC substrate).
- Pipetting devices.
- For washing: squirt (wash or squeeze) bottle without sprout. More information on the washing procedure at www.ucytech.com/directions-washing-plates.
- CO_2 incubator (37 °C, 100% humidity, 5% CO_2).
- A reflected light microscope or an automated ELISPOT reader for spot counting.

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 ELISPOT plate: prepare 1 L PBS.

Wash buffer

PBS containing 0.05% Tween-20.

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

Note: Use PBS only (without Tween-20) as wash buffer in the Human IL-21 T cell ELISPOT assay.

Blocking buffer (1x)

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

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

Coating antibody

Reconstitute the lyophilized antibody 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 ELISPOT plate (2-plate kit): 100 µl is mixed gently but thoroughly with 5 ml PBS-I.

For 1 ELISPOT plate (5-plate kit): 50 µl is mixed gently but thoroughly with 5 ml PBS-I.

Detection antibody

Reconstitute the lyophilized antibody 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 ELISPOT plate: 100 µl 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 ELISPOT plate: 100 µl is mixed gently but thoroughly with 10 ml dilution buffer (1x).

Coloring system

The AEC coloring system consists of two items: a concentrated AEC stock solution* and a concentrated substrate buffer.

For 1 ELISPOT plate: mix 1 ml Substrate buffer (10x) thoroughly with 4.2 ml 70% ethanol and 4.8 ml demineralized water to reach a final concentration of substrate buffer (1x) in 30% ethanol. Add 660 µl AEC stock solution (toxic: use a fume hood) and mix thoroughly. After mixing the solution should be clear.

This AEC solution should be used within 30 min after preparation.

* AEC stock solution must not come into contact with polystyrene pipettes and vials.

Assay controls

Before starting an ELISPOT experiment, appropriate 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 ELISPOT 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 ELISPOT plate to avoid confluent or poorly defined spots.
- It is recommended to test the samples in triplicate and in serial dilutions in the ELISPOT 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 ELISPOT 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 ELISPOT plate is 100 µl/well.
- More information at www.ucytech.com/t-cell-elispot-assay-guidelines.

T cell ELISPOT assay procedure

Note: 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 suspensions, which should be ready for step 9, and plan accordingly.

Read www.ucytech.com/cell-sample-preparation for more information.

Sterile conditions

1. Prewet the PVDF membrane of each well of the ELISPOT 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 coating antibody solution into each well of the ELISPOT plate.
4. Cover the plate with a lid and incubate overnight at 4 °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 ELISPOT plate. Add 100 µl/well.
10. Cover ELISPOT 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, protein 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 detection antibody into each well.
14. Seal the plate with an adhesive cover slip and incubate 2 h at RT (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 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 freshly prepared AEC solution into each well.
20. Cover plate with lid and incubate for 30 min at RT protected from light.
21. Stop the reaction by emptying the plate and thoroughly rinse both sides of the PVDF membrane with demineralized water.
22. Air-dry the plate at RT (protected from light).
23. Count spots by using a reflected light microscope or an ELISPOT reader.

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

* More information on cell preparation, cell incubation times, stimuli, washing and troubleshooting can be found at www.ucytech.com/t-cell-elispot-assay-guidelines.

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

Note: CTxxx-PR2 are 2-plate format kits and CTxxx-PR5 are 5-plate format kits.

Analyte	Human	Old World Monkey	New World Monkey	Mouse	Rat
IFN-γ	CT230-PR2 CT230-PR5	CT121-PR2 CT121-PR5	CT939-PR2 CT939-PR5	CT317-PR2 CT317-PR5	CT079-PR2 CT079-PR5
IL-1β	CT242-PR2 CT242-PR5	CT123-PR2 CT123-PR5			
IL-2	CT231-PR2 CT231-PR5	CT127-PR2 CT127-PR5		CT435-PR2 CT435-PR5	
IL-4	CT232-PR2 CT232-PR5	CT128-PR2 CT128-PR5		CT319-PR2 CT319-PR5	CT081-PR2 CT081-PR5
IL-5	CT233-PR2 CT233-PR5	CT129-PR2 CT129-PR5		CT321-PR2 CT321-PR5	
IL-6	CT234-PR2 CT234-PR5	CT130-PR2 CT130-PR5			
IL-10	CT235-PR2 CT235-PR5	CT131-PR2 CT131-PR5		CT320-PR2 CT320-PR5	
IL-12/23p40		CT135-PR2 CT135-PR5	CT942-PR2 CT942-PR5		
IL-12p70	CT240-PR2 CT240-PR5				
IL-13	CT236-PR2 CT236-PR5	CT132-PR2 CT132-PR5	CT943-PR2 CT943-PR5		
IL-17A	CT416-PR2 CT416-PR5	CT401-PR2 CT401-PR5	CT944-PR2 CT944-PR5		
IL-17F	CT418-PR2 CT418-PR5	CT403-PR2 CT403-PR5			
IL-21	CT419-PR2 CT419-PR5				
G-CSF	CT680-PR5	CT122-PR5			
GM-CSF	CT241-PR2 CT241-PR5	CT124-PR2 CT124-PR5			
Granzyme B	CT229-PR2 CT229-PR5				
Perforin	CT681-PR2 CT681-PR5	CT136-PR2 CT136-PR5			
TNF-α	CT237-PR2 CT237-PR5	CT133-PR2 CT133-PR5	CT938-PR2 CT938-PR5	CT322-PR2 CT322-PR5	

If you require assistance, information or have any questions, please contact our Customer Service by e-mail: cs@ucytech.com.