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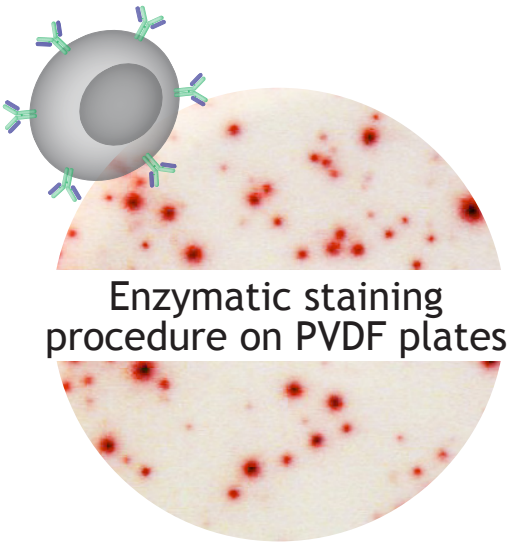
Instruction Manual
B cell ELISPOT kit



Enzymatic staining
procedure on PVDF plates

5-plate format

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

Contents

Abbreviations

AEC	3-Amino-9-EthylCarbazole
ASC	Antibody Secreting Cells
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-linked ImmunoSPOT
FCS	Fetal Calf Serum
HRP	HorseRadish Peroxidase
Ig	Immunoglobulin
min	minute(s)
PBS	Phosphate Buffered Saline
PBS-I	Sterile and Pyrogen-free PBS
PBMC	Peripheral Blood Mononuclear Cell
PVDF	Polyvinylidene fluoride
RT	Room Temperature (temperature between 20 °C and 26 °C)
sec	seconds

Catalogue numbers B cell ELISPOT kits

This manual applies to the following B cell ELISPOT kits

Analyte	Human	Old World Monkey	Mouse
IgG	CT780-PR5	CT785-PR5	CT790-PR5
IgG_1			CT791-PR5
IgG_{2a}			CT792-PR5
IgG_{2b}			CT793-PR5
IgE	CT781-PR5		CT794-PR5

NOTE: The colors pink and green in this manual refer to the IgG and IgE B cell ELISPOT assays, respectively.

NOTE: The accompanying 'Typical data' and 'Addendum B cell ELISPOT assay', which contains guidelines and troubleshooting for ELISPOT analyses are available on our website (www.ucytech.com/manuals).

Introduction

The B cell ELISPOT (Enzyme-linked ImmunoSPOT) assay makes it possible to investigate the presence of antibody secreting cells (ASC) in blood or tissue samples.

The traditional method to monitor a B cell response generated after immunization or infection is to quantify specific antibody titers in serum by ELISA. The ELISA method is a straightforward method to measure serum antibody titers. However, the assay does not provide any information about the number and location of the ASCs. The B cell ELISPOT assay has proven particularly useful to identify and determine the number of individual ASCs in single cell suspensions.

Memory B cells, which have a long lifespan, play a central role in the humoral immune response. Under natural conditions these B cells do not proliferate or produce antibodies until they are activated by re-exposure to a specific antigen. While being inactive, the presence of these cells can not be determined by ELISA. A major advantage of the B cell ELISPOT is its ability to activate *ex vivo* antigen-specific memory B cells, whereafter they can be detected.

The B cell ELISPOT is the assay of choice to determine the magnitude and longevity of (vaccine-induced) protection against a certain infection. The B cell ELISPOT assay is useful in different fields of biomedical research including vaccine development, infection research, drug treatment, autoimmune diseases and allergy.

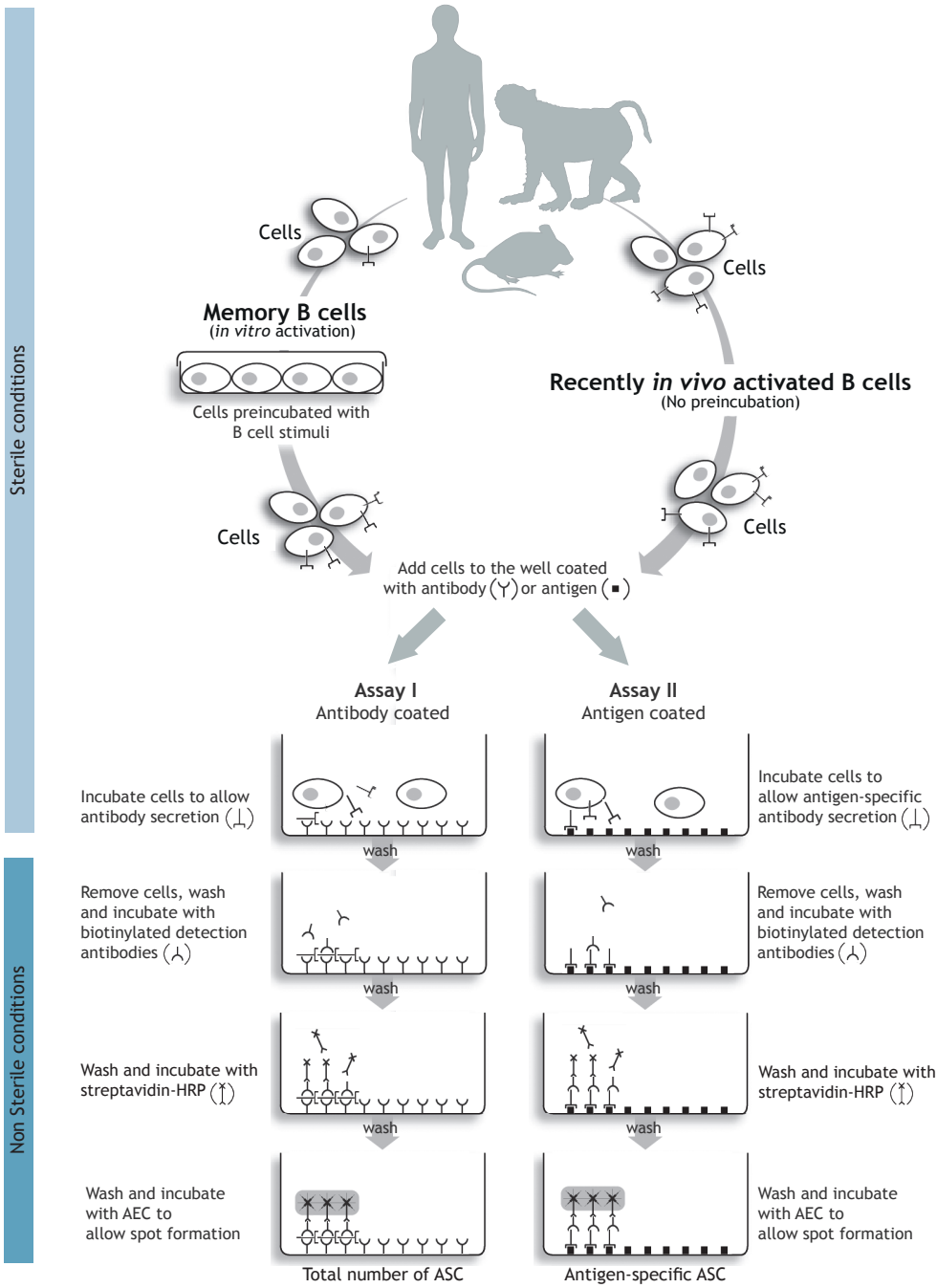
Principle of the test

The performance of the B cell ELISPOT is based on two assays:
Assay I : enumeration of total immunoglobulin secreting B cells.
Assay II: enumeration of antigen-specific immunoglobulin secreting B cells.

The different steps of the ELISPOT assays are illustrated in the "Flow diagram B cell ELISPOT" on page 5. A cell suspension of activated B cells are brought into the wells of the ELISPOT plate coated with antibodies directed to species-specific immunoglobulins (Assay I) or coated with an antigen of interest (Assay II). Antibodies released by the B cells are captured by the coated antibody or antigen. After incubation, cells are washed away and areas in which secreted antibodies have been bound, are detected by the sequential addition of a biotinylated antibody and an enzyme conjugate. The last step in the assay is the addition of AEC substrate revealing the sites of antibody secretion (footprints of individual ASC). These footprints (spots) represent either the total number of ASC (Assay I) or antigen-specific ASC (Assay II).

Please note that it might be necessary to activate the B cells *in vitro* first. This requires a preincubation step prior to adding the B cells into the ELISPOT plate. See "Cell sample preparation" on page 13 for more information.

Flow diagram B cell ELISPOT



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

Warning:
AEC (3-amino-9-ethylcarbazole) stock solution is classified as dangerous and Dilution buffer R is classified as irritation according to Regulation (EC) no. 1272/2008 and its amendments:

AEC stock solution: 

Acute toxicity, oral (Category 4), Carcinogenicity (Category 1A), Skin iritation (Catogory 2), Eye irritation (Category 2), Specific target organ toxicity - single exposure (Category 3).

Hazard statements: Harmful if swallowed (H302), Causes skin irritation (H315), Causes serious eye irritation (H319), May cause respiratory irritation (H335), May cause cancer (H350).

The AEC stock solution should be handled only in a chemical fume hood. Use only non-sparking tools and keep away from open flames and hot surfaces.

Dilution buffer R: 

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).

AEC stock solution and Dilution buffer R:
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 (5-plate format)	Storage conditions
Coating antibody* for Assay I	1 vial	4°C
Biotinylated detection antibody*	1 vial	4°C
Streptavidin-HRP conjugate*	1 vial	4°C
Recombinant IL-2*	1 vial	-20°C
R848 (Resiquimod)	0.25 ml	-20°C in the dark
Recombinant IL-4*	1 vial	-20°C
Anti-CD40*	1 vial	4°C
AEC coloring system:		
I. AEC stock solution	4 ml	4 °C in the dark
II. Substrate buffer capsules	5 pieces	RT
Blocking stock solution (10x)	10 ml	4°C
Dilution buffer R (10x)	10 ml	4°C
Tween-20	5 ml	RT

* Lyophilized

NOTE: The colors pink and green in this table mark the items that exclusively belong to either the IgG or the IgE B cell ELISPOT assays

Storage and stability

Coating antibody and biotinylated detection antibody
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 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 (stable for at least two years).

IgG

Recombinant IL-2
The vial with lyophilized recombinant IL-2 can be safely stored at ≤ -20°C until the expiry date (indicated on the vial). After reconstitution, the solution should be stored in small aliquots at ≤ -70°C for single use (stable for at least 12 months).

R848
The vial with R848 is stable until the expiry date (indicated on the vial) when stored at ≤ -20°C in the dark. It is strongly recommended to divide the solution into small aliquots for single use. These aliquots should be stored at ≤ -20°C in the dark (stable for at least 12 months).

IgE

Recombinant IL-4
The vial with lyophilized recombinant IL-4 can safely be stored at ≤ -20°C until the expiry date (indicated on the vial). After reconstitution, the solution should be stored in small aliquots at ≤ -70°C for single use (stable for at least 12 months).

Anti-CD40
The vial with lyophilized anti-CD40 antibody can be safely stored at 4°C until the expiry date (indicated on the vial). 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 (stable for at least two years).

Streptavidin-HRP conjugate
The vial with lyophilized Streptavidin-HRP conjugate is stable until the expiry date (indicated on the vial) when stored at ≤ -20°C in the dark. After reconstitution, the reagent is stable for at least 2 months at 4°C when kept sterile and protected from light. However, it is strongly recommended to divide the solution into small aliquots for single use. These aliquots should be stored at ≤ -20°C in the dark (stable for at least 12 months).

AEC stock solution
The vial with AEC stock solution is stable until the expiry date (indicated on the vial) when stored at ≤ -20°C in the dark. It is strongly recommended to divide the solution into small aliquots for single use (polypropylene vials only). These aliquots should be stored at ≤ -20°C in the dark (stable for at least 12 months).

Substrate buffer capsules
The Substrate buffer capsules are stable until the expiry date (indicated on the vial) when stored at RT in a moisture-free environment.

Blocking stock solution (10x) and Dilution buffer R (10x)
Blocking stock solution (10x) and Dilution buffer R (10x) 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 until the expiry date (indicated on the vial).

Materials and equipment (required but not provided)

- 96-well PVDF membrane-bottomed plates: Millipore cat. no. MSIP S4510 is recommended.
- Tubes and containers/plates to prepare the solutions.
- Tissue culture plates for preincubation (optional).
- Antigen of interest for coating.
- Sterile distilled water.
- 70% ethanol.
- PBS pH 7.4 (home-made). For washing purposes only.
- Sterile and pyrogen-free PBS pH 7.4 (PBS-I): 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). Please note, do not use human, non-human primate or rodent serum as growth supplements.
  - RPMI-1640: Thermo Fisher Scientific cat. no. 52400 (Gibco®).
  - L-Glutamine: Thermo Fisher Scientific cat. no. 25030-081.
  - 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®).
- Pipetting devices.
- Squirt (wash or squeeze) bottle with wide sprout for washing.
- CO_2 incubator (37 °C, 100% humidity, 5% CO_2).
- 37 °C incubator.
- A reflected light microscope or an Immunospot image analyzer for spot counting.

Preparation solutions and reagents

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

Coating antibody (Assay I)
Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently for approximately 15 sec and allow the vial to stand for 5 min at RT.
For one ELISPOT plate: 50 µl is gently but thoroughly mixed with 5 ml PBS-I.

Antigen of interest (for coating)
For one ELISPOT plate: dilute the antigen to a suitable concentration (0.5-15 µg/ml) in 5 ml PBS-I. (Determine the optimal concentration first).

Blocking buffer (1x)
Dilute Blocking stock solution (10x) in PBS-I.
For one ELISPOT 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 ELISPOT plate: 2 ml is gently but thoroughly mixed with 18 ml PBS-I.

Biotinylated detection antibody
Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently for approximately 15 sec and allow the vial to stand for 5 min at RT.
For one ELISPOT plate: 100 µl is gently but thoroughly mixed with 10 ml Dilution buffer R (1x).

Streptavidin-HRP conjugate
Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently for approximately 15 sec and allow it to stand for 5 min at RT.
For one ELISPOT plate: 100 µl is gently but thoroughly mixed with 10 ml Dilution buffer R (1x).

AEC coloring system
The AEC coloring system consists of two items: a concentrated AEC stock solution* and a substrate buffer capsule. For preparing the AEC substrate solution, the contents of one capsule is dissolved in 57 ml water. After complete dissolution, 43 ml 70% ethanol is added to reach a final concentration of 30% ethanol. 10 ml of this solution is thoroughly mixed with 660 µl AEC stock solution (toxic, use a fume hood). After mixing, the solution should be clear. This amount is sufficient for one ELISPOT plate and should be used within 30 min after preparation. The remaining substrate buffer solution can be discarded.

* Do not bring AEC stock solution in contact with polystyrene pipettes and vials.

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 ELISPOT plate: make 1 L PBS.

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

IgG

Reagents for *in vitro* activation of IgG memory B cells
Reconstitute the recombinant IL-2 by injecting the appropriate volume (indicated on the vial) of sterile distilled water into the vial. Mix gently for approximately 15 sec and allow the vial to stand for 5 min at RT.
The vial with R848 is thawed for 10 min at RT and then gently mixed. Combine both reagents in culture medium at a final concentration of 1/100 for recombinant IL-2 and 1/200 for R848.

IgE

Reagents for *in vitro* activation of IgE memory B cells
Reconstitute the recombinant IL-4 and anti-CD40 by injecting the appropriate volume (indicated on the vial) of sterile distilled water into each vial. Mix gently for approximately 15 sec and allow the vials to stand for 5 min at RT. Combine both reagents in culture medium at a final concentration of 1/100.

Cell sample preparation

Both fresh and cryopreserved cells can be used for ELISPOT analysis. Guidelines for specimen collection and handling are described in the Addendum B cell ELISPOT assay (www.ucytech.com/manuals).

Recently *in vivo* activated B cells

In vivo activated B cells, for instance after vaccination, actively produce antibodies. The peak of the antibody production is 3 to 9 days post-vaccination and this can be directly analyzed in the ELISPOT assay (Assay II).

For the ELISPOT procedure, cells are diluted to the desired cell concentration in culture medium without stimuli and transferred to the ELISPOT plate (100 µl/well). Check both Table 2 and 3 for more information on cell concentration and stimuli.

Activation of memory B cells *in vitro*

Memory B cells do not produce antibodies in significant quantities unless they are activated for several days *in vitro* with appropriate polyclonal stimuli. These stimuli are supplied with the kit. Since memory B cells also expand during activation, the ratio of both antigen-specific ASC (Assay II) and total ASC (Assay I) is used to determine memory B cell responses.

Memory B cell responses are obtained when cells are preincubated in culture medium supplied with stimuli at 37°C with 5% CO_2 in a humidified atmosphere. For recommended incubation times, cell concentrations and stimuli, see Table 1.

After preincubation, cells are washed twice with fresh culture medium without stimuli (two centrifugation/resuspension steps; 8 min, 200x g, RT). Thereafter cells are counted and diluted to the desired cell concentration in culture medium and transferred to the ELISPOT plate (100 µl/well). Check both Table 2 and 3 for more information on cell concentration and stimuli.

NOTE: Stimulation with IL-4 + anti-CD40 induces immunoglobulin class switching (Hasbold, J. *et al*. Eur. J. Immunol. 1998, 28: 1040-1051). Results with cells stimulated with these reagents may therefore not be representative for the situation *in vivo*.

Table 1: Guidelines preincubation

Cell type	Assay	Incubation	Cell density	Stimuli
Human PBMC	IgG	3-5 days	$2x10^6$ cells/ml	IL-2 + R848
	IgE	4-5 days	$2x10^6$ cells/ml	IL-4 + anti-CD40
Old World Monkey PBMC	IgG	3-5 days	$2x10^6$ cells/ml	IL-2 + R848
Murine spleen cells	IgG	2-3 days	$5x10^6$ cells/ml	IL-2 + R848
	IgE	3-5 days	$5x10^6$ cells/ml	IL-4 + anti-CD40

The above mentioned incubation times are guidelines. When other cell types are used, other incubation times may have to be considered.

B cell ELISPOT procedure

All solutions should be at RT prior to use. Before starting the ELISPOT procedure, read "Cell sample preparation", page 13. Steps 1 till 11 should be performed under sterile conditions.

1. Pre-wet 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. Immediately thereafter, wells are rinsed 2x with 200 µl PBS-I. The plate is subsequently emptied and tapped on tissue paper.
3. Add 50 µl of diluted coating antibody or 50 µl of a specific antigen into individual wells (Assay I or Assay II, respectively). At least 3 wells are filled with 50 µl/well PBS-I. See Table 2 for more details.
4. Cover the plate with a lid and incubate overnight at 4 °C.
5. Remove solution from wells and rinse each well 3x with 200 µl PBS-I. The plate is subsequently emptied.
6. Add 200 µl Blocking buffer (1x) to each well.
7. Cover the plate with a lid and incubate for at least 1 hour at 37 °C. During this incubation step start preparing the cell sample preparations (see page 13).
8. If the cell preparations are ready, decant the blocking solution from wells (do not wash the wells).
9. Bring the cell preparations into the wells of the ELISPOT plate. Add 100 µl/well.

Sterile conditions

Table 2: Guidelines coating and cell concentration

Response	Coating per well	Cells/well
Background response (negative control)	50 µl PBS-I (no coating)	$1x10^5$-$3x10^5$
Number of total immunoglobulin secreting cells (Assay I)	50 µl diluted coating antibody	$2x10^3$-$1x10^5$
Number of antigen-specific immunoglobulin secreting cells (Assay II)	50 µl diluted antigen (0.5-15 µg/ml)	$1x10^5$-$3x10^5$

The above mentioned cell concentrations are guidelines. It is recommended to analyze a series of dilutions to determine the optimal cell concentration first. A maximum of $3x10^5$ cells can be put into a well of a 96-well plate. However, it can be important to use a lower cell concentration in order to obtain individual spots. It is recommended to run the above mentioned combinations in triplicate.

10. Cover the plate with a lid and incubate at 37 °C, 5% CO_2, 100% humidity. For recommended incubation times see Table 3.
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.

12. Wash the plate 5x with 250 µl Wash buffer/well. Consult "Addendum B cell ELISPOT assay" (www.ucytech.com/manuals) for directions on washing.
13. Add 100 µl of diluted biotinylated detection antibody into each well.
14. Seal the plate with adhesive cover slip and incubate 1 hour at 37 °C (or overnight at 4 °C).
15. Empty plate. Remove and discard the underdrain from the bottom of the plate.
16. Wash both sides of the PVDF membrane 5x with Wash buffer.
17. Add 100 µl diluted Streptavidin-HRP conjugate into each well.
18. Seal the plate with an adhesive cover slip and incubate 1 hour at 37 °C.
19. Empty plate and wash both sides of the PVDF membrane 5x with Wash buffer.
20. Add 100 µl freshly prepared AEC substrate solution into each well.
21. Cover plate with lid and incubate for 30-60 min at RT in the dark.
22. Stop color development by emptying the plate and rinse thoroughly both sides of the PVDF membrane with demineralized water.
23. Air-dry the plate at RT (in the dark).
24. Count spots by use of a reflected light microscope or an Immunospot image analyzer.

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

Table 3: Guidelines incubation time

Cell type	Assay	Incubation	Stimuli
Human PBMC	IgG	16-24 hours	No
	IgE	16-24 hours	No
Old World Monkey PBMC	IgG	16-24 hours	No
Murine spleen cells	IgG	5-6 hours	No
	IgE	24-48 hours	Yes

The above mentioned incubation times are guidelines. When other cell types are used, other incubation times may have to be considerd.

Technical assistance

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

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On our website (www.ucytech.com/manuals) you can find: Manuals, Typical data, Addendum and MSDS of our B cell ELISPOT kits.