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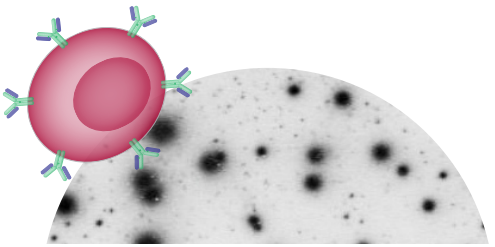
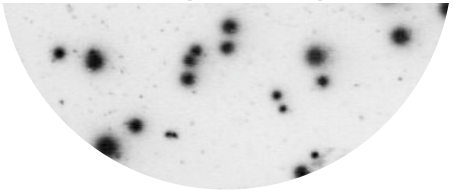
Instruction Manual
B cell ELISPOT kit



Silver staining procedure
on transparent plates



5-plate format

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

Contents

Abbreviations

ASC	Antibody secreting cells
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-linked immunoSPOT
FCS	Fetal Calf Serum
GABA	Gold-labeled Anti-Biotin Antibodies
IgG	Immunoglobulin G
min	minute(s)
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-I	Sterile and Pyrogen-free PBS
PS	Polystyrene
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-T5	CT785-T5	CT790-T5
IgG_1			CT791-T5
IgG_{2a}			CT792-T5
IgG_{2b}			CT793-T5

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 is designed for this purpose and 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. 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 (e.g. 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.

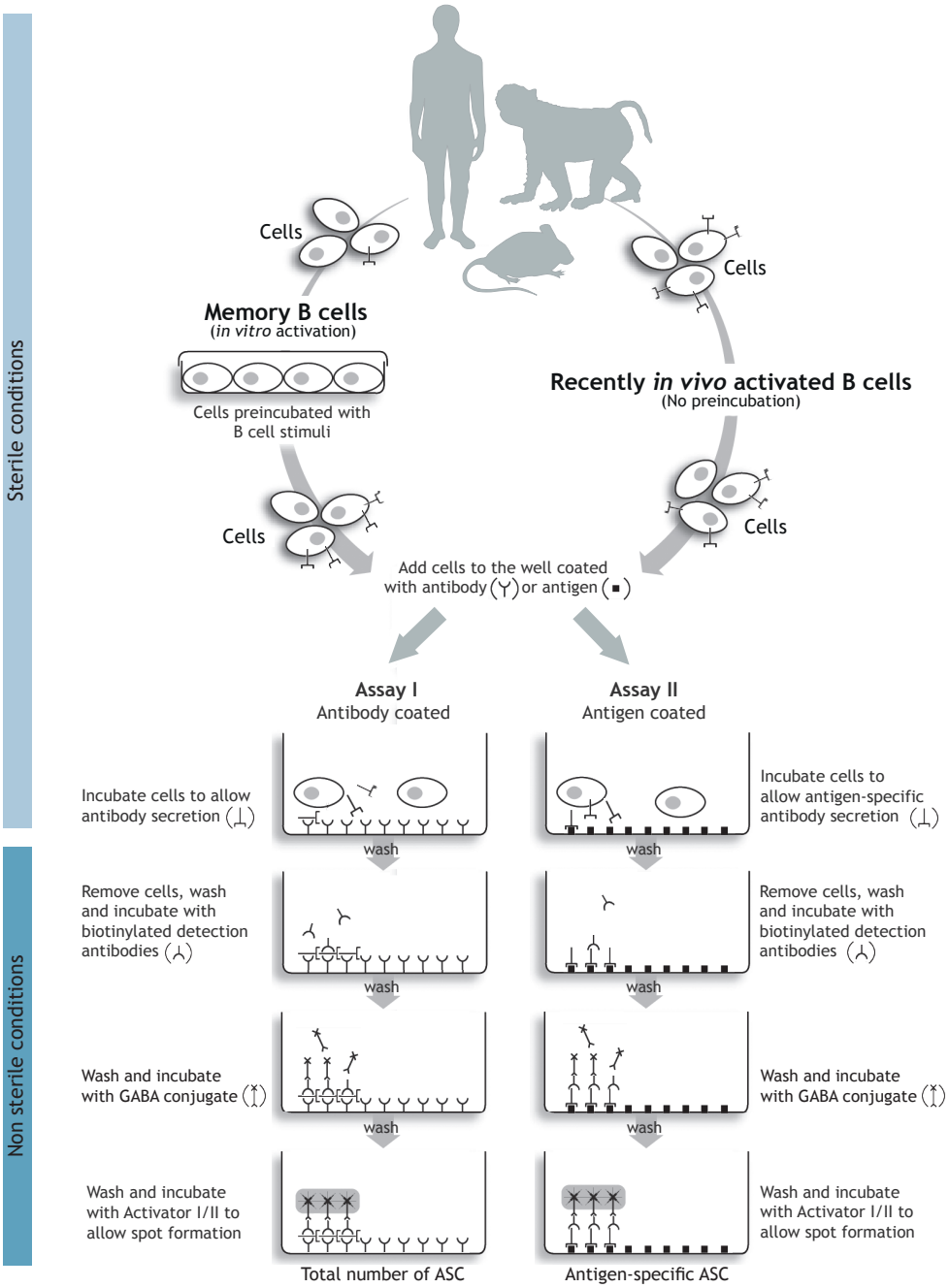
Principle of the test

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 GABA conjugate (anti-biotin antibody). In the last step of the assay, Activator is added which allows silver to precipitate revealing the antibody secretion sites (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 onto the ELISPOT plate. See "Cell 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 Activator I+II solutions, the items in this kit are not classified as dangerous according to Regulation (EC) no. 1272/2008 and its amendments.

Activator I + II: 

Warning:
Activator I+II solutions are classified as dangerous according to Regulation (EC) no. 1272/2008 and its amendments: Serious eye damage (Category 1), Skin sensitization (Category 1) and Chronic aquatic toxicity (Category 1).

Hazard statement: May cause an allergic skin reaction (H317), Causes serious eye damage (H318), Very toxic to aquatic life with long lasting effects (H410).

The Activator I+II solutions should be kept away from acids and sources of ignition; use only non-sparking tools. Keep away from light, air and heat. In case of contact with skin, wash with soap and water and remove contaminated clothing and shoes. Upon ingestion rinse mouth (if person is conscious) and call physician immediately. Do not induce vomiting. In case of contact with eyes, irrigate 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 and 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
GABA conjugate*	1 vial	4°C
Recombinant IL-2*	1 vial	-20 °C
R848 (Resiquimod)	0.25 ml	-20 °C in the dark
Activator I	9.5 ml	4°C
Activator II	9.5 ml	4°C
Blocking stock solution (10x)	10 ml	4°C
Dilution buffer T (10x)	8 ml	4°C
Tween-20	5 ml	RT
96-well ELISPOT plate with lid (Transparent PS-bottomed Nunc MaxiSorp)	6	RT
Adhesive cover slip	10	RT

* Lyophilized

Storage and stability

Coating antibody and biotinylated detection antibody
The vials with lyophilized coating antibody 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).

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).

GABA conjugate
The vial with lyophilized GABA conjugate can be safely stored at 4°C 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. However, it is strongly recommended to divide the reconstituted reagent into small aliquots for single use. These aliquots should be stored at ≤ -20°C (stable for at least 12 months).

Activator I and II
The Activators I and II should be stored at 4°C and are stable until the expiry date (indicated on the vials)*. It is strongly recommended to divide the solutions into small aliquots for single use. These aliquots should be stored at ≤ -20°C in the dark (stable for at least two years).

*Avoid exposure to light and air and tightly close the vials after use.

Blocking stock solution (10x) and Dilution buffer T (10x)
Blocking stock solution (10x) and Dilution buffer T (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 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).
- Antigen of interest for coating.
- Sterile distilled water.
- 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). 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.
- For washing: squirt (wash or squeeze) bottle with wide sprout or (automated) washing device, see also Addendum B cell ELISPOT assay.
- CO_2 incubator (37°C, 100% humidity, 5% CO_2).
- 37°C incubator.
- A transmitted 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 T (1x)
Dilute Dilution buffer T (10x) in PBS-I.
For one ELISPOT plate: 1.5 ml is gently but thoroughly mixed with 13.5ml 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 T (1x)

GABA 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 ELISPOT plate: 100 µl is gently and thoroughly mixed with 5 ml Dilution buffer T (1x).

Activator I and II
Bring the two bottles of Activator to RT prior to use. Shake the bottles gently but intensively before mixing 1.8 ml of Activator I with 1.8 ml Activator II (for one ELISPOT plate). Use immediately thereafter.

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.

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.

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 Table 2 for more information on cell concentrations.

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 and cell concentrations, 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 without stimuli and transferred to the ELISPOT plate (100 µl/well). Check Table 2 for more information on cell concentrations in the ELISPOT plate.

Table 1: Guidelines preincubation

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

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 9 should be performed under sterile conditions.

Sterile conditions

1. 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. See Table 2 for more details. Fill each well up to 100 µl/well with PBS-I.
2. Cover the plate with a lid and incubate overnight at 4°C.
3. Remove solution from wells and rinse each well 3x with 200 µl PBS-I. The plate is subsequently emptied.
4. Add 200 µl Blocking buffer (1x) to each well.
5. 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 without stimuli (see page 13).
6. If the cell sample preparations are ready, decant the blocking buffer from wells (do not wash the wells).
7. Bring the cell preparations into the wells of the ELISPOT plate. Add 100 µl/well.

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, lower cell concentrations are often more optimal to obtain individual spots. Run the above mentioned combinations in triplicate.

8. Cover the plate with a lid and incubate 4 to 7 hours (valid for PBMCs from Humans and Old World Monkeys and for Murine spleen cells) at 37°C, 5% CO_2, 100% humidity.
9. 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.

10. Wash the plate 6x with 250 µl Wash buffer/well. Consult “Addendum B cell ELISPOT assay” (www.ucytech.com/manuals) for directions on washing.

11. Add 100 µl of diluted biotinylated detection antibody into each well.

12. Seal the plate with adhesive cover slip and incubate 1 hour at 37°C (or overnight at 4°C).

13. Empty plate and wash 6x with Wash buffer.

14. Add 50 µl diluted GABA conjugate into each well.

15. Seal the plate with an adhesive cover slip and incubate 1 hour at 37°C.

16. Empty plate and wash 6x with Wash buffer (after the last wash, wells should not contain residual Wash buffer).

17. Add 35 µl freshly prepared Activator I/II solution into each well. Distribute the Activator I/II uniformly over the bottom of the well.

18. Cover plate with lid and incubate for 30-60 min at RT. Monitor spot development by light microscope.

19. When clear spots have developed, stop the reaction by emptying the plate and rinse with demineralized water.

20. Air-dry the plate at RT.

21. Count spots by use of a transmitted light microscope or an Immunospot image analyzer.

Visually, spots have a grayish color, microscopically they are black. Silver-stained spots are highly stable and spot quality is preserved indefinitely when the plate is stored at a dry place.

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.