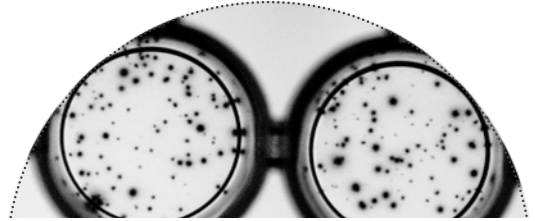


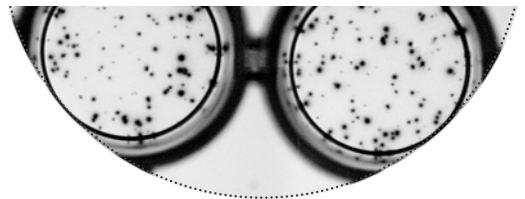


Manufactured By: U-CyTech Biosciences

## Instruction Manual IgG 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.



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This B cell ELISPOT manual applies for the following U-CyTech B cell ELISPOT kits

(please find below the catalogue number of the B cell ELISPOT kit)

Analyte	Species	
	Human	Old World Monkey
IgG	ACT780-T5	ACT785-T5

### Intended use

The B cell ELISPOT (Enzyme-linked Immunospot) assay has been designed to identify and enumerate individual antibody secreting cells (ASC) in single cell suspensions of (most commonly) peripheral blood mononuclear cells (PBMC).

### Brief description of B cell ELISPOT assay

The performance of the B cell ELISPOT is based on two separate assays:

I : wells of a 96-well ELISPOT plate are coated with species-specific immunoglobulines (Ig).

II: wells of a 96-well ELISPOT plate are coated with an antigen of interest.

A cell suspension with B cells are brought into the wells of the coated ELISPOT plate and incubated for 5-7 hour at 37°C. Antibodies released by the B cells are captured by the coated Ig or antigen. After incubation, cells are washed away and areas in which secreted antibodies are bound are detected by the sequential addition of biotinylated anti-isotype specific antibodies and  $\phi$ -labeled anti-biotin antibodies (GABA). The last step in the assay is the addition of a reagent allowing the precipitation of silver on  $\phi$  revealing the sites of antibody secretion (footprints of individual ASC). These footprints (spots) represent either the total number of ASC (in Ig-coated wells) or antigen-specific ASC (in antigen-coated wells).

The different steps of the assay are illustrated in the Flow diagram (page 2).

### Activation of B cells

*In vivo* activated B cells, for instance after vaccination, actively produce antibodies and do not need pre-stimulation. Antigen-specific ASC can be found in the circulation 6 to 9 days post-vaccination. These cells can directly be detected in the ELISPOT assay (assay II).

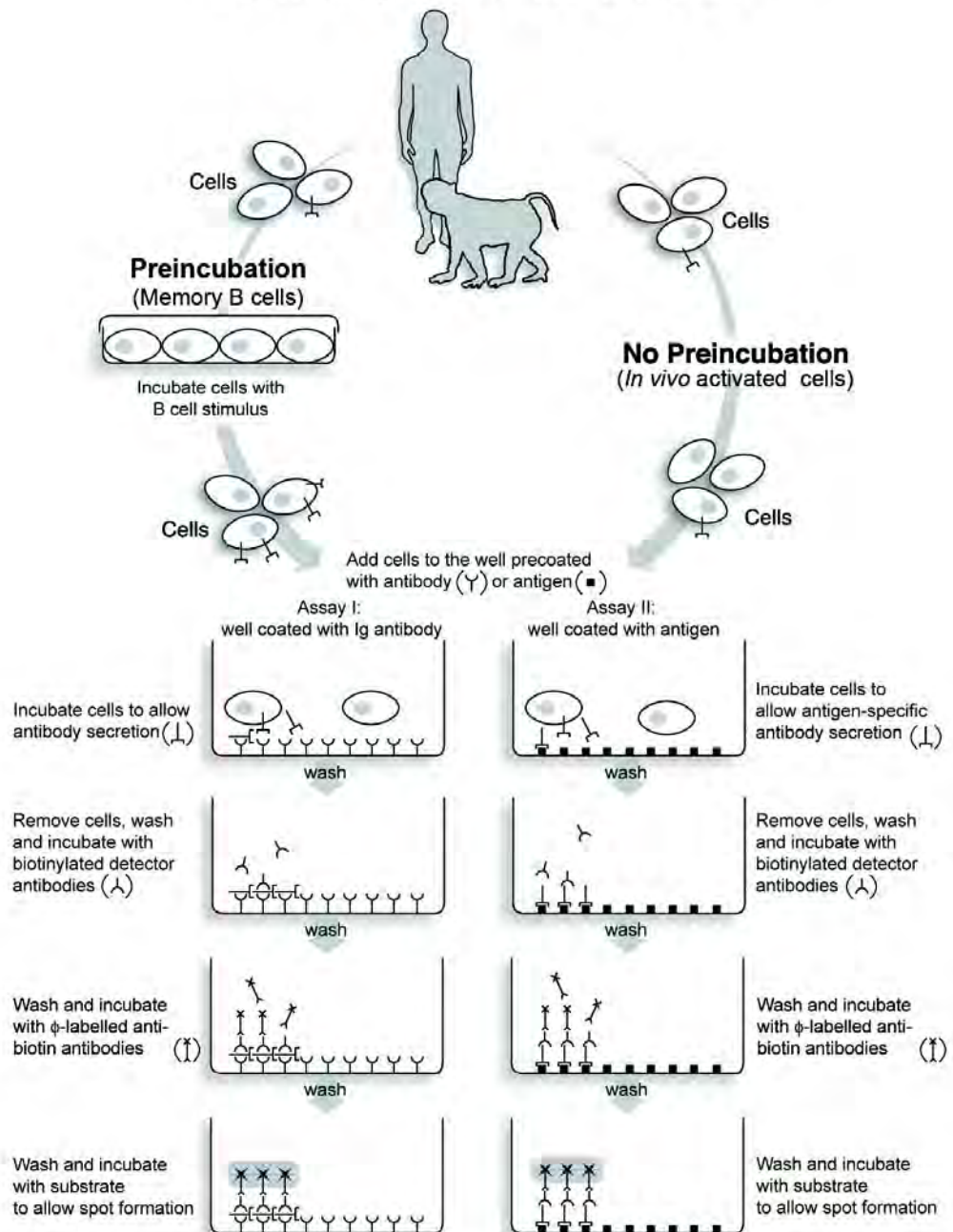
Activation and expansion of memory B cells requires a specific stimulatory reagent and several days of stimulation under appropriate conditions. Since memory B cells expand *in vitro* the frequency of antigen-specific ASC (assay II) is normally compared to the total number of ASC (assay I) found after stimulation.

Optimal B cell responses for PBMCs are obtained when cells are cultured with B cell stimulus (supplied with the kit) for 5 days at 37°C with 5-7% CO<sub>2</sub> in a 100% humidified atmosphere at a density of 2x10<sup>6</sup>

cells per ml. After stimulation, cells are washed twice (two gentle centrifugation steps) and are resuspended in fresh culture medium before they are transferred to the ELISPOT plate.

To a 96-well ELISPOT plate,  $0.5-2 \times 10^5$  cells/well is added for the detection of antigen-specific B cell responses (assay II), whereas  $2-4 \times 10^3$  cells/well is required for the enumeration of the total number of ASC (assay I).

## Flow diagram B cell ELISPOT



## Contents of kit

Items	Quantity (5-plate format)
Coating antibodies (lyophilized) for assay I	1 vial
Biotinylated detector antibodies (lyophilized)	1 vial
B cell stimulus (lyophilized)	2 vials
φ-labeled anti-biotin antibodies (GABA) (lyophilized)	1 vial
Activator I	9.5 ml
Activator II	9.5 ml
Blocking stock solution B (10x)	10 ml
Dilution buffer T (10x)	8 ml
Tween-20	5 ml
96-well ELISPOT plate* with lid	6
Adhesive cover slip	10

\* Transparent polystyrene-bottomed Nunc MaxiSorp plates.



### Hazard information

#### Warning:

Activator I+II solutions are classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EC and its amendments:

Serious eye damage (Category 1)

Skin sensitization (Category 1)

Chronic aquatic toxicity (Category 1)

#### Hazard statements:

H317: May cause an allergic skin reaction. H318: Causes serious eye damage.

H410: Very toxic to aquatic life with long lasting effects.

#### Precaution statements:

P273: Avoid release to the environment.

P280: Wear protective gloves/protective clothing/eye protection/face protection.

P305 + P 351 + P338: IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.

P501: Dispose of contents/container to an approved waste disposal plant.

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

Toxic to aquatic organisms; avoid release in the environment.

Other kit components are not classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EC or 1999/45/EC and their amendments.

Please find the Material Safety Data Sheet on [www.ucytech.com/manuals](http://www.ucytech.com/manuals).

## Reagents/materials required but not provided

- Sterile distilled water.
- Phosphate buffered saline (PBS): home-made, filter-sterilize or autoclave. For washing purposes only.
- Wash buffer: PBS containing 0.05% Tween-20.
- Sterile and pyrogen-free PBS (PBS-I): Invitrogen cat. no. 10010-015 is recommended.
- Antigen of interest for coating.
- Pipetting devices.
- Culture medium: see Addendum\*.
- Plate washer: automated or manual, see Addendum\*.
- CO<sub>2</sub>-incubator (37°C, 100% humidity, 5% CO<sub>2</sub>).
- Tissue culture plates for pre-stimulation (optional).
- An inverted microscope or an immunospot image analyzer for spot counting.

\* The accompanying Addendum B cell ELISPOT assay contains guidelines and troubleshooting for B cell ELISPOT analyses. The Addendum B cell ELISPOT assay is also available on our website ([www.ucytech.com](http://www.ucytech.com)) or contact U-CyTech biosciences ([order@ucytech.com](mailto:order@ucytech.com)).

## Storage reagents

- The vials with lyophilized coating antibodies, biotinylated detector antibodies and GABA antibodies can safely be stored at 4°C until the expiry date (indicated on the vials). After reconstitution, the antibodies are stable for minimal 12 months at 4°C when kept sterile. The reconstituted antibodies can also be stored frozen ( $\leq -20^{\circ}\text{C}$ ) in small aliquots for single use. Frozen antibodies are stable for minimal two years.
- The vials with lyophilized B cell stimulus can be stored at 4°C until the expiry date (indicated on the vial). After reconstitution, the reagent is stable for minimal 1 month at 4°C when kept sterile. When stored at  $\leq -20^{\circ}\text{C}$  the reconstituted stimulus is stable for minimal 6 months (avoid repeated cycles of freezing and thawing).
- The Activators I and II should be protected from light and stored at 4°C until the expiry date (indicated on the vials). Since the reagents are susceptible to oxidation by air, it is important that after use, the vials are tightly closed. It is recommended to divide the solutions into small aliquots for single use. These aliquots should be stored at  $\leq -20^{\circ}\text{C}$  in the dark. Frozen samples are stable for at least two years.
- Blocking stock solution B (10x) and Dilution buffer T (10x) should be stored at 4°C until the expiry date (indicated on the vials). After opening these solutions are stable for minimal 6 months when kept sterile.
- Tween-20 can best be stored at room temperature until the expiry date (indicated on the vials).

## Preparation kit reagents

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

### 1. Coating antibodies for assay I

Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently and allow the vial to stand for minimal 2 minutes at room temperature. For one B cell ELISPOT plate 50 µl is required. Mix 50 µl with 5 ml PBS-I.

### 2. B cell stimulus (to activate memory B cells)

Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently and allow the vial to stand for minimal 2 minutes at room temperature. Working dilution: 100x in cell culture medium.

### 3. Blocking buffer B (1x)

Dilute Blocking stock solution B (10x) in PBS-I. For one ELISPOT plate, 2 ml is thoroughly mixed with 18 ml PBS-I.

### 4. Dilution buffer T (1x)

Dilute Dilution buffer T (10x) in PBS-I. For one ELISPOT plate, 1.5 ml is thoroughly mixed with 13.5 ml PBS-I.

### 5. Biotinylated detector antibodies

Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently and allow the vial to stand for 2 minutes at room temperature. For one ELISPOT plate, 100 µl is thoroughly mixed with 10 ml Dilution buffer T (1x).

### 6. GABA (φ-labeled anti-biotin antibodies)

Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on vial) of sterile distilled water. Mix gently and allow it to stand for 2 minutes at room temperature. For one ELISPOT plate, 100 µl is thoroughly mixed with 5 ml Dilution buffer T (1x).

### 7. Activators

For one ELISPOT plate, mix gently but thoroughly 1.8 ml of Activator I with 1.8 ml Activator II. Keep temperature at 4°C during mixing. Use immediately thereafter.

## Procedure

Use ELISPOT plates and reagents under aseptic conditions (e.g. Laminar Flow Hood) for steps 1 to 6.

1. Pipet 50 µl of properly diluted coating antibodies or 50 µl of a specific antigen to individual wells (total number of ASC and antigen-specific ASC, respectively) and fill up to 100 µl/well with PBS-I. The optimal concentration of an antigen differs but usually varies between 0.5-2 µg/ml. At least 3 wells are filled with 50 µl/well PBS to determine background responses. Cover the plate with a lid and incubate overnight at 4°C.
2. Decant solution from wells. Wash each well 3x with 200 µl PBS-I. Subsequently add 200 µl Blocking buffer B (1x) to each well. The plate is covered with a lid and incubated for 1 h at 37°C.
3. Prepare cell suspension (see Addendum B cell ELISPOT assay). If applicable, preincubate cells for 5 days with B cell stimulus to convert memory B cells into ASC (see "Activation of B cells").
4. Decant the Blocking buffer B from the wells (do not wash the wells). Bring cells in the wells of the ELISPOT plate (100 µl/well).

Triplicates of  $0.5-2 \times 10^5$  cells/well are recommended to assess antigen-specific responses. To determine the total amount of antibody secreting cells a significant lower number of cells should be used ( $2-4 \times 10^3$  cells/well is recommended).

5. Cover ELISPOT plate with a lid and incubate 5 to 7 hours at 37°C, 5% CO<sub>2</sub>, 100% humidity.
6. Remove the bulk of cells with a firm shake-out action and wash 2x with PBS-I of room temperature (200 µl/well). Thereafter wells are washed 6x with 250 µl Wash buffer/well (see Addendum B cell ELISPOT assay).
7. Discard Wash buffer and add 100 µl of properly diluted biotinylated detector antibodies to each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C or preferably overnight at 4°C.
8. Decant solution from wells. Wash wells 6x with 250 µl Wash buffer/well. Bring 50 µl of properly diluted GABA solution into each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C.
9. Decant solution from wells. Wells are washed 6x with 250 µl Wash buffer/well and subsequently emptied by a firm 'shake-out' action (wells should not contain residual Wash buffer).
10. Add 35 µl of freshly prepared Activator I/II solution to each well. Uniformly distribute the Activator I/II solution over the well. Cover plate with lid and incubate at room temperature in the dark.
11. Monitor spot development by light microscopy (from 30 to 40 minutes). When clear spots have developed, stop the reaction by rinsing the wells with demineralised water.
12. Air dry the plate at room temperature and count spots by use of an inverted microscope or an immunospot image analyzer.

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