

## IgG B cell ELISPOT Kit

**Catalog No.** CKH339

**Quantity:** 5 x 96 tests

**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:** In this manual two different procedures are described:  
A. procedure for enzymatic staining on PVDF plates;  
B. procedure for silver staining on transparent plates.

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 a defined length of time 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 an enzyme conjugate or  $\phi$ -labeled anti-biotin antibodies (GABA). The last step in the assay is the addition of AEC substrate (3-amino-9-ethylcarbazole) or 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).

**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, 0.5-2x10<sup>5</sup> cells/well is added for the detection of antigen-specific B cell responses (assay II), whereas 2-4x10<sup>3</sup> cells/well is required for the enumeration of the total number of ASC (assay I).



## A. Procedure for enzymatic staining on PVDF Plates

### Contents of kit with reagents for enzymatic staining on PVDF plates

Items	Quantity (5-plate format)
Coating antibodies (lyophilized) for assay I	1 vial
Biotinylated detector antibodies (lyophilized)	1 vial
B cell stimulus	2 vials
Streptavidin-HRP conjugate (lyophilized)	1 vial
AEC coloring system:	
I. AEC stock solution	4 ml
II. Substrate buffer capsules	5
Blocking stock solution R (10x)	10 ml
Dilution buffer R (10x)	10 ml
Tween-20	5 ml



### Hazard information

AEC (3-Amino-9-ethylcarbazole) stock solution is classified as harmful according to Directive 1999/45/EC and its amendments.

Risk phrases: R22/R36 Safety phrases: S26/S39/S45

The AEC stock solution should be handled only in a chemical fume hood.

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

Other kit components are not classified as dangerous according to Directive 67/548/EC or 1999/45/EC and their amendments.

### Reagents/materials required but not provided:

- PVDF membrane-bottomed plates: Millipore cat. no. MSIP S4510 is recommended.
- Sterile distilled water.
- 70% ethanol.
- 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.
- Culture medium: see Addendum\*.
- Pipetting devices.
- Squirt (wash or squeeze) bottle with wide spout for washing, see Addendum\*.
- CO<sub>2</sub>-incubator (37°C, 100% humidity, 5% CO<sub>2</sub>).
- Tissue culture plates for pre-stimulation (optional).
- A dissecting microscope or an immunospot image analyzer for spot counting.

The accompanying B cell ELISPOT Addendum contains guidelines and troubleshooting for B cell ELISPOT analysis.

- Storage reagents:**
- The vials with lyophilized coating antibodies and biotinylated detector antibodies can safely be stored at 4°C until the expiry date (indicated on the vials). After reconstitution, the



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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 vial with lyophilized Streptavidin-HRP conjugate should be stored at  $\leq -20^{\circ}\text{C}$  until the expiry date (indicated on the vial). After reconstitution, the reagent is stable for minimal 2 months at 4°C when kept sterile. However, it is strongly recommended to divide the reconstituted conjugate into small aliquots for single use. These aliquots should be stored at  $\leq -20^{\circ}\text{C}$  (stable for minimal one year). The reconstituted Streptavidin-HRP rapidly loses activity when kept at room temperature.
- The AEC stock solution should be protected from light and is stable for at least 12 months at  $\leq -20^{\circ}\text{C}$ . Prepare aliquots for single use in polypropylene vials and store these at  $\leq -20^{\circ}\text{C}$ .
- The Substrate buffer capsules are stable for an indefinite length of time when stored at room temperature in a moisture-free environment.
- Blocking stock solution R (10x) and Dilution buffer R (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  $\mu\text{l}$  is required. Mix 50  $\mu\text{l}$  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 R (1x) Dilute Blocking stock solution R (10x) in PBS-I. For one ELISPOT plate, 2 ml is thoroughly mixed with 18 ml PBS-I.
4. Dilution buffer R (1x) Dilute Dilution buffer R (10x) in PBS-I. For one ELISPOT plate, 2 ml is thoroughly mixed with 18 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  $\mu\text{l}$  is thoroughly mixed with 10 ml Dilution buffer R (1x).
6. Streptavidin-HRP conjugate 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 B cell ELISPOT plate, 100  $\mu\text{l}$  is thoroughly mixed with 10 ml Dilution buffer R (1x).
7. 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 content 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 330  $\mu\text{l}$  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 minutes after preparation.

## Procedure (enzymatic staining on PVD plates)

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

1. Pre-wet the PVDF membranes by adding 25  $\mu$ l of 70% ethanol to each well. Incubate for 1 minute at room temperature.
2. Aspirate or firmly shake-out the ethanol. Immediately thereafter, wells are rinsed 2x with PBS-I. The plate is subsequently emptied and tapped on tissue paper.
3. Pipet 50  $\mu$ l of properly diluted coating antibodies or 50  $\mu$ l of a specific antigen to individual wells (total number of ASC and antigen-specific ACS, respectively). The optimal concentration of an antigen differs but usually varies between 0.5-2  $\mu$ g/ml. At least 3 wells are filled with 50  $\mu$ l/well PBS to determine background responses. Cover the plate with a lid and incubate overnight at 4°C.
4. Decant solution from wells. Wash each well 3x with 200  $\mu$ l PBS-I. Subsequently add 200  $\mu$ l Blocking buffer R (1x) to each well. The plate is covered with a lid and incubated for 1 h at 37°C.
5. Prepare cell suspension (see Addendum). If applicable, pre-incubate cells for 5 days with B cell stimulus to convert memory B cells into ACS (see "Activation of B cells").
6. Decant the Blocking buffer R from the wells (do not wash the wells). Bring cells in the wells of the ELISPOT plate (100  $\mu$ l/well). Triplicates of 0.5-2x10<sup>5</sup> cells/well are recommended to assess antigen-specific responses. To determine the total amount of antibody secreting cells a much lower number of cells should be used (2-4x10<sup>3</sup> cells/well is recommended).
7. Cover ELISPOT plate with a lid and incubate 5 to 7 hours at 37°C, 5% CO<sub>2</sub>, 100% humidity.
8. Remove the bulk of cells with a firm shake-out action and wash 2x with PBS-I of room temperature (200  $\mu$ l/well). Thereafter wells are washed 5x with 250  $\mu$ l Wash buffer/well (see Addendum).
9. Discard Wash buffer and add 100  $\mu$ l 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 overnight at 4°C.
10. Decant solution from wells. Remove and discard the underdrain from the bottom of the plate and wash both sides of the PVDF membrane 5x with Wash buffer. Discard Wash buffer and bring 100  $\mu$ l of properly diluted Streptavidin-HRP solution into each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C.
11. Decant solution from wells. Wash both sides of the PVDF membrane 5x with Wash buffer.
12. Discard Wash buffer and add 100  $\mu$ l AEC substrate solution to each well. Cover plate with lid and incubate for 45 minutes at room temperature in the dark.
13. Stop color development by thoroughly rinsing both sides of the PVDF membrane with de-mineralized water.
14. Air dry the plate at room temperature and count spots by use of a dissecting microscope or an immunospot image analyzer.

To prevent bleaching of spots store the plate at a dry place protected from light.



## B. Procedure for silver staining on transparent plates

### Contents of kit with reagents for silver staining on transparent plates

Items	Quantity (5-plate format)
Coating antibodies (lyophilized) for assay I	1 vial
Biotinylated detector antibodies (lyophilized)	1 vial
B cell stimulus	2 vials
$\varphi$ -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

Activator I and II are classified as harmful according to Directive 1999/45/EC and its amendments. Risk phrases: R22, R36/38, R52/53 Safety phrases: S36/37/39, S46, S61  
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 minutes. Assure adequate flushing by separating the eyelids. Seek medical advice immediately.

Other kit components are not classified as dangerous according to Directive 67/548/EC or 1999/45/EC and their amendments.

### 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. nr. 10010-015 is recommended.
- Antigen of interest for coating.
- Culture medium: see Addendum\*.
- Pipetting devices.
- 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 B cell ELISPOT Addendum contains guidelines and troubleshooting for B cell ELISPOT analyses.



- 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 are stable for at least 12 months at 4°C. Since the reagents are susceptible to oxidation by air, it is important that after use, the vials are tightly closed.
  - 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  $\mu\text{l}$  is required. Mix 50  $\mu\text{l}$  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  $\mu\text{l}$  is thoroughly mixed with 10 ml Dilution buffer T (1x).
6. GABA ( $\phi$ -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  $\mu\text{l}$  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 (silver staining on transparent plates)

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

15. Pipet 50  $\mu$ l of properly diluted coating antibodies or 50  $\mu$ l of a specific antigen to individual wells (total number of ASC and antigen-specific ASC, respectively) and fill up to 100  $\mu$ l/well with PBS-1. The optimal concentration of an antigen differs but usually varies between 0.5-2  $\mu$ g/ml. At least 3 wells are filled with 50  $\mu$ l/well PBS to determine background responses. Cover the plate with a lid and incubate overnight at 4°C.
16. Decant solution from wells. Wash each well 3x with 200  $\mu$ l PBS-I. Subsequently add 200  $\mu$ l Blocking buffer B (1x) to each well. The plate is covered with a lid and incubated for 1 h at 37°C.
17. Prepare cell suspension (see Addendum). If applicable, pre-incubate cells for 5 days with B cell stimulus to convert memory B cells into ASC (see "Activation of B cells").
18. Decant the Blocking buffer B from the wells (do not wash the wells). Bring cells in the wells of the ELISPOT plate (100  $\mu$ l/well). Triplicates of 0.5-2x10<sup>5</sup> 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 (2-4x10<sup>3</sup> cells/well is recommended).
19. Cover ELISPOT plate with a lid and incubate 5 to 7 hours at 37°C, 5% CO<sub>2</sub>, 100% humidity.
20. Remove the bulk of cells with a firm shake-out action and wash 2x with PBS-I of room temperature (200  $\mu$ l/well). Thereafter wells are washed 6x with 250  $\mu$ l Wash buffer/well (see Addendum).
21. Discard Wash buffer and add 100  $\mu$ l 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.
22. Decant solution from wells. Wash wells 6x with 250  $\mu$ l Wash buffer/well. Bring 50  $\mu$ l of properly diluted GABA solution into each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C.
23. Decant solution from wells. Wells are washed 6x with 250  $\mu$ l Wash buffer/well and subsequently emptied by a firm 'shake-out' action (wells should not contain residual Wash buffer).
24. Add 35  $\mu$ l 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.
25. Monitor spot development by light microscopy (from 30 to 40 minutes). When clear spots have developed, stop the reaction by rinsing the wells with de-mineralized water.
26. 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.

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