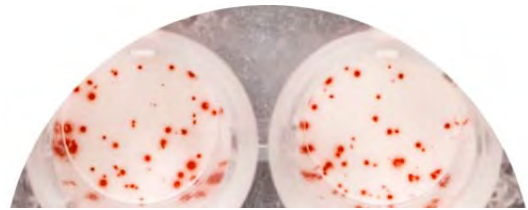


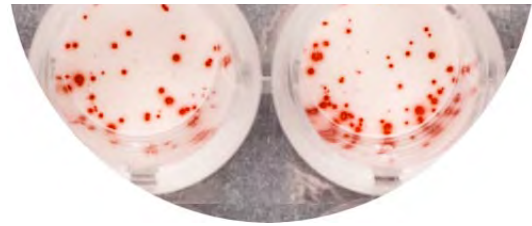


Manufactured By: U-CyTech Biosciences

Instruction Manual IgG 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.

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-PR5	ACT785-PR5

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 16-24 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 an enzyme conjugate. The last step in the assay is the addition of AEC substrate (3-amino-9-ethylcarbazole) 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).

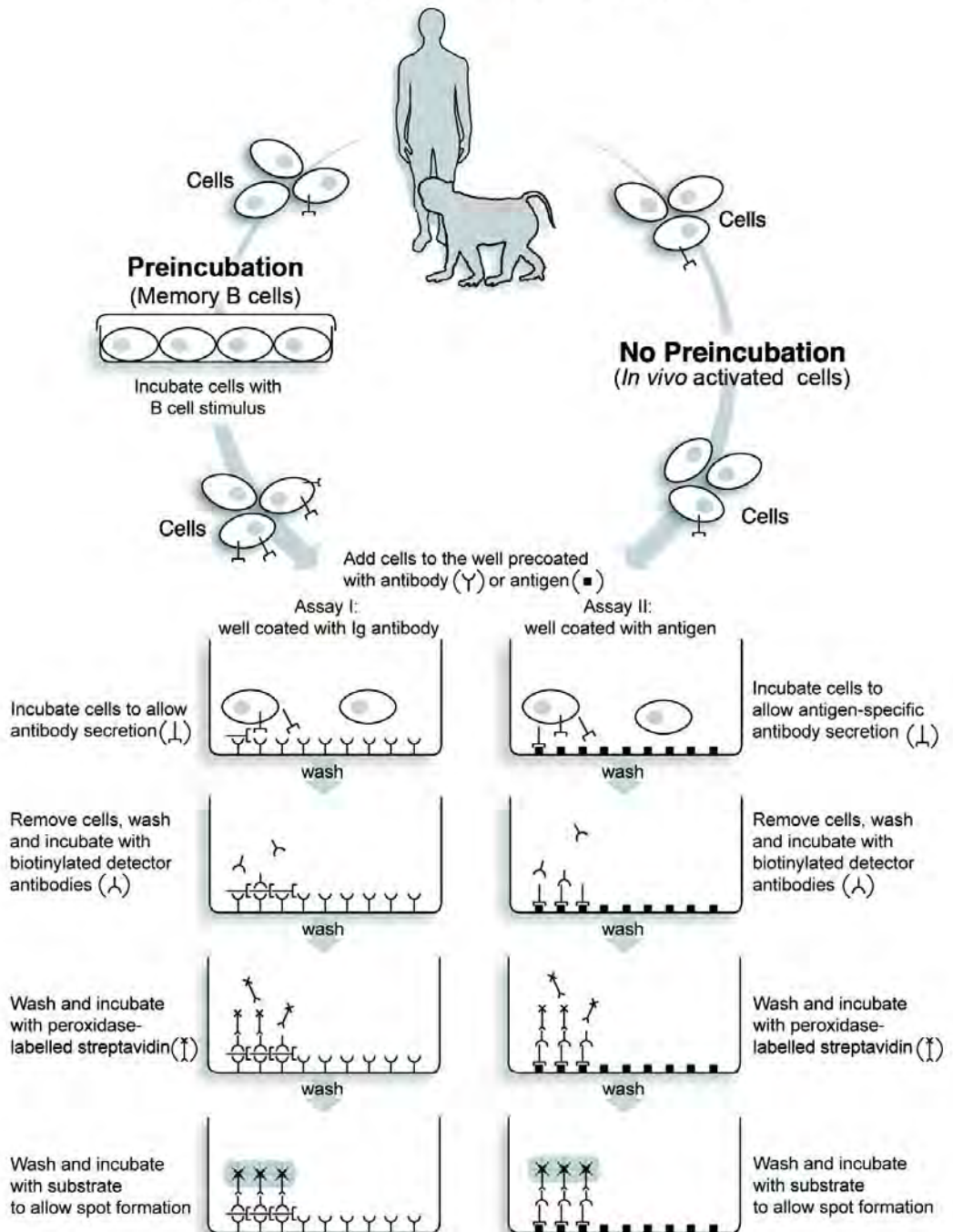
Activation and expansion of memory B cells requires a specific stimulatory reagent and several days of stimulation under appropriate conditions. Since memory B cells expand *in vitro* the frequency of antigen-specific ASC (assay II) is normally compared to the total number of ASC (assay I) found after 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% CO₂ in a 100% humidified atmosphere at a density of 2x10⁶

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
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

Warning:

AEC (3-Amino-9-ethylcarbazole) stock solution is classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EC and its amendments.

Hazard statements:

H302: Harmful if swallowed.
H315: Causes skin irritation.
H319: Causes serious eye irritation.
H335: May cause respiratory irritation.
H350: May cause cancer

Precaution statements:

P201: Obtain special instructions before use.
P210: Keep away from heat/sparks/open flames/hot surfaces. - No smoking.
P261: Avoid breathing dust/fume/gas/mist/vapors/sprays.
P280: Wear protective gloves/ protective clothing/eye protection/face protection.
P301 + P312: IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.
P302 + P352: IF ON SKIN: Wash with plenty of soap and water
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.
P308 + P313: IF exposed or concerned: Get medical advice/attention.

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.

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 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.

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*.
- CO₂-incubator (37°C, 100% humidity, 5% CO₂).
- Tissue culture plates for pre-stimulation (optional).
- A dissecting 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) or contact U-CyTech biosciences (order@ucytech.com).

Storage reagents

- The vials with lyophilized coating antibodies and biotinylated detection antibodies can be safely stored at 4°C until the expiry date (indicated on the vials). After reconstitution, the reagents are stable for minimal 6 months at 4°C when kept sterile. However, it is strongly recommended to divide the reconstituted antibody preparations into small aliquots for single use. These aliquots should be stored at ≤ -20°C. Under these conditions the reagents are stable for minimal one year.
- 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 ≤ -20°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 ≤ -20°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 ≤ -20°C. Under these conditions the reagent is 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 should be stored at ≤ -20°C until the expiry date (indicated on the vial). It is recommended to divide the solution into small aliquots for single use in polypropylene vials. These aliquots should be stored at ≤ -20°C protected from light.
- The Substrate buffer capsules are stable until the expiry date (indicated on the vial) when stored at room temperature and 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 μ l is required. Mix 50 μ 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 μ 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 μ 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 μ 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

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

1. Prewet the PVDF membranes by adding 25 μ 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 μ l of properly diluted coating antibodies or 50 μ 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 μ g/ml. At least 3 wells are filled with 50 μ 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 μ l PBS-I. Subsequently add 200 μ 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 B cell ELISPOT assay). If applicable, preincubate 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 μ l/well).

Triplicates of 0.5-2x10⁵ 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 (2-4x10³ cells/well is recommended).

7. Cover ELISPOT plate with a lid and incubate 5 to 7 hours at 37°C, 5% CO₂, 100% humidity.
8. Remove the bulk of cells with a firm shake-out action and wash 2x with PBS-I of room temperature (200 μ l/well). Thereafter wells are washed 5x with 250 μ l Wash buffer/well (see Addendum B cell ELISPOT assay).
9. Discard Wash buffer and add 100 μ 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 μ 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 μ 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 demineralised 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.