

# Human IgM ELISpot<sup>BASIC</sup>

Product Code: 3840-2H

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## **CONTENTS:**

### **Vial 1 (red top)**

Monoclonal anti-IgM antibody (800 µl)

Concentration: 0.5 mg/ml

### **Vial 2 (yellow top)**

Anti-IgM antibody, biotinylated (100 µl)

Concentration: 0.5 mg/ml

### **Vial 3 (white top)**

Streptavidin-Horseradish Peroxidase (500 µl)

### **Vial 4 (black top)**

Polyclonal activator: R848 (100 µl)

Concentration: 1 mg/ml

### **Vial 5 (blue top)**

Lyophilised recombinant human IL-2 (1 µg)

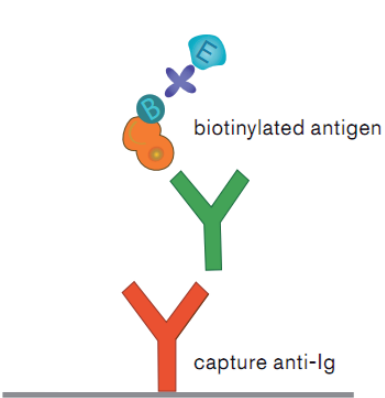
Antibodies are supplied in sterile filtered (0.2 µm) PBS with 0.02% sodium azide. Streptavidin-HRP is supplied in PBS with 0.002% Kathon CG. R848 is supplied in sterile filtered (0.2 µm) PBS containing 1% DMSO. Vials have been overfilled to ensure recovery of the specified amount.

## **STORAGE:**

Shipped at ambient temperature. On arrival antibodies and Streptavidin-HRP should be stored refrigerated at 4-8°C. R848 and IL-2 should be stored at -20°C.

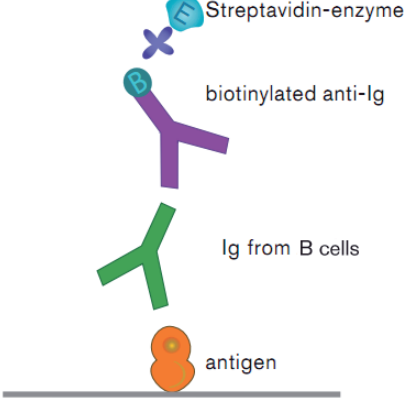
# Schematic illustration of the different protocols

PROTOCOL I



Antigen-specific Ig ELISpot

PROTOCOL II



Total Ig ELISpot

# Kit description

Please read through before starting the assay

This kit contains reagents for detection and enumeration of B cells secreting IgM antibodies. B cells secreting antigen-specific IgM as well as B cells secreting IgM irrespective of antigen specificity (total IgM) can be measured.

## Detection of antigen-specific B cells

Detection of antigen-specific IgM-secreting B cells, visualised as spots, can be made using either of two protocols (Protocol I or II). The choice of protocol will depend on several factors such as the available amount and molecular nature of the antigen. The use of biotinylated antigen (Protocol I) may be more sensitive and result in spots of higher quality. In addition, the antigen is used in smaller quantities and is not present during cultivation.

In **Protocol I**, antibodies from all secreting B cells are captured by anti-IgM antibodies coated on the ELISpot plate. Cells producing specific antibody are then visualised by the addition of biotinylated antigen followed by Streptavidin-enzyme and substrate. Please note that antigen must be biotinylated prior to starting the experiment for this protocol.

In **Protocol II**, representing the conventional way of performing B cell ELISpot, the antigen is coated on the ELISpot plate and antigen-specific antibodies secreted by the B cells bind to the immobilised antigen. Spots are detected after addition of biotinylated anti-IgM followed by Streptavidin-enzyme and substrate.

## Detection of total IgM-producing B cells

Both protocols allow determination of the total number of IgM-secreting B cells, used as a positive control or for establishing the proportion of antigen-specific B cells.

# Hints and comments

Please read through before starting the assay

These suggestions are based on the detection of antigen-specific immune responses using PBMC. If using B-cell clones, mixtures of separated cell fractions etc., other protocols may have to be considered.

## Plates

We recommend the use of PVDF-based membrane plates. Maximal antibody binding capacity of these plates is obtained by a brief treatment with ethanol. It is essential that the membrane is not allowed to dry after the treatment. If this occurs the treatment step (A2-A3) needs to be repeated before adding the coating antibody.

## Plate washing

Washing of plates can be done using a multi-channel micropipette. In washing steps not requiring sterile conditions (C1-C5), a regular ELISA plate washer can also be used, provided that the washing head is adapted to the ELISpot plates. Avoid getting liquid on the underside of the membrane as this may cause leakage due to capillary drainage. If using MAIPSWU plates, always remove the plate from the plate tray before manually emptying the plate.

## Cells

Both fresh and cryopreserved cells may be used with good results. However it is recommended that the latter are rested for at least one hour to allow removal of cell debris before addition to the plate. Triplicates or duplicates of 100,000-500,000 cells per well are often used to assess antigen-specific responses. For detection of total IgM, less cells (e.g. 25,000-50,000 cells per well) may be used to avoid confluent spot formation.

## *In vivo* activated B cells

B cells that have been activated *in vivo*, for instance as a result of vaccination, may be analysed directly in the ELISpot wells without prior stimulation. Typically, cells secreting antigen-specific antibodies can be detected in the circulation 6 to 9 days after vaccination.

## Memory B cells - *in vitro* activation

Memory B cells may require polyclonal stimulation before secreting detectable amounts of antibody. Pre-stimulation can be made with a mixture of R848 (1 µg/ml) and rhIL-2 (10 ng/ml) (both included) in separate tubes/plates for 24-72 hours. After pre-stimulation, wash the cells to ensure removal of any secreted antibodies. Resuspend the cells in medium and add the cell suspension to the ELISpot plate.

Reconstitution of rhIL-2: Add 1 ml PBS to obtain 1 µg/ml. Leave at room temperature for 15 minutes and then vortex. Use immediately or store in aliquots at -20°C.

## **Serum**

The serum should be selected to support cell culture and give low background staining. We recommend the use of fetal calf serum. Alternatively serum-free medium evaluated for cell culture can be used. Human serum is not recommended as it may contain antibodies which can interfere with the assay.

## **Buffers**

PBS for washing and dilution should be filtered (0.2  $\mu\text{m}$ ) for optimal results. Although possible to use, we do not recommend the inclusion of Tween or other detergents in the washing and incubation buffers.

## **Substrate development**

Develop until distinct spots are visible in positive wells (usually 10-30 minutes). A general darkening of the membrane may occur but disappears after drying. Preferably use deionized water to stop the plates since some ions may cause fading of TMB spots.

## **Biotinylation of antigen**

If biotinylated antigen will be used for detection, (Protocol I), this should be labeled before starting the ELISpot experiment. A suitable reagent for biotinylation is e.g. Sulfo-NHS-LC-Biotin (Pierce product code 21335). Further information on biotin and biotin conjugation may be found at [www.piercenet.com](http://www.piercenet.com)

-The protein antigen to be biotinylated with Sulfo-NHS-LC-Biotin should be in a suitable amine-free buffer (e.g. PBS, pH 7.4). If solution contains sodium azide, this should first be removed e.g. by dialysis. For small volumes (< 200  $\mu\text{l}$ ) a MINI-dialysis unit Slide-A-Lyser (10.000 MWCO, Pierce) can be used. The same dialysis unit can be used for removal of free biotin (see below). Conjugation is normally performed using a 20- to 50-fold molar excess of biotin to protein where the higher ratio is for protein at low concentration.

-The Sulfo-NHS-LC-Biotin should be dissolved in pure  $\text{H}_2\text{O}$  and immediately added to the antigen solution. After incubation for 30 minutes at room temperature, free biotin may be removed by dialysis against PBS or other buffer.

The concentration of the biotinylated antigen in the assay is typically 0.01-1  $\mu\text{g}/\text{ml}$  but depends on the size of the antigen and the degree of biotinylation and should be established separately.

# Protocol I

## (using biotinylated antigen)

### A Preparation of ELISpot plate (sterile conditions)

1. Dilute the coating anti-IgM antibody to 10 µg/ml in sterile PBS, pH 7.4.
2. PVDF plates need to be treated (activated) with ethanol before coating. PVDF plates from Millipore, type MAIPSWU should be treated with 50 µl 70% ethanol/well for maximum 2 minutes. PVDF plates, type MSIP should be treated with 15 µl 35% ethanol/well for maximum 1 minute. After ethanol treatment, immediately proceed to plate washing.
3. Wash plate 5 times with sterile water, 200 µl/well.
4. Add 100 µl/well of the antibody solution and incubate overnight at 4-8°C.

### B Incubation of cells in plate (sterile conditions)

**See Hints and Comments section for details on cell stimulation.**

1. Wash plate 5 times with sterile PBS, 200 µl/well, to remove excess antibody.
2. For blocking, add 200 µl/well of medium containing 10% of the same serum as used for the cell suspensions. Incubate for at least 30 minutes at room temperature.
3. For analysis of *in vivo* activated B cells, remove the medium and add the cell suspension to the ELISpot plate. For analysis of memory B cells, the cells can be pre-activated separately in tubes prior to addition to the plates. See further Hints and Comments/*In vivo* activated B cells and Memory B cells.
4. Put the plate in a 37°C humidified incubator with 5% CO<sub>2</sub> and incubate for 16-24 hours. Do not move the plate during this time and take measures to avoid evaporation (e.g. by wrapping the plate in aluminium foil).

### C Detection of spots

1. To remove the cells, empty the plate and wash 5 times with PBS, 200 µl/well.

#### 2a. Antigen-specific IgM

Dilute biotinylated antigen to a suitable concentration (0.01-1 µg/ml) in PBS-0.5% fetal calf serum (FCS). Add 100 µl/well and incubate for 2 hours at room temperature.

#### 2b. Total IgM

Dilute the detection antibody (anti-IgM-biotin) to 1 µg/ml in PBS-0.5% FCS. Add 100 µl/well and incubate for 2 hours at room temperature.

3. Wash plate as above (step C1).
4. Dilute the Streptavidin-HRP\* in PBS-0.5% FCS and add 100 µl/well. Incubate for 1 hour at room temperature.

\*Streptavidin-HRP may require different dilutions depending on the substrate used. For AEC substrates dilution 1:100 is usually suitable. For other substrates (e.g. TMB) dilution 1:500 - 1:1000 may be required. *Please note that sodium azide used in buffers will inhibit HRP activity.*

5. Wash plate as above (step C1).
6. Add 100 µl/well of substrate solution (e.g. TMB) and develop until distinct spots emerge.
7. Stop colour development by washing extensively in deionized water. If desirable, remove the plate from the plate tray or the underdrain and rinse the underside of the membrane.
8. Leave the plate to dry. Inspect and count spots in an ELISpot reader or in a dissection microscope.
9. Store plate in the dark at room temperature.

# Protocol II

## (using coated antigen)

### A Preparation of ELISpot plate (sterile conditions)

- |  |   |
|--|---|
| 1a. <b>Antigen-specific IgM</b><br>Dilute the antigen to suitable concentration (1-50 µg/ml in sterile PBS, pH 7.4). | 1b. <b>Total IgM</b><br>Dilute the coating anti-IgM antibody to 10 µg/ml in sterile PBS, pH 7.4 |
|--|---|
- PVDF plates need to be treated (activated) with ethanol before coating. PVDF plates from Millipore, type MAIPSWU should be treated with 50 µl 70% ethanol/well for maximum 2 minutes. PVDF plates, type MSIP should be treated with 15 µl 35% ethanol/well for maximum 1 minute. After ethanol treatment, immediately proceed to plate washing.
  - Wash plate 5 times with sterile water, 200 µl/well.
  - Add 100 µl/well of the antigen or antibody solution and incubate overnight at 4-8°C.

### B Incubation of cells in plate (sterile conditions)

**See Hints and Comments section for details on cell stimulation.**

- Wash plate 5 times with sterile PBS, 200 µl/well, to remove excess antibody
- For blocking, add 200 µl/well of medium containing 10% of the same serum as used for the cell suspensions. Incubate for at least 30 minutes at room temperature.
- For analysis of *in vivo* activated B cells, remove the medium and add the cell suspension to the ELISpot plate. For analysis of memory B cells, the cells can be pre-activated separately in tubes prior to addition to the plates. See further Hints and Comments/*In vivo* activated B cells and Memory B cells.
- Put the plate in a 37°C humidified incubator with 5% CO<sub>2</sub> and incubate for 16-24 hours. Do not move the plate during this time and take measures to avoid evaporation (e.g. by wrapping the plate in aluminium foil).

### C Detection of spots

- To remove the cells, empty the plate and wash 5 times with PBS, 200 µl/well.
- Dilute the detection antibody (anti-IgM-biotin) to 1 µg/ml in PBS-0.5% fetal calf serum (PBS-0.5% FCS). Add 100 µl/well and incubate for 2 hours at room temperature.
- Wash plate as above (step C1).
- Dilute the Streptavidin-HRP\* in PBS-0.5% FCS and add 100 µl/well. Incubate for 1 hour at room temperature.

\*Streptavidin-HRP may require different dilutions depending on the substrate used. For AEC substrates dilution 1:100 is usually suitable. For other substrates (e.g. TMB) dilution 1:500 - 1:1000 may be required. *Please note that sodium azide used in buffers will inhibit HRP activity.*

- Wash plate as above (step C1).
- Add 100 µl/well of substrate solution (e.g. TMB) and develop until distinct spots emerge.
- Stop colour development by washing extensively in deionized water. If desirable, remove the plate from the plate tray or the underdrain and rinse the underside of the membrane.
- Leave the plate to dry. Inspect and count spots in an ELISpot reader or in a dissection microscope.
- Store plate in the dark at room temperature.

MABTECH AB  
Augustendalsvägen 19  
Box 1233, SE-131 28 Nacka Strand  
Sweden  
Tel: +46 8 716 27 00  
Fax: +46 8 716 27 01  
E-mail: [mabtech@mabtech.com](mailto:mabtech@mabtech.com)

MABTECH Inc  
M.E.B. 220, 3814 West Street  
Cincinnati, OH 45227  
USA  
Toll free: +1 866 ELI-SPOT  
Tel: +1 513 871 4500  
Fax: +1 513 871 7353  
E-mail: [mabtech.usa@mabtech.com](mailto:mabtech.usa@mabtech.com)

MABTECH AUSTRALIA Pty Ltd  
Australia  
Tel: +61 3 9470 4704  
Fax: +61 3 8678 3216  
E-mail: [mabtech.au@mabtech.com](mailto:mabtech.au@mabtech.com)

MABTECH AB Büro Deutschland  
Germany  
Tel: +49 40 4135 7935  
Fax: +49 40 4135 7945  
E-mail: [mabtech.de@mabtech.com](mailto:mabtech.de@mabtech.com)

MABTECH AB Bureau de liaison  
France  
Tel: +33 (0)4 92 38 80 70  
Fax: +33 (0)4 92 38 80 71  
E-mail: [mabtech.fr@mabtech.com](mailto:mabtech.fr@mabtech.com)

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