ELISpot^{PLUS} for Human IgA

Product Code: 3860-2HW-Plus

CONTENTS:

Vial 1 (blue top)
Monoclonal antibody MT57 (1.2 ml)
Concentration: 0.5 mg/ml

Vial 2 (red top)
Biotinylated monoclonal antibody MT20 (100 µl)
Concentration: 0.5 mg/ml

Vial 3 (yellow top)
Biotinylated monoclonal antibody MT57 (100 µl)
Concentration 0.5 mg/ml

Vial 4 (white top)
Streptavidin-Horseradish Peroxidase (50 µl)

Vial 5 (black top)
Polyclonal activator: R848 (100 µl)
Concentration: 1 mg/ml

Vial 6 (blue top)
Lyophilised recombinant human IL-2 (1 µg)

To ensure total recovery of stated quantity, vials have been overfilled.

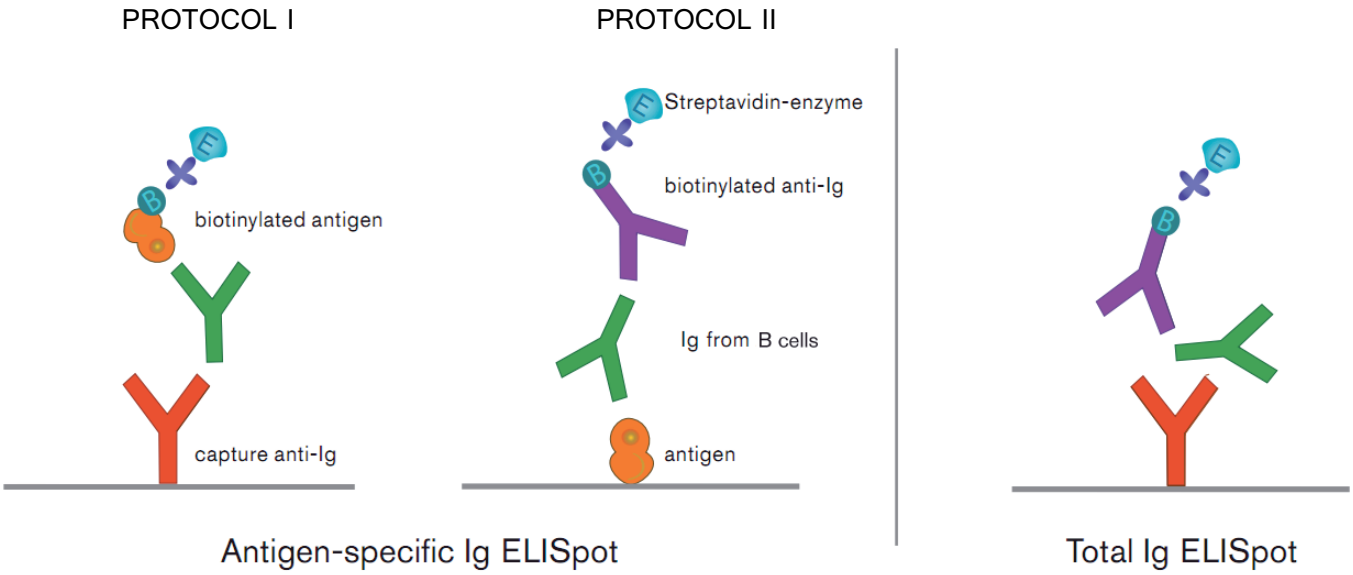
ELISpot plates (4 PVDF plates)

TMB substrate (2 x 25 ml)

STORAGE:

Shipped at ambient temperature. On arrival antibodies, streptavidin-HRP and substrate should be stored refrigerated at 4-8°C. R848 and IL-2 should be stored at -20°C. Plates may be kept at room temperature. Antibodies are supplied in sterile filtered (0.2 µm) PBS with 0.02% sodium azide. Streptavidin-HRP is supplied in PBS with 0.15% Kathon CG. R848 is supplied in sterile filtered (0.2 µm) PBS containing 2% DMSO.

Schematic illustration of the different protocols



	Antigen-specific IgA	**Total IgA**
Protocol I		
coating	MT57	MT57
detection	biotinylated antigen	MT20-biotinylated
Protocol II		
coating	antigen	MT57
detection	MT57-biotinylated	MT20-biotinylated

Kit description

Please read through before starting the assay

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

Detection of antigen-specific IgA-secreting B cells can be made using either biotinylated antigen (Protocol I) or using the antigen as coated (Protocol 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.

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

Protocol I: Detection of B cells secreting antigen-specific IgA (using biotinylated antigen) and total IgA. IgA from all secreting B cells are captured by capture mAb MT57. The secretion of specific IgA is then detected by the addition of biotinylated antigen and total IgA by mAb MT20-biotin. Spots are further visualised by Streptavidin-enzyme and substrate.

Please note that antigen must be biotinylated prior to starting the experiment.

Protocol II: Detection of B cells secreting antigen-specific IgA (using coated antigen) and total IgA. In the conventional way of performing B cell ELISpot, the antigen is coated on the ELISpot plate and antigen-specific IgA secreted by the B cells bind to the immobilised antigen. Spots are detected after addition of MT57-biotin followed by Streptavidin-enzyme and substrate. Total IgA is detected as in Protocol I

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

To obtain maximal antibody binding capacity, the plates need to first be activated 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 (A2-3) needs to be repeated before adding the coating antibody.

Plate washing

Always remove the plate from the plate tray before manually emptying the plate. 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.

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 IgA, 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 the kit) in separate tubes/plates for 48-72 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 μm) 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.

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

-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 μl) 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 H_2O 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 μg/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. **Antigen-specific IgA and total IgA**
   Dilute the coating mAb MT57 to 15 µg/ml in sterile PBS, pH 7.4.
2. Remove the ELISpot plate (type MAIPSWU included in this kit) from the package and pre-wet the membrane by adding 50 µl 70% ethanol per well for maximum 2 minutes.
3. Wash plate 5 times with sterile water, 200 µl/well. To avoid leakage, always remove the plate from the plate tray before manually emptying the plate.
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 section.
4. Put the plate in a 37°C humidified incubator with 5% CO_2 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 IgA**
Dilute biotinylated antigen to a suitable concentration (e.g. 0.01-1 µg/ml) in PBS containing 0.5% fetal calf serum (FCS). Add 100 µl/well and incubate for 2 hours at room temperature.

2b. **Total IgA**
Dilute the detection mAb MT20-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 (1:1000) in PBS-0.5% FCS and add 100 µl/well. Incubate for 1 hour at room temperature.*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 ready-to-use TMB substrate solution and develop until distinct spots emerge.
7. Stop colour development by washing extensively in tap water. Remove the plate from the plate tray 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. **Antigen-specific IgA**
Dilute the antigen to suitable concentration (e.g. 1-50 µg/ml in sterile PBS, pH 7.4).

1b. **Total IgA**
Dilute the coating mAb MT57 to 15 µg/ml in sterile PBS, pH 7.4

2. Remove the ELISpot plate (type MAIPSWU included in this kit) from the package and prewet the membrane by adding 50 µl 70% ethanol per well for maximum 2 minutes.
3. Wash plate 5 times with sterile water, 200 µl/well. To avoid leakage, always remove the plate from the plate tray before manually emptying the plate.
4. 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.

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 section.
4. Put the plate in a 37°C humidified incubator with 5% CO_2 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.
2. **Antigen-specific IgA**
Dilute the detection mAb MT57-biotin to 1 µg/ml in PBS containing 0.5% fetal calf serum (PBS-0.5% FCS). Add 100 µl/well and incubate for 2 hours at room temperature.
Total IgA
Dilute the detection mAb MT20-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 (1:1000) in PBS-0.5% FCS and add 100 µl/well. Incubate for 1 hour at room temperature.*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 ready-to-use TMB substrate solution and develop until distinct spots emerge.
7. Stop colour development by washing extensively in tap water. Remove the plate from the plate tray 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.

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