

Human TNF- α ELISpot^{BASIC} kit

Product Code: 3510-2H

CONTENTS, kit for 4 plates:

Vial 1 (red top)

Monoclonal antibody TNF3/4, a combination of two different antibodies (1.2 ml)

Concentration: 0.5 mg/ml

Vial 2 (blue top)

Biotinylated monoclonal antibody TNF5 (50 μ l)

Concentration: 0.5 mg/ml

Vial 3 (white top)

Streptavidin-Horseradish Peroxidase (HRP) (500 μ l)

STORAGE:

Shipped at ambient temperature. On arrival all reagents should be stored refrigerated at 4-8 °C. The expiry date indicates how long unopened products, stored according to instructions, are recommended for use. Antibodies are supplied in sterile filtered (0.2 μ m) PBS with 0.02% sodium azide. Streptavidin-HRP is supplied in PBS with 0.002% Kathon CG. Vials have been overfilled to ensure recovery of stated quantity.

Guidelines for Human TNF- α ELISpot^{BASIC}

Please read through before starting the assay

A Preparation of ELISpot plate (sterile conditions)

1. Dilute the coating antibody (TNF3/4) to 15 $\mu\text{g}/\text{ml}$ in sterile PBS, pH 7.4.
2. Remove the ELISpot plate from the package and if using a PVDF plate, pre-wet the membrane by adding ethanol. PVDF-plates from Millipore Corp., MAIPSWU, should be treated with 50 μl 70% ethanol per well for 2 minutes. PVDF-plates, type MSIP, should be treated with 15 μl 35% ethanol per well for maximum 1 minute.
3. Wash plate 5 times with sterile water, 200 $\mu\text{l}/\text{well}$.
4. Add 100 $\mu\text{l}/\text{well}$ of the antibody solution and incubate overnight at 4-8°C.

B Incubation of cells in plate (sterile conditions)

1. Remove excess antibody and wash plate 5 times with sterile PBS, 200 $\mu\text{l}/\text{well}$.
2. Add 200 $\mu\text{l}/\text{well}$ of medium containing 10% of the same serum as used for the cell suspensions. Incubate for at least 30 minutes at room temperature.
3. Remove the medium and add the stimuli followed by the cell suspension. Alternatively cells and stimuli can be mixed before addition to the plate.
4. Put the plate in a 37°C humidified incubator with 5% CO₂ and incubate for 12-48 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. Remove the cells by emptying the plate and wash 5 times with PBS, 200 $\mu\text{l}/\text{well}$.
2. Dilute the detection antibody (TNF5-biotin) to 0.5 $\mu\text{g}/\text{ml}$ in PBS containing 0.5% fetal calf serum (PBS-0.5% FCS). Add 100 $\mu\text{l}/\text{well}$ and incubate for 2 hours at room temperature.
3. Wash as above (step C1).
4. Dilute the Streptavidin-HRP* in PBS-0.5% FCS and add 100 $\mu\text{l}/\text{well}$. Incubate for 1 hour at room temperature.

*Please, note that the HRP conjugate may require different dilutions depending on the choice of substrate in the final step. Thus, for AEC a dilution of 1 in 100 is usually suitable whereas a higher dilution (1 in 500-1000) may be required if using other substrates (e.g. TMB). Please, also note that HRP-conjugates should not be used with buffers containing sodium azide as this compound will inhibit enzyme activity.

5. Wash as above (step C1).
6. Add 100 $\mu\text{l}/\text{well}$ 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 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.

Hints and comments

These suggestions are based on the detection of antigen-specific immune responses using PBMC. If using T-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 (A2-3) needs to be repeated before adding the coating antibody.

Plate washing

Always remove the plate MAIPSWU 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 freshly prepared and cryopreserved cells may be used in the assay. However it is recommended that the latter are rested for at least one hour to allow removal of cell debris. To decrease background TNF- α spots, a subsequent resting overnight is recommended after removal of cell debris. Triplicates or duplicates of 250,000 cells per well are often used to assess antigen-specific responses. For polyclonal activators, the cell number may have to be reduced to avoid confluent spot formation. Protocols with other incubation times have to be established by the user.

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 heterophilic antibodies or intrinsic analyte which may interfere with the assay.

Detection antibody

To reduce unspecific background it is recommended to filter (0.2 μm) the working dilution of detection mAb.

Assay controls

The number of cells responding to stimulation is often compared to the number of cells spontaneously producing the cytokine, which is determined by incubating the same number of cells in the absence of stimuli. A polyclonal activator such as phytohemagglutinin (1-10 $\mu\text{g/ml}$) is often used as a control for cell viability and functionality of the test system.

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. Preferably use deionized water to stop the plates since some ions may cause fading of TMB spots.

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