References:
Zuber B, *et al.* Detection of human perforin by ELISpot and ELISA: *Ex vivo* identification of virus-specific cells. *J. Immunol. Methods 302: 13, 2005*

NOTE; for research use only.

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ELISpot for Human Perforin

Product Code: 3465-2H

CONTENTS, kit sufficient for ≥ 4 plates:

- ❑ Vial 1 (yellow top)
  Monoclonal antibodies Pf-80/164 (1.2 ml)
  Concentration: 1 mg/ml
- ❑ Vial 2 (blue top)
  Biotinylated monoclonal antibody Pf-344 (50 µl)
  Concentration: 1 mg/ml
- ❑ Vial 3 (white top)
  Streptavidin-Horseradish Peroxidase (500 µl)

STORAGE:
All reagents should be stored refrigerated at 4-8°C. 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®.

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

Guidelines for Human Perforin ELISpot

This assay is based on the use of a combination of two coating antibodies, Pf-80 and Pf-164, for optimal results. The assay also detects perforin from rhesus and cynomolgus macaques.

A Preparation of ELISpot plate *(sterile conditions)*

1. Dilute the coating antibody (Pf-80/164) to 30 µg/ml in sterile PBS, pH 7.4.
2. Remove the ELISpot plate from the package and if using a PVDF plate, prewet the membrane by adding ethanol. PVDF-plates from Millipore Corp., type ELIIP, should be activated with 50 µl 70% ethanol per well for 2 minutes. PVDF-plates, type MSIP, should be activated with 15 µl 35% ethanol per well for maximum 1 minute.
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)*

1. Remove excess antibody and wash plate 5 times with sterile PBS, 200 µl/well.
2. Add 200 µl/well of medium containing 10% of the same serum as used for the cell suspensions. Incubate for ≥30 minutes at room temperature.
3. Remove the medium and add the cell suspension including possible stimulatory agents such as antigen (final volume 100-150 µl/well).
4. Put the plate in a 37°C humidified incubator with 5% CO_2 and incubate for 18-48 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 µl/well.
2. Dilute the detection antibody (Pf-344-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.
3. Wash 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.

   **Please, note that the peroxidase 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 µl/well of substrate solution and develop until distinct spots emerge.
7. Stop colour development by washing extensively in tap water. On ELIIP plates, remove the plate tray, and on MSIP plates the underdrain (the plastic under the plate), and rinse the back of the membranes.
8. Leave the plate to dry. Inspect and count spots in a dissection microscope (x40) or in an ELISpot reader.
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 human peripheral blood mononuclear cells (PBMC). If using T-cell clones, mixtures of separated cell fractions etc., other protocols may have to be considered.

Perforin system

Carefully follow the recommended protocol to achieve optimal and reliable results. The spots from perforin secreting cells will be small and distinct and are hard to detect without microscope. The perforin spots normally demands longer substrate development time than e.g. human IFN-γ spots.

Important

Important in all steps: Avoid getting liquid on the backside of the membrane or in the underdrain as this may cause leakage due to capillary drainage.

Plates

Several types of plates can be used in the ELISpot. However, for optimal results we recommend the use of PVDF-based membrane plates type ELIIP (MAIPSWU10 from Millipore Corp). To obtain maximal antibody binding capacity these plates need to first be activated by a brief treatment with ethanol or methanol. It is essential that the membrane is not allowed to dry after the activation. If this occurs the activation step (A2-3) needs to be repeated before adding the coating antibody.

Plate washing

Always remove the ELIIP 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.

Cells

Both freshly prepared and cryopreserved cells may be used in the assay with good results. 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 in order to avoid confluent spot formation. A serum that is good for cell culture and gives low background staining should be chosen; fetal calf serum is normally recommended.

Cell incubation time

Antigen-specific stimulation of cells can result in detectable spots after 18 hours of incubation. If desired, the incubation time can be extended up to 48 hours. Protocols with shorter incubation times have to be developed and evaluated by the user.

Assay controls

The number of cells responding to antigen stimulation is often compared to the number of cells spontaneously producing the cytokine. Spontaneous production is determined by incubating the same number of cells in the absence of antigen. The spontaneous production may be higher for perforin than for the human IFN-γ system. A polyclonal activator such as phytohemagglutinin (1-10 µg/ml) is often included 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

Development is made until distinct spots are seen in positive wells (usually 20-50 minutes). A general darkening of the membrane may occur during development but disappears after drying.