IFNγ-ELISpot, Peptide stimulation

INTRODUCTION

Known since 1983 (Czerkinsky et al.), the ELISPOT assay (enzyme-linked immunospot assay) is one of the most sensitive functional assays for immune monitoring. Originally developed as a method to detect antibody-secreting B cells, the assay was adapted to quantify antigen-specific T cells via secretion of effector molecules such as cytokines, granzyme B or perforin at the single cell level. In general it does not require \textit{in vitro} stimulation or exogenous cytokines and it works with fresh and frozen cells.

The assay is based on the principle of an ELISA. Peptide or whole cells are applicable for stimulation of the cells directly on the microtiter plates coated with a detection antibody against the effector molecule of interest. Following a stimulation period, the cytokine or perforin will be visualized by an enzyme-labeled secondary antibody and its corresponding substrate. In the following, a short 2-day protocol for IFN-γ ELISPOT with peptide antigens is described in detail.
MATERIALS

REAGENTS:
- PBMCs following Ficoll separation from heparinized blood
- Phosphate buffered saline (PBS), pH 7.4, sterile
- PBS/0.01% Tween®20 (pH 7.4, 1:5 diluted; SIGMA; P-3563); (Mabtech recommended no Tween for new types of precoated plates)
- destilled/demineralized water
- heat-inactivated fetal calf serum (FBS-Gold #A15-151, PAA Laboratories GmbH)
- 35% (55 %) methanol or ethanol (depends on type of microtiter plate), not necessary for precoated plates
- ELISpotPRO (PRecoated One-step detection)-Kit (Mabtech #3420-2APT-10 for 10 transparent plates); for the 2-day protocol
- basic ELISpot-Kit (Mabtech, #3420-2A or 3420-2H; without substrate) or ELISpotPLUS-Kit (Mabtech #3420-2AW-Plus or 3420-2HW-Plus; with substrate and plates) for the 3-day protocol
- recommended substrates: BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Moss Inc. #NBTH-100) for alkaline phosphatase (ALP) or TMB (tetramethylbenzidine; Moss Inc. #TMB-H-100) for HRP (horseradish peroxidase)
- Benzonase Nuclease (purity <99%, Novagen Merck Biosciences # 71206-3)
- C.T.L. wash™ supplement (Cellular Technology Ltd. Europe #CTLW-010)
- C.T.L. Test™ medium (Cellular Technology Ltd. Europe #CTLT-010)

EQUIPMENT:
- recommended plates: 96-well MultiscreenHTS filter plates, sterile, transparent with a validated Immobilon®-P (PVDF, polyvinylidene fluoride) membrane (Millipore #MSIP S4510)
- hemacytometer “Neubauer improved”
- 10 and 25 ml graded serological pipets
- variable pipets (10-1000 µl)
- pipette tips (sterile, pyrogen free)
- variable multichannel pipette (100-200 µl) with reservoir
- filter, sterile (0.22 µm pore size)
- 15 and 50 polypropylene tubes (graded, sterile, pyrogen free)
- 50 ml syringe
- paper towels
- refrigerator, adjusted to +4-8°C
- humidified CO₂-incubator
- “class II” biological safety hood
- humidified metal box or aluminium foil
- light microscope (200x-400x magnification)
- dissecting microscope (or magnifying glass for evaluation of staining)
- ELISpot automatic reader system (e.g. ELISpot Reader System AID Autoimmun Diagnostika GmbH; ELR03 with software version 3.2.3 or 4.0)
- optional: ELI-Puncher Kit (Millipore #MELIPUNCH) for removal of membrane
- optional: a vacuum manifold for washing (Caution: should not be used for precoated plates!). A regular ELISA washer can be used after incubation and removal of the cells if the washing head is adapted to the ELISpot plates. We recommend washing with a multichannel micropipette.

**REAGENT SETUP:**

- **Coating antibody (clone 1-D1K, Mabtech, stock 1 mg/ml):** dilute to 15 µg/ml in sterile PBS, pH7.4, not necessary for pre-coated plates
- **Biotinylated detection antibody (clone 7-B6-1, Mabtech, stock 1 mg/ml):** dilute to 1 µg/ml in filtered PBS/0.5% FCS or use the one-step detection reagent ALP-conjugated antibody: to 1.5 µg/ml in filtered PBS/0.5% FCS (200x).
- **Streptavidin-ALP or –HRP (Mabtech):** dilute 1:1000 in filtered PBS/0.5% FCS
- **Ready-to-use substrate solutions BCIP/NBT (for ALP) or TMB (for HRP):** should be freshly made and filtered
- **Blocking medium: RPMI III** (= RPMI-1640 + 2mM L-glutamine, 1mM sodium pyruvate, penicillin/streptomycin (100U/ml) supplemented with 10% human AB serum (BioWhittaker). *Serum batches must be pretested for background or alternatively use the C.T.L. -Test™ medium (it contains enough protein for blocking)*
- **Culture medium: serum-free C.T.L.-Test™ medium**

Storage at 4°C, protected from light; should be supplemented with 1% fresh glutamine and pre-warmed prior to use
- **C.T.L.-Wash™ supplement medium**
  Storage at 4°C, protected from light; should be diluted 1+9 with RPMI-1640 asupplemented with 1% fresh glutamine prior to use; sterile filtered and use warm for washing PBMC

- **Tumor-associated peptide antigens**: small aliquots of lyophilized peptides should be stored at -20°C or -80°C until usage (stocks 40 mg/ml), then dissolved in DMSO and diluted with culture medium. Final concentration for each peptide: 2-10 µg/ml (optimal concentration should be tested before)

- **CEF peptide pool HLA-A2 (PANATecs GmbH #PA-CEF-004)**: CMVpp65 (NLVPMVATV, aa495-503), EBV-BMLF1 (GLCTLVAML, aa280-288), EBV-LMP-2 (CLGGLLTMV, aa426-434), influenza M1 protein (GILGFVFTL, aa58-66) and influenza RNA polymerase PA (FMYSDFHFI, aa46-54) useful as an internal positive control.
  
  Dilute stock solution (20 µg of each peptide/ml) 1:5 with culture medium to a concentration of 4 µg of each peptide/ml and freeze in 50 µl aliquots. Final concentration: 0.2 µg of each peptide per well.

- alternatively the 23-(or 32) viral CEF peptide pools with different HLA class-I restricted epitopes can be used

- **negative controls**: medium only, PBMC only, PBMC with an irrelevant peptide antigen

**IFNγ-ELISpot**

for quantification of peptide-specific T cells

**2-Day Protocol:**

with ELISpot®PRO-Kit: contains PVDF membrane plates pre-coated with the capture antibody, the ALP-conjugated detection antibody and the ready-to-use substrate BCIP/NBT-Plus.

*Each batch should be tested using a control experiment. A test plate can be ordered from the company free of charge. Carefully add 2-5 x 10⁴ PBMC from a healthy donor to each well of the plate +/- OKT3 (20 ng/ml).*
A) Preparation and blocking of pre-coated plates:

*Work under the laminar flow in sterile conditions!*

Prepare the PBMC according to the standard SOP ([link to the protocols for thawing, overnight resting and Benzonase treatment](#))

Remove plates from the sealed package and wash with sterile PBS (4x 150 µl/well); carefully shake excess liquid from the plate and pat the bottom with absorbent paper towels

Block with medium containing pretested 10% human AB serum or with the C.T.L.-Test™ medium (50 µl/well)

![Hourglass] at least 1 hour at 37°C or room temperature

B) Stimulation of PBMC:

Decant blocking medium and wash again once with 50 µl sterile PBS, carefully shake excess liquid from the plate and pat the bottom with absorbent paper

Distribute 50 µl of each peptide solution according to the experimental set up (see pipetting scheme as an example, use at least triplicates).

*Carefully* add 1-2 (-4) x10⁵ PBMC in 100 µl culture medium per well (final volume: 150 µl). The optimal cell number for each patient should ideally be tested before and depends also on the type of antigen and the type of cytokine.

Put the plates in a humidified metal box or wrap the plates in aluminium foil and incubate in a CO₂ incubator (*Caution! Do not move the plates during incubation and control the level to prevent uneven spot distribution!*)

![Hourglass] 18-24 hours at 37°C

C) Detection:

Decant the cells and wash thoroughly first with PBS/0.01% Tween®20 (5x 150 µl, 3 min. each times) and then with PBS (3x 150 µl, 3 min. each times); shake excess liquid from the plate and pat the bottom with absorbent paper
Dilute (200x) and filter the ALP-conjugated detection antibody 7-B6-1 solution (final conc.: 1.5 mg/ml in PBS/0.5% FCS). Failure to filter the antibody solution may result in non-specific spot formation due to protein aggregates!

Add 100 µl to each well and incubate plate

max. 2 hours at room temperature

Wash again thoroughly with PBS (5x 150 µl, 3 min. each time); shake excess liquid from the plate and pat the bottom with absorbent paper

Filter the BCIP/NBT-Plus solution (10 ml per plate) and add 100 µl of the substrate to each well

Develop until spots emerge. Caution! Not exceed 10-15 min. because of background staining! Control with a magnifying glass!

Stop the colour development by washing in tap water using a squirt bottle. Remove the underdrain of the plate and rinse the back of the membranes.

Remove all excess liquid even from the back of the wells with a paper towel. Leave the plates to dry over night or for two hours at 37°C in the dark.

Optional: Remove the membrane filters with the ELI-Puncher Kit (see protocol on the Millipore homepage)

Count spots with a dissection microscope or as recommended use a computer-assisted video image analysis system with a security and quality control system (links to evaluation of ELISpot plates e.g. by using an automatic reader system and to the trouble shootings).