LUMC-ECIT 008 Research

IFNγ-ELISPOT

MEASUREMENT OF ANTIGEN-SPECIFIC CYTOTOXIC T-CELLS
IN PERIPHERAL BLOOD MONONUCLEAR CELLS

This method defines how to detect antigen specific IFNγ-producing human PBMC. After culturing PBMC with antigen in plates coated with antibodies to IFNγ, a 2nd biotinylated antibody to IFNγ is added. To this a streptavidin conjugated horseradish peroxidase molecule is added followed by substrate. Where the antigen stimulated IFNγ-producing cells lay, a blue spot occurs which can be counted. This gives a frequency of antigen specific IFNγ–producing T-cells. **Version 6** is made to replace the medium with serum for medium without serum (X-vivo) based on our profiency panel results.

Materials:

- Exact MHC class I binding peptides binding to HLA-A/B/C?
- Nitrocellulose backed multiscreen 96-well plates (Millipore MSHA S4510)
- Phytoheamagglutinin (PHA) Murex Biotech HA16
- Sterile Phosphate buffered saline (PBS) Delivered by LUMC
- BCIP/NBT-Alkaline phosphatase substrate Sigma B-5655
- Extravidin-ALP Sigma E-2636
- Anti human IFN gamma Mab-1-D1K MabTech 3420-3-1000
- Anti human IFN gamma Mab-7-B6-1 biotinylated MabTech 3420-6-1000
- Human AB serum (ABS) PAA C15-021 or Greiner 758099S175
- IMDM+ (according to appendix 01) with + 10% Human AB serum (ABS): IMDM-ABS.
- X-vivo 15 (Lonza BE04-418F)
- 50 ml tube
- MQ fresh from millipore tap or bottle (Braun bottle supplied by pharmacist LUMC))

Method:

Day 1

- Note down the ID no. of the test (LUMC-ECIT 008-n, n=1….+1)
- Depending on the number of donor PBMC test samples and the variety of antigens (peptide, proteins, and controls) select the appropriate number of Multiscreen plates for use.
- For each Multiscreen plate dilute 50µl of mAb 1-D1K diluted in 10ml of PBS (5µg/ml). Contents are mixed well using a vortex.
- Incubate each well of a Nitrocellulose backed Multiscreen plates with 100µl of coating antibody overnight at 4^{o}C.
- PBMC samples. For each sample thaw vials that will provide enough PBMC in order to add $5x10^5$/well for each antigen in triplicate. Depending on cell quality and patient status calculate in a loss of 25% of cells.
  Number of test Ag x 3 (triplicate wells)x($5x10^5$) PBMC = Number of PBMC required.

- Thaw the PBMC of the pre-vaccination sample according to LUMC-ECIT 011.
- Thaw the PBMC of the post-vaccination sample according to LUMC-ECIT 011.
- Note down the number of cells recovered for each visit.

- Resuspend PBMC in 5-10 ml IMDM-ABS (maximal $2x10^6$ PBMC/ml) .
- Incubate PBMC in the 50 ml tube with cap not completely closed at 37^oC, 5% CO_2 in a humidified incubator overnight.

Day 2

- *Note: incubation with coating antibody can also be done for 4 hours at 37^oC, 5% CO_2 in a humidified incubator.*
- Aspirate the coating antibody and wash plates 4 times with 100µl PBS (keep it sterile).
- Add 100 µl of X-vivo to each well and incubate plates for at least 1 hour at 37^oC, 5% CO_2 in a humidified incubator.

- Make working dilution of antigens (proteins and peptides) in X-vivo to 3 times the final concentration (1 µg/ml for CTL peptides, 10 µg/ml for proteins, 1 µg/ml for PHA).

- Resuspend the PBMC in the 50 ml tube.
- Count cell yield and viability using trypan blue dye and hemacytometer.
- Note down the number of living and death cells.
- Make a concentration of $5x10^6$ PBMC/ml with X-vivo.

- Aspirate the X-vivo from the Multiscreen plates.
- Add 50 µl/well of working antigen solutions to the appropriate wells of the coated Multiscreen plate in triplicate. As a negative control make sure at least one triplicate of wells is set up where 50 µl of X-vivo containing no antigen is added to each plate. As a positive control use PHA (0.5 µg/ml).
- Add 100 µl of PBMC suspension ($5x10^6$/ml) to all wells except for those which have PHA. They receive 50µl of PBMC in each well, and 50µl of medium.
- Culture plates overnight (20-26 hours) at 37^oC, 5% CO_2 in a humidified incubator. *Note: Make sure that the plates are not moved anymore until staining the next day, since movement of cells in the wells during the IFNγ production may result in vague spots or even overlapping spots.*

Day 3

- Discard contents of all Multiscreen plate wells into the sink, by turning plates upside down. Use a 10 ml or 25 ml pipette or squeezy bottle to fill the wells with PBS/Tween 0.05%.
- Discard PBS/Tween 0.05% into the sink.
- Wash Multiscreen plate for a second time with PBS/Tween 0.05%.
- Remove back cover of plate and wash the back of each well twice with PBS/Tween 0.05% including the back covering the plate.
- Wash the wells again 4 times with PBS/Tween 0.05%.
- Replace back of Multiscreen plate, ensuring it is firmly attached.
- Dilute mAb (7-B6-1-biotin) 1:3000 (0.3 µg/ml) in PBS. Add 100 µl of diluted biotinylated mAb 7-B6-1 biotin to each well of the Multiscreen plate.
- Cover with evaporation lid and incubate for 2 hours at room temperature.
- Wash plate 5 times (also back of the wells) with PBS/Tween 0.05%.

- Dilute Extravidin-ALP 1:1000 in PBS. Add 100 µl of diluted Extravidin-ALP to each well and incubate for 1 hour at room temperature.
- Prepare substrate solution (at least 30 minutes before use) by dissolving one tablet of BCIP/NBT ALP substrate in 10 ml of MilliQ (keep it in dark until use).
- Wash plate 6 times (also back of the wells) with PBS/Tween 0.05% as described above.
- Dispense 100 µl/well of substrate solution and incubate for 1-20 minutes at room temperature.
  Note: When substrate has been added, incubate in the dark at room temperature. Stop the colour process at the point that spots are clearly visible in the positive control and no spots are seen in the negative (medium) control.
- Wash thoroughly with tap water to stop colorimetric reaction also wash back of the plate with tap water
- Remove the cover of the back of the Multiscreen plate.
- Allow the plates to dry.
- Measure the number of spots per well using the ELISPOT reader (according to the manual of the ELISPOT reader).
- Store Multiscreen plates in dark to prevent the degradation of the spot intensity.

Protocol: LUMC-ECIT 008, version 6 Signature:

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Date: 08-02-2011 LUMC, The Netherlands