

## Addendum

### *Guidelines and troubleshooting for ELISPOT analyses*

#### **Assay performance**

There are two ways: either the assay is performed *directly* in the ELISPOT well or *indirectly* by introducing a preincubation step in the procedure. The rationale of preincubation is that exogenous proteins must be internalized, processed and presented by antigen presenting cells (APC) via MHC class I/II molecules to CD8<sup>+</sup>/CD4<sup>+</sup> T cells. It has been shown that a preincubation step in a tube prior to analyzing the cells in the ELISPOT assay is required for optimal antigen presentation<sup>1</sup>.

#### **Isolation and handling of human and non-human primate blood cells**

Venous or arterial blood should be collected from humans or non-human primates, fasted for at least 6 hours and using heparin as anti-coagulant. After being drawn, blood is kept at room temperature for maximally 16 hours.

Peripheral blood mononuclear cells (PBMCs) are isolated from venous blood by density gradient centrifugation and washed twice in medium (RPMI 1640 + L-glutamin + antibiotics).

Specimen collection from humans and non-human primates should be carried out in accordance with NCCLS document M29-T2. No known test method can offer complete assurance that human- or non-human primate-derived blood or tissue samples will not transmit infection. Therefore, all human and non-human primate specimens should be considered potentially infectious.

#### **Directions for cell culture of human or non-human primate PBMCs**

Optimal conditions for the generation of cells releasing cytokines or other effector molecules in heterogeneous cell populations should be determined empirically. However it should be realized that different cell types, producing the same effector molecules, require different conditions for stimulation. For instance, the optimal conditions for the detection of IFN- $\gamma$  secreting CD8<sup>+</sup> T cells in PBMC preparations differ considerably from those for the detection of IFN- $\gamma$  secreting CD4<sup>+</sup> T cells<sup>1</sup>. Moreover, the production of cytokines, such as TNF- $\alpha$ , IL-6 and IL-10, is not restricted to T cells and a high frequency of these cytokine secreting cells can also be attributable to activated monocytes/macrophages. Adherence of these cells to the surface of an ELISPOT well may already be sufficient to trigger IL-6 and TNF- $\alpha$  release.

When following the *direct* ELISPOT procedure, it should be realized that cell contact is critical for an optimal immune response. For that reason, 1-3x10<sup>6</sup> PBMCs/ml should be brought into the well<sup>2</sup> (100  $\mu$ l/well). *Polyclonal* stimulation of this high number of PBMCs usually leads to more than 500 spot forming cells (SFC)/well interfering with the formation of individual spots. Therefore for polyclonal stimuli, the cell number in the ELISPOT plate should be reduced to less than 5x10<sup>5</sup> cells/ml (100  $\mu$ l/well). On the other hand, *antigen-specific* stimulation generally yields less than 100 SFC/10<sup>5</sup> PBMCs making this *direct* approach highly suited for *antigen-specific* responses. However, it should be realized that a preincubation step has been recommended for optimal antigen presentation by APC<sup>1</sup>.

In the *direct* procedure, cells are suspended in culture medium with an appropriate stimulus and brought into the well of the ELISPOT plate and incubated for 16 to 24 hours to allow spot formation. For optimal ELISPOT responses with human PBMCs, serum-free medium (AIM-V) has proven to be the best choice. However, RPMI 1640 medium supplemented with 2 mM L-glutamine, antibiotics and 10% FCS (fetal calf serum) is a good medium for various other cell types. No more than 3x10<sup>5</sup> cells/well should be suspended in the ELISPOT plate.

When following the *indirect* procedure, consistent results are obtained if the cells are preincubated in culture medium with an appropriate stimulus for 16 to 42 hours at  $4 \times 10^6$  cells/ml in a tissue culture plate (100% humidity, 37°C and 5-7% CO<sub>2</sub>). It is important that during preincubation cell density is high. Therefore a minimum of 1 ml medium containing  $4 \times 10^6$  cells is brought in a well of a 24-well plate, 0.5 ml in a well of a 48-well plate or 100 µl in a well of a 96-well plate.

After preincubation, the non-adherent cells are collected and washed twice with culture medium to avoid the carryover of cytokines produced during the preincubation step. This involves two centrifugation/resuspension steps (8 min, 200 x g, room temperature). Then the cells are suspended in culture medium with an appropriate stimulus at  $1-3 \times 10^6$  cells/ml (antigen-specific responses). Hundred µl of this cell suspension is transferred to the well of the ELISPOT plate and incubated for 5 to 24 hours to allow spot formation. For polyclonal stimuli, the cell number needs to be reduced to  $\leq 5 \times 10^5$  cells/ml.

For the *indirect* procedure, AIM-V medium is dissuaded.

### **Directions for the isolation and cell culture of rodent cells**

For rodent cells, the same culturing conditions can be applied as described for human cells. However, most data so far were obtained with rodent spleen cells and only the collection, preparation and culture conditions of mouse or rat spleen cells are described in this section.

Spleens are aseptically removed from rodents and collected in RPMI 1640 medium. A single cell suspension is prepared by gently teasing the spleen tissue through a sterile stainless steel or nylon screens into RPMI 1640 medium and then washed twice with RPMI 1640 medium. This involves two centrifugation/resuspension steps (8 min, 200 x g, room temperature). Cells are cultured in culture medium (RPMI 1640 medium + 2 mM L-glutamine + antibiotics + 10% FCS) supplemented with an appropriate stimulus for triggering cytokine production. Consistent results are obtained if the splenocytes are prestimulated for 24 hours at  $2 \times 10^6$  cells in a volume of 0.5 ml in the wells of a 48-well tissue culture plate in a humidified atmosphere at 37°C with 5-7% CO<sub>2</sub>. Subsequently, the non-adherent cells are collected by two gentle washing steps using prewarmed (37°C) culture medium. Cells are centrifuged (200 x g) for 5 minutes at room temperature and resuspended in 500 µl culture medium with the same supplements as present during stimulation (including antigen/mitogen). Thereafter varying concentrations of cells (starting at  $3 \times 10^6$  cells/ml in triplicate with 1:3 serial dilutions down to  $\pm 10^3$  cells/ml, final volume: 100 µl/well) are transferred to the ELISPOT plate for a further incubation of 5-16 hours to allow spot formation.

### **Stimuli and their concentrations**

For antigen-specific stimulation, the optimal antigen concentration should be determined experimentally but generally varies between 1 and 10 µg/ml of protein or peptide.

As antigen-specific positive control in the human IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13 and granzyme B ELISPOT assay the ICE peptide pool (1 µg of each peptide/ml) can be used. This pool consist of 23 peptides of Influenza A virus (flu), Cytomegalovirus (CMV) and Epstein Barr virus (EBV) epitopes which are recognized by CD8<sup>+</sup> T cells and presented by 11 class I HLA-A and HLA-B alleles prevalent among Caucasian individuals<sup>3</sup>.

As polyclonal stimuli for human and non-human primate PBMCs, concanavalin A (conA; 6-10 µg/ml), a combination of PMA (50 ng/ml) plus ionomycin (1 µg/ml), PHA (10 µg/ml) or anti-CD3/CD28 antibodies can be used. Whereas the first three stimuli can be used in all the different cytokine ELISPOT assays, anti-CD3/CD28 is effective only for the IFN-γ, IL-4, IL-10 and granzyme B ELISPOT assays. For rodent splenocytes, conA (4 µg/ml) or a combination of PMA (50 ng/ml) plus ionomycin (1 µg/ml) have shown to be effective polyclonal stimuli.

### Directions for washing of polystyrene-bottomed plates

- All washing must be performed with Wash buffer (PBS containing 0.05% Tween-20).
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into each well. Take care not to scratch the bottom of the well. After aspiration, fill the wells with at least 250 µl of wash buffer and then aspirate the liquid. After washing, the wells of the plate are emptied by a firm shake-out action.
- Alternatively, the wash buffer may be put into a squirt (wash, squeeze) bottle (use a squirt bottle with a wide spout). If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After washing, the wells of the plate are emptied by a firm shake-out action.
- If using an automated washing device, the operating instructions should carefully be followed.

### Directions for washing of PVDF membrane-bottomed plates

- All washing must be performed with Wash buffer (PBS containing 0.05% Tween-20).
- For effective washing of PVDF membranes, a squirt bottle with a wide spout has shown to produce the best results. The bottle should be used to thoroughly flush all wells of the plate with Wash buffer. While flushing, the wells are completely filled with Wash buffer and subsequently emptied by a firm 'shake-out' action. After washing, the plate is emptied by tapping both sides on absorbent tissue.
- Additional washing of the underside of the PVDF membrane is needed after the incubation steps with detection antibody and conjugates to further reduce background staining. To do so, remove the plastic underdrain of the plate and use the squirt bottle to flood the underside of the membrane with Wash buffer. After washing, the Wash buffer is removed by a gentle 'shake-out' action.

### Recommended reagents

- Ficoll-Paque: Amersham biosciences (GE Healthcare) cat. nr. 17-1440-02 (for isolation of PBMCs by density gradient centrifugation)
- RPMI 1640 medium: Invitrogen cat. nr. 52400-025
- L-glutamine: Invitrogen cat. nr. 35030-024
- Penicillin/Streptomycin: Invitrogen cat. nr. 15140-122
- AIM-V medium: Invitrogen cat. nr. 31035-025
- ICE peptide pool: U-CyTech biosciences cat. nr. CT370
- Concanavalin A (ConA): Amersham biosciences (GE Healthcare) cat. nr. 17-0450-01
- Phorbol 12-myristate 13-acetate (PMA): Sigma Aldrich cat. nr. P8139
- Ionomycin: Sigma Aldrich cat. nr. I0634
- Phytohemagglutinin (PHA): Sigma Aldrich cat. nr. L8902
- Anti-CD3/CD28 for human PBMCs: U-CyTech biosciences cat. nr. CT372

### Troubleshooting

#### *No or low frequency of spots*

- Polyclonal stimulation of cells sometimes leads to a low frequency of spot forming cells as a consequence of apoptotic/necrotic cell death. If the culture medium turns yellow during stimulation, cell death is likely to occur. No such phenomenon occurs with antigenically stimulated cells.
- Clumping of cells during preincubation (particularly prominent with a polyclonal stimulus) may lead to underestimation of spot forming cells and inconsistent results. It is therefore critical that before the cells are transferred to the ELISPOT plate, they are thoroughly resuspended to obtain a single cell suspension (*indirect* procedure only).
- PBMCs isolated from blood kept for more than 16 hours at room temperature may produce less spot forming cells.

#### *Faintly stained spots*

- PBS tablets should not be used for the preparation of the coating antibody solution. The filler in the tablets interferes with the coating process.
- The AEC stock solution can lose activity when it is exposed to light or prolonged stored at temperatures  $\geq 0^{\circ}\text{C}$  (enzymatic staining procedure).
- For optimal coloring, the AEC substrate solution can be best applied to the wells at temperatures of 25-30°C (enzymatic staining procedure).
- The Activator I and II solutions can lose activity when they are exposed to air and/or light, are not properly stored or have been cross-contaminated (silver-staining procedure).

#### *Artifactual spots and/or high background staining*

- Just prior to spot counting, it is important to clean the underside of the polystyrene-bottomed wells with 70% ethanol and to remove dust particles by blowing 4-5 bar compressed air into the wells (dust particles can be the cause of spot-like structures).
- The reconstituted antibody solutions should not be used if there is an indication of bacterial growth or if the solutions have become turbid.
- Bacterial or fungal infections in PBMC preparations or culture medium can produce spot-like structures in the well.
- Inadequate post-coating of the ELISPOT well or insufficient washing between the different incubation steps may be the cause of artifactual spots or high background staining.
- When the *indirect* ELISPOT procedure step is followed, wash the cells thoroughly before they are transferred to the ELISPOT plate to avoid the carryover of cytokines released in the preincubation medium.
- Complete drying of the PVDF membranes (overnight at room temperature and in the dark) after the completion of the assay, is important for obtaining optimal spot intensity and low background staining.
- PBMCs from HTLV-1-infected humans and STLV-infected monkeys may contain a high frequency of spontaneously secreting IFN- $\gamma$  producing cells<sup>4</sup>.

#### *Other*

- Do not stack the plates during incubation.
- Do not puncture the PVDF membrane by pipetting/washing procedures. The membrane is fragile and may easily be damaged.
- To identify the optimal cell concentration for spot formation, include a wide range of cell concentrations in the first experiment.
- Spots may become irregular and ambiguous when the ELISPOT plate is moved during incubation. Even minor vibrations caused by closing the door of the incubator can affect spot formation.
- Granulocytes have a negative impact on spot formation<sup>5</sup>.
- During incubation with blocking solution, membrane-leakage occasionally occur. This phenomenon, however, does not negatively affect assay results (PVDF membrane-bottomed plates only).

#### References

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4. Van der Meide, P.H. *et al.* 1995. J. Med. Primatol. 24: 271.
5. De Rose, R. *et al.* 2005. J. Immunol. Meth. 297: 177.