

Addendum FluoroSpot

Guidelines and troubleshooting for FluoroSpot analyses

Assay performance

In the FluoroSpot assay it is critical to include a **preincubation** step for optimal results (indirect method). The rationale of preincubation is to optimize conditions for antigen uptake, processing and presentation to CD8⁺/CD4⁺ T cells¹. Omitting this step may lead to small-sized spots.

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- γ secreting CD8⁺ T cells in PBMC preparations differ considerably from those for the detection of IFN- γ secreting CD4⁺ T cells¹. Moreover, the production of cytokines, such as TNF- α , 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- α release.

A maximal frequency of spot forming cells is obtained when cells are preincubated in RPMI culture medium containing 2 mM L-glutamine, antibiotics and 10% FCS (fetal calf serum) and an appropriate stimulus for 16 to 42 hours at 4x10⁶ cells/ml (100% humidity, 37°C and 5-7% CO₂). It is important that during preincubation cell density is high. Therefore a minimum of 1 ml medium containing 4x10⁶ cells is brought in a well of a 24-well cell culture plate, 0.5 ml in a well of a 48-well plate or 100 μ l 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 $1\text{-}3 \times 10^6$ cells/ml (antigen-specific responses). Hundred μl of this cell suspension is transferred to the well of the FluoroSpot plate and incubated for 20 hours to allow spot formation. For polyclonal stimuli, the cell number needs to be reduced to 1×10^5 cells/ml or less.

Stimuli and their concentrations

For antigen-specific stimulation, the optimal antigen concentration should be determined experimentally but generally varies between 1 and 10 $\mu\text{g/ml}$ of protein or peptide.

As antigen-specific positive control in the FluoroSpot 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⁺ T cells and presented by 11 class I HLA-A and HLA-B alleles prevalent among Caucasian individuals².

As polyclonal stimuli for human and non-human primate PBMCs, concanavalin A (conA; 6-10 $\mu\text{g/ml}$), a combination of PMA (50 ng/ml) plus ionomycin (1 $\mu\text{g/ml}$), PHA (10 $\mu\text{g/ml}$) or anti-CD3/CD28 antibodies can be used.

Directions for washing of Fluorospot plates

All washing must be performed with Wash buffer (PBS containing 0.05% Tween-20).

For effective washing of Fluorospot plates, 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 Fluorospot plate is needed after the incubation steps with detector antibodies and Fluorescent-labeled conjugate to further reduce background signal. To do so, remove and discard 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. no. 17-1440-02
- (for isolation of PBMCs by density gradient centrifugation)
- RPMI 1640 medium: Invitrogen cat. no. 52400-025
- L-glutamine: Invitrogen cat. no. 35030-024
- Penicillin/Streptomycin: Invitrogen cat. no. 15140-122
- ICE peptide pool: U-CyTech biosciences cat. no. CT370
- Concanavalin A (ConA): Amersham biosciences (GE Healthcare) cat. no. 17-0450-01
- Phorbol 12-myristate 13-acetate (PMA): Sigma Aldrich cat. no. P8139
- Ionomycin: Sigma Aldrich cat. no. I0634
- Phytohemagglutinin (PHA): Sigma Aldrich cat. no. L8902
- Anti-CD3/CD28 for human PBMCs: U-CyTech biosciences cat. no. 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 FluoroSpot plate, they are thoroughly resuspended to obtain a single cell suspension.
- PBMCs isolated from blood kept for more than 16 hours at room temperature may produce less spot forming cells.

Faint fluorescent 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.

Artificial spots and/or high background signal

- 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 FluoroSpot well or insufficient washing between the different incubation steps may be the cause of artificial spots or high background signal.
- Wash the cells thoroughly before they are transferred to the FluoroSpot plate to avoid the carryover of cytokines released in the preincubation medium.
- Complete drying of the Fluorospot plates after the completion of the assay, is important for obtaining optimal spot intensity.
- PBMCs from HTLV-1-infected humans and STLV-infected monkeys may contain a high frequency of spontaneously secreting IFN- γ producing cells³.
- Dust particles produce bright artificial fluorescent spots, which have both a green and the red emission signal.
- The PVDF membrane-bottomed plates supplied with the kit are specially developed for FluoroSpot analyses. Other PVDF membrane-bottomed plates may result in autofluorescence interference.
- The use of serum-free medium (AIM-V) may lead to high background signals.

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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 FluoroSpot 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 formation⁴.
- During incubation with blocking solution, membrane-leakage occasionally occur. This phenomenon, however, does not negatively affect assay results.

References

1. Schmittl, A. *et al.* 2001. J. Immunol. Methods 247: 17.
2. Currier J.R. *et al.* 2002. J. Immunol. Meth. 260: 157.
3. Van der Meide, P.H. *et al.* 1995. J. Med. Primatol. 24: 271.
4. De Rose, R. *et al.* 2005. J. Immunol. Meth. 297: 177.

For further information, please visit our website (www.ucytech.com) and consult 'Frequently asked questions (FAQs)' or contact U-CyTech biosciences (info@ucytech.com).



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