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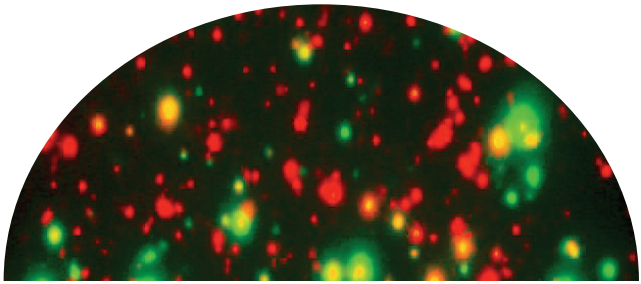
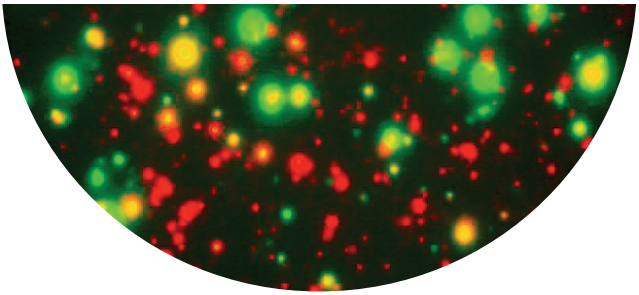
Addendum FluoroSpot assay



Guidelines and troubleshooting



Cell collection and handling

PBMCs derived from human and non-human primates

Whole blood samples should be collected from humans or non-human primates, using heparin or citrate as anti-coagulant. After being drawn, blood samples can be kept at room temperature (RT) for up to 8 hours. Peripheral blood mononuclear cells (PBMCs) are isolated by density gradient centrifugation (using e.g. Ficoll) and washed twice in culture medium. Washing involves two centrifugation/resuspension steps (8 min, 200 x g, RT). PBMCs can be used directly or frozen for later use in the FluoroSpot assay.

NOTES: 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.

The period between blood draw and PBMC isolation should be as short as possible (up to 8 hours).[1-4] If blood samples have been stored >20 hours the PBMC preparations may have become contaminated with activated granulocytes. This will result in a significant reduced T cell response. Methods to address this effect:

- Dilution of the blood sample 1:1 in PBS or RPMI-1640 medium prior to RT storage.[4]
- Depletion of granulocytes shortly after blood draw.

PBMCs from HTLV-1-infected human and STLV-infected monkeys may contain a high frequency of spontaneously IFN-γ producing cells.[5]

Cryopreservation of PBMCs

PBMCs can be safely frozen in culture medium supplemented with 10% dimethyl sulfoxide (DMSO) and 30% to 90% Fetal Calf Serum (FCS) with a recovery of more than 80%. The recommended cell concentration is ≥ $2x10^7$ cells/ml. Cool the freezing medium to 0 °C before use. Freeze the cells by using a Nalgene™ cryo 1°C freezing container and by placing the container in a -80 °C freezer overnight. The vials with cells are subsequently stored in liquid nitrogen. Alternatively, also serum-free cell freezing medium can be used for the FluoroSpot (e.g. BAMBANKER™ is recommended).[6]

The thawing procedure is stressful to frozen cells. Using a proper and fast procedure ensures that a high proportion of cells will survive. In brief, the cells are rapidly thawed by gently swirling the cryovial in a 37 °C water bath until there is just a small bit of ice left. Dropwise add 1 volume of fresh cell culture medium containing 50% FCS. Subsequently, 10 volumes of culture medium containing 10% FSC is added (slowly and under constant swirling) for the first wash step. To remove DMSO the cells are washed twice. This involves two centrifugation/resuspension steps (8 min, 200 x g, RT) with fresh culture medium containing 10% FCS.

Resting of cryopreserved human PBMCs

Different research groups have shown that detection of antigen-specific T cell responses by the ELISPOT/FluoroSpot using cryopreserved PBMCs is improved when cells are rested overnight prior to assay (both in human as non-human primates).[3,7,8,9] This resting procedure should help to eliminate apoptotic cells, and allows more accurate counts of viable and functional cells. However, resting of cryopreserved PBMCs does not always improve the ELISPOT/FluoroSpot performance.[10] Therefore, it cannot be said unambiguously whether resting will benefit the performance of the ELISPOT/FluoroSpot assay since it strongly depends on the internal laboratory procedure, clinical subject cohorts and the antigens used.

References

1. Afonso G *et al.* (2010). Critical parameters in blood processing for T-cell assays: Validation on ELISpot and tetramer platforms. J Immunol Meth 359: 28–36.
2. Bull M *et al.* (2007). Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. J Immunol Methods 322: 57-69.
3. Kierstead LS *et al.* (2007). Enhanced Rates and Magnitude of Immune Responses Detected against an HIV Vaccine: Effect of Using anOptimized Process for Isolating PBMC. Aids Res Hum Retrov 23: 86-92.
4. McKenna KC *et al.* (2009). Delayed processing of blood increases the frequency of activated CD11b+ CD15+ granulocytes which inhibit T cell function. J Immunol Meth 341(1-2): 68-75.
5. Meide van der PH *et al.* (1995). Enumeration of lymphokine-secreting cells as a quantitative measure for cellular immune responses in rhesus macaques. J Med Primatol 24: 271-281.
6. Filbert H *et al.* (2013). Serum-free freezing media support high cell quality and excellent ELISPOT assay performance across a wide variety of different assay protocols. Cancer Immunol Immunother 62: 615–627.
7. Janetzki S *et al.* (2008). Results and harmonization guidelines from two large-scale international ELISPOT proficiency panels conducted by the cancer vaccine consortium (cvc/svi). Cancer Immunol Immunother 57: 303–315.
8. Santos R *et al.* (2015). Improvement of IFNγ ELISPOT performance following overnight resting of frozen PBMC samples confirmed through rigorous statistical analysis. Cells 4: 1-18.
9. Donaldson MM *et al.* (2012). Optimization and qualification of an 8-color intracellular cytokine staining assay for quantifying T cell responses in rhesus macaques for pre-clinical vaccine studies. J Immunol Methods 14; 386: 10–21.
10. Kuerten S *et al.* (2012). Resting of cryopreserved PBMC does not generally benefit the performance of Antigen-Specific T Cell ELISPOT Assays. Cells 1: 409-427.

Recommended reagents

- BAMBANKER™: Nippon Genetics Europe cat. no. BB03
- Culture medium RPMI-1640 medium: Thermo Fisher Scientific cat. no. 52400.
- Fetal calf serum: Life Technologies cat. no. 16000-044.
- Ficoll-Paque PLUS: GE Healthcare cat. no. 17-1440-02 (for isolation of PBMCs by density gradient centrifugation).
- L-glutamine: Thermo Fisher Scientific cat. no. 25030-081.
- Penicillin/Streptomycin: Thermo Fisher Scientific cat. no. 15140-122.
- Trypan Blue Solution: Sigma-Aldrich cat. no. T8154.

The recommended culture medium is RPMI-1640 supplemented with 2 mM L-glutamin and 100 units/ml Penicillin and 100 µg/ml Streptomycin.

Directions for washing of FluoroSpot plates

All washing steps 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 emptied 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. Repeat this as many times as mentioned in the instruction manual. After washing, the plate is emptied by tapping it upside down on absorbent tissue paper.

Additional washing of the underside of the PVDF membrane is needed after the incubation steps with detection antibody and conjugate to further reduce background staining. 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.

NOTE: Do not puncture the PVDF membrane by pipetting/washing procedures. The membrane is fragile and may easily be damaged.

During incubation with blocking buffer, membrane-leakage occasionally occurs. This phenomenon, however, does not negatively affect assay results.

Overview general cell stimuli for use in FluoroSpot

Stimuli	Type of stimulation	Activated cell type	Stimulates secretion of	Recommended cell concentration*	Recommended stimulus concentration*
ICE peptide pool (Influenza A, CMV and EBV epitopes) (cat. no. CT387)	antigen specific	Human $CD8^+$ T cells (Caucasian)	IFN-γ, IL-2, Granzyme B	$2x10^5$ PBMC/well	1 µg of each peptide/ml
Tetanus toxoid (TT)	antigen specific	Human $CD4^+$ T cells	IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A	$2x10^5$ PBMC/well	0.5 LF/ml
Phytohaemagglutinin (PHA)	polyclonal	T cells	IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, Granzyme B, TNF-α	$2x10^3$ to $1x10^5$ PBMC/well	10-30 µg/ml
Phorbol 12-myrstate 13-acetate (PMA) + ionomycin	polyclonal	T cells	IFN-γ, IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, Granzyme B, TNF-α	$2x10^3$ to $1x10^5$ cells/well	PMA: 50 ng/ml + Ionomycin: 1 µg/ml
Concanavalin A (ConA)	polyclonal	T cells	IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, Granzyme B, TNF-α	$2x10^3$ to $1x10^5$ cells/well	6-10 µg/ml
anti-CD3/CD28 monoclonal antibodies (cat. no. CT372)	polyclonal	Human T cells	IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, Granzyme B	$2x10^3$ to $5x10^4$ PBMC/well	0.05 µg/ml

* The above mentioned concentrations are guidelines. It is recommended to analyze a series of dilutions to determine the optimal concentration first. A maximum of $3x10^5$ cells can be put into a well of a 96-well plate. However, it is important that the number of cells per well is not too high in order to facilitate counting of spots (50 to 100 spots/well).

Troubleshooting FluoroSpot

Problem	Possible cause	Solution
High background signal or artifactual spots	Incomplete washing	- Follow the "Directions for washing" in this Addendum carefully.
	Too many cells in FluoroSpot well	- Lower cell concentrations in the wells, by making a series of dilutions that will result in formation of distinct spots (optimally 50-100 spots/well).
	Aspecific binding	- Serum in the culture medium should be selected on low background staining.
	Contaminated working solutions or cell culture	- Solutions should not be used when they have become turbid or if there is an indication of bacterial or fungal growth. - Use a clean container for the transfer of solutions into the wells of the Fluorospot plate.
	Wrong type of PVDF membrane-bottomed plate used	- The IPFL PVDF membrane-bottomed plate (Millipore; cat. no. S5EJ104107) which are supplied with the kit are specifically developed for the FluoroSpot. Other PVDF plates may result in autofluorescence interference.
	Improper incubation period of cells in the Fluorospot plate	- Decrease incubation time of cells on the FluoroSpot plate.
	Carryover of secreted proteins (i.e. cytokines) produced during the preincubation step	- Ensure proper washing of collected cells after preincubation and before adding them to the FluoroSpot plate.
	Incomplete drying of PVDF membranes after completion of the FluoroSpot assay	- Allow the PVDF membranes to dry completely (at 37°C in the dark) prior to spot counting.
	Dust particles present in the wells	- Dust particles poduce bright artificial fluorescent spots, which can have both a green and red emission signal. Remove dust particles by blowing 4-5 bar compressed air into the wells.
Faint fluorescent spots	Incorrect incubation periods or temperature	- Ensure correct incubation times and temperature. - Reagent solutions should reach RT before use.
	Use of PBS tablets for preparing coating antibody or antigen solution	- The filler in tablets interferes with the coating process. Use sterile liquid PBS instead. Thermo Fisher Scientific cat. no. 10010 is recommended.
	Drying out of the PVDF membrane	- Do not allow PVDF membrane to dry during the procedure. If this occurs directly after pre-wetting, repeat pre-wetting step.
	No complete drying of the PVDF membrane after the completion of the assay	- Dry the plate further in a 37°C incubator (in the dark)
Small spot size	Inadequate incubation time	- Prolong incubation period of cells in the FluoroSpot wells.
Large spot size	Inadequate incubation time	- Shorten incubation time of cells in the FluoroSpot wells.
Confluent spots or poorly defined spots	Moving Fluorospot plate during cell incubation	- Prevent the plate from being moved during the cell incubation step. Even minor vibrations caused by closing the door of the incubator can affect spot formation.

Problem	Possible cause	Solution
No or low frequency of spots	Clumping of cells	- Resuspend cells gently but thoroughly, to gain a good homogeneous cell suspension, before they are brought into the wells of the FluoroSpot plate.
	Reduced viability of cells	- Improper freezing/thawing procedure.
	Too many activated granulocytes present before PBMC isolation	- The maximum time period between blood draw and PBMC isolation should be 8 hours.
	Cell concentration in FluoroSpot wells too low	- Increase cell concentration in the FluoroSpot wells, by making a series of dilutions that will result in formation of distinct spots (optimally 50-100 spots/well).
	Inadequate incubation time	- Determine the optimal (pre)incubation time of the cells by increasing or decreasing the (pre)incubation time.
Poor consistency of replicates	Inaccurate pipetting	- Ensure accurate pipetting. - Check pipettes.
	Clumping of cells	- Resuspend cells gently but thoroughly, to gain a good homogeneous cell suspension, before they are brought into the wells of the FluoroSpot plate.
	Evaporation of solutions	- Ensure proper sealing of the plate.
	Inaccurate temperature distribution during incubation steps	- Do not stack plates during incubation.
	Inadequate washing	- Follow the “Directions for washing” in this Addendum carefully.
Blank areas	Cells are unevenly distributed	- Resuspend cells gently but thoroughly, to gain a good homogeneous cell suspension, before they are brought into the wells of the FluoroSpot plate.
	Inaccurate pre-wetting of the PVDF membrane	- Do not allow PVDF membrane to dry after pre-wetting with ethanol. If this occurs, repeat pre-wetting step.
	Foam formation during washing	- The spout of the squirt bottle may be too narrow and should be enlarged.

Abbreviations

EBV	Epstein-Barr virus
HTLV	Human T-cell Lymphotropic Virus
PVDF	Polyvinylidene Fluoride
RT	Room Temperature (temperature between 20°C and 26 °C)
STLV	Simian T-cell Lymphotropic virus

Technical assistance

If you require assistance, information or have questions, please contact our company:

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On our website (www.ucytech.com/manuals) you can find: Manuals, Typical data, Addendum and MSDS of our FluoroSpot kits.