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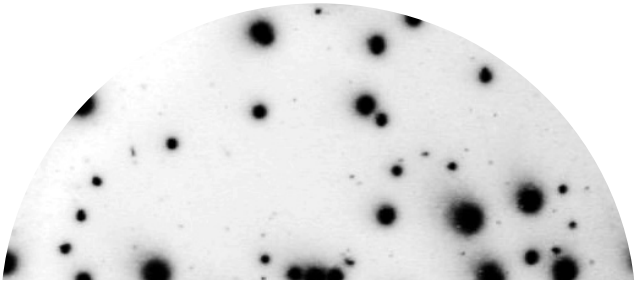
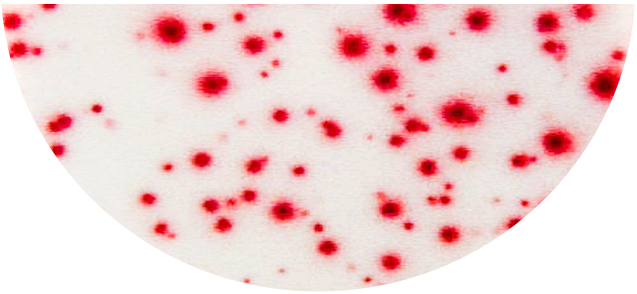
Addendum
T cell ELISPOT assay



Guidelines and troubleshooting



Contents

Abbreviations

AEC	3-amino-9-ethylcarbazole
CMV	Cytomegalovirus
ConA	Concanavalin A
DMSO	Dimethyl Sulfoxide
EBV	Epstein-Barr virus
ELISPOT	Enzyme-Linked ImmunoSPOT
FCS	Fetal Calf Serum
HRP	Horse Radish Peroxidase
HTLV	Human T-cell Lymphotropic Virus
LPS	Lipopolysaccharide
min	minutes
PBMC	Peripheral Blood Mononuclear Cell
PHA	Phytohaemagglutinin
PMA	Phorbol 12-Myristate 13-Acetate
PVDF	Polyvinylidene Fluoride
RT	Room Temperature
STLV	Simian T-cell Lymphotropic virus
TT	Tetanus Toxoid

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 are 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 T cell ELISPOT 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% DMSO and 30% to 90% 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 ELISPOT (e.g. BAMBANKER™).[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 and cold cell culture medium containing 50% FCS (4 °C). Subsequently, 10 volumes of cold culture medium containing 10% FSC (4 °C) is added (slowly and under constant swirling) for the first wash step (8 min, 200 x g, 4 °C). Thereafter, the cells are washed with fresh culture medium (RT) containing 10% FCS (8 min, 200 x g, RT).

Resting of cryopreserved PBMCs

Different research groups have shown that detection of antigen-specific T cell responses by the ELISPOT 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 performance.[10] Therefore, it cannot be said unambiguously whether resting will benefit the performance of the ELISPOT assay since it strongly depends on the internal laboratory procedure, clinical subject cohorts and the antigens used.

Cells from rodent origin

For rodent PBMCs, the same conditions can be applied as described for human cells. However, most ELISPOT data so far are obtained with mouse or rat spleen cells. It is recommended to start isolating spleen cells within 8 hours after spleen collection.

Spleens are aseptically removed from rodents and collected in culture medium. A single cell suspension is prepared by gently teasing the spleen tissue through a sterile stainless steel or nylon screens into culture medium and by washing with culture medium. Washing involves two centrifugation/resuspension steps (8 min, 200 x g, RT). Spleen cells can now be used in the ELISPOT assay.

Crypreservation of rodent spleen cells

Crypreservation of rodent spleen cells is complicated and has in general a negative impact on cell viability and function. By using BAMBANKER™ serum-free cell freezing medium, it is possible to obtain a recovery of 70-80% viable cells and T cell function at levels similar to freshly isolated spleen cells. To thaw the cells, follow the procedure described in the section “Cryopreservation of PBMCs”. Gad *et al.* (2013) reported that resting of cryopreserved murine spleen cells before ELISPOT did not improve T cell responses.[11] Overall, it is recommended to work with fresh rodent spleen cells in the ELISPOT.

Recommended reagents

- BAMBANKER™: Nippon Genetics Europe cat. no. BB03
- Culture medium RPMI-1640 medium: Thermo Fisher Scientific cat. no. 52400.
- Dimethyl sulfoxide (DMSO): Sigma-Aldrich cat. no. D2650.
- Fetal Calf Serum (FCS): Life Technologies cat. no. 16000.
- 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-glutamine and 100 units/ml Penicillin and 100 µg/ml Streptomycin.

Overview general cell stimuli commonly used in ELISPOT

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, Perforin	$2x10^5$ 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, IL-21, G-CSF, Granzyme B, Perforin, 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, IL-17F IL-21, G-CSF, GM-CSF, Granzyme B, Perforin, 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, IL-21, G-CSF, GM-CSF, Granzyme B, TNF-α	$2x10^3$ to $1x10^5$ cells/well	human/non-human primate PBMC: 6-10 µg/ml rodent splenocytes: 4 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
Lipopolysaccharide (LPS) + IFN-γ	polyclonal	Human monocytes/ dendritic cells	IL-1ß, IL-6, IL-10, IL-12, IL-23, GM-CSF	$1x10^3$ to $5x10^4$ PBMC/well	LPS: 100 ng/ml + IFN-γ: 10-100 units/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).

Directions for washing of polystyrene-bottomed plates

All washing steps must be performed with Wash buffer.

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. Repeat this as many times as mentioned in the instruction manual. After washing, the wells of the plate are emptied by a firm shake-out action followed by tapping the plate upside down on absorbent tissue paper.

Alternatively, the Wash buffer may be put into a squirt bottle (use a squirt bottle with a wide spout). If a squirt bottle is used, empty the wells by a firm 'shake-out' action and then flood the plate with Wash buffer, completely filling all wells. Repeat this as many times as mentioned in the instruction manual. After washing, the wells of the plate are emptied by a firm 'shake-out' action followed by tapping the plate upside down on absorbent tissue paper.

When using an automated washing device, the operating instructions should be carefully followed.

Directions for washing of PVDF membrane-bottomed plates

All washing steps must be performed with Wash buffer.

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 solution, membrane-leakage occasionally occurs. This phenomenon, however, does not negatively affect assay results.

Troubleshooting ELISPOT

Problem	Possible cause	Solution
High background	Incomplete washing	- Follow the “Directions for washing” in this Addendum carefully.
	Too many cells in ELISPOT well	- Lower cell concentration on ELISPOT plate, 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 ELISPOT plate.
	Overdeveloped plate	- Reduce incubation time of color development.
	Improper incubation period of cells in the ELISPOT plate	- Decrease incubation time of cells on the ELISPOT plate or follow the procedure with a preincubation step.
	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 ELISPOT plate.
	Incomplete drying of PVDF membranes after completion of the ELISPOT assay Ω	- Allow the PVDF membranes to dry completely (at RT in the dark) prior to spot counting.
Faintly stained 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.
	Improper handling of reconstituted Streptavidin-HRP conjugate ●	- Avoid exposure to light and heat. - Avoid prolonged storage of vial at RT.
	Improper handling of AEC stock solution ●	- Avoid exposure to light or prolonged storage at ≥ 0 °C. - Do not bring this solution in contact with polystyrene plastics.
	Improper handling of Activator I and II solutions Δ	- Avoid exposure to light and/or air. - Avoid cross contamination between the two vials. - Ensure gently, but thorough shaking of the vials before use.
	Drying out of the PVDF membrane Ω	- Do not allow PVDF membrane to dry during the procedure. If this occurs directly after pre-wetting, repeat pre-wetting step.
	Poor color development	- Increase time for color development.
	Bleaching of enzymatic stained spots ●	- Store ELISPOT plates at a dry place in the dark. Enzymatic stained spots will always bleach eventually.
Small spot size	Inadequate incubation time	- Prolong incubation period of cells on ELISPOT plate.
Large spot size	Inadequate incubation time	- Shorten incubation time of cells on ELISPOT plate.

Problem	Possible cause	Solution
Confluent spots or poorly defined spots	Cell concentration in ELISPOT wells too high	- Lower cell concentration on ELISPOT plate, by making a series of dilutions that will result in formation of distinct spots (optimally 50-100 spots/well).
	Moving ELISPOT 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.
	Dust particles ‡	- Prior to spot counting, clean the underside of the plate (polystyrene-bottomed) wells with 70% ethanol and remove dust particles by blowing 4-5 bar compressed air into the wells.
Low spot frequency	Clumping of cells	- Resuspend cells gently but thoroughly, to gain a good homogeneous cell suspension, before they are brought into the wells of the ELISPOT 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 ELISPOT wells too low	- Increase cell concentration in ELISPOT plate, 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 ELISPOT 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 ELISPOT plate.
	Inaccurate pre-wetting of the PVDF membrane Ω	- 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 is too narrow and should be wider, or the automated washing device does not operate properly and should be adjusted.
	Washing problem ‡	- The aspiration tubes of the washing device may be too close to the bottom of the ELISPOT wells and/or the flow rate may be too high. Change settings.

• Accounts only for enzymatic staining procedure on PVDF membrane-bottomed plates.
Δ Accounts only for silver staining procedure.
Ω Accounts only for procedure with PVDF membrane-bottomed plates.
‡ Accounts only for silver staining procedure on transparent polystyrene-bottomed plates.

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Notes

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 ELISPOT kits.