



Four Color ImmunoSpot® Assays for Identification of Effector T-Cell Lineages

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Abstract

Single color IFN- γ ELISPOT assays have evolved as a highly sensitive T cell immune monitoring platform. By detecting individual T cells that secrete IFN- γ in response to antigen exposure, these assays permit the measurement of the frequency of antigen-specific T cells among white blood cells. These assays therefore are well suited to assess clonal expansions, that is, whether a (Th1) T cell response has been induced to an antigen in a test subject. Single color IFN- γ ELISPOT assays are not suited, however, to provide information on the Th2/Th17 quality of the T cell response, nor do they provide insights into the differentiation state of CD8 cells. Recently it has been established that co-expression profiles of IL-2, TNF- α , and granzyme B along with IFN- γ permit to identify CD8 cell subpopulations. Naïve CD8 cells, central CD8 memory cells, CD8 terminal effector cells, polyfunctional CD8 cells, stem-cell like CD8 memory cells, dysfunctional- and senescent CD8 cells all differ in the extent they produce these molecules upon antigen re-encounter. We therefore have developed, and introduce here, a four color T cell ELISPOT assay in which the co-expression levels of IFN- γ , IL-2, TNF- α , and granzyme B can be established for individual antigen-specific CD8 cells, thereby identifying the activation/differentiation state of these cells.

Key words Central CD8 memory cells, CD8 terminal effector cells, Polyfunctional CD8 cells, Stem-cell like CD8 memory cells, Dysfunctional CD8 cell, Senescent CD8 cell, IFN- γ , IL-2, TNF- α , Granzyme B, Co-expression, ELISPOT, Multiplexing, Tumor immunity, Autoimmunity, Immune monitoring

1 Introduction

The immune system protects from various infections and tumors, but can also cause allergies, autoimmunity, and transplant rejection. Over the last decades, it has become clear that in each of these cases different types of effector T cell lineages play a role, the major ones for CD4 cells being Th1, Th2, and Th17 [1]. Th1 cells operate via the secretion of IFN- γ that shuts down viral replication in infected cells and induces MHC expression on such cells preparing them for T cell recognition. Th2 cells produce IL-4 that is involved in the regulation of immune globulin class switching, and a sub-lineage of Th2 cells is central to anti-parasite defense by activation of eosino-

phils via the secretion of IL-5. Finally, the Th17 cell type mediates DTH (delayed type hypersensitivity) by secreting IL-17 [2]. In DTH, macrophages are attracted and activated to protect against intracellular pathogens.

The Th1, Th2, and Th17 effector cell lineages emerge through instructed differentiation [3–6]. Once this differentiation is completed, the cytokine profiles of these cells are imprinted to be mutually exclusive [7]. Upon antigen re-encounter, Th1 cells will produce IFN- γ (but no IL-4 nor IL-17), Th2 cells will secrete IL-4 or IL-5 (but no IFN- γ and no IL-17), and Th17 cells will produce IL-17 in the absence of IFN- γ , IL-4, or IL-5 [8, 9]. The relative frequency of Th1/Th2/Th17 cells within the antigen-specific CD4 cell repertoire defines the quality of the immune response, that is, whether it is suitable for the different types of antigen encounters. Therefore, unambiguous measurements of Th1, Th2, and Th17 cell frequencies are critical for immune monitoring.

Antigen-specific Th1, Th2, and Th17 cells can be readily detected in ELISPOT assays that measure the cytokines that these cells produce after antigen stimulation. While IFN- γ release by CD4 cells peaks at 24 h after T cell receptor stimulation, IL-17 secretion by Th17 cells does not even start within 48 h, and peaks at 96 h [10]. It takes Th2 cells 48 h to engage in maximal IL-4 production, and their IL-5 secretion does not reach maximal levels until 96 h after antigen encounter [11]. Due to these fundamentally different secretion kinetics of TH1-, Th2- and TH17 cytokines, the CD4 effector lineages are best studied in single color ELISPOT assays that account for the optimal time point for each cytokine. While an ELISPOT test kit for the simultaneous detection of IFN- γ /IL-4/IL-17 can be readily manufactured, due to the underlying CD4 cell biology, such an IFN- γ /IL-4/IL-17 multiplex assay could provide inaccurate and false negative frequency measurements for antigen-specific Th1, Th2, and Th17 cells.

Like CD4 cells, also CD8 cells can differentiate into sublineages that produce IL-4 (Tc2) or IL-17 (Tc17), or function as regulatory cells [12]. The unique contribution of CD8 cells to host defense is, however, the ability to kill infected target cells. Naïve CD8 cells neither kill nor produce cytokine (Fig. 1A), but within 5 days after having been engaged in an immune response, they will develop into terminal effector CD8 cells that express high levels of perforin and granzyme B enabling them to kill target cells [13]. Terminal effector CD8 cells excel in the secretion of IFN- γ and TNF- α , but they produce little to no IL-2 (Fig. 1B) [14]. If the antigen is successfully eliminated, the effector cells return to a quiescent state in which they are called central memory cells. In the absence of antigen, it takes approximately 3 weeks for the terminal effector cell to become a resting memory cell [15]. Central CD8 memory cells will secrete IFN- γ within 24 h of re-antigen encounter, but they have lost their ability to secrete instantly granzyme B and perforin, and they will

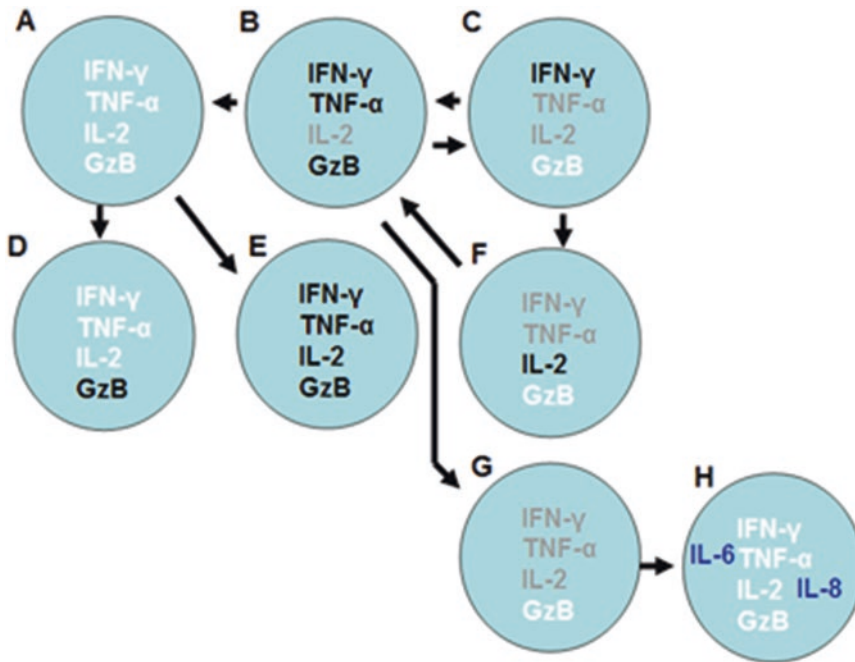


Fig. 1 CD8 cell activation and differentiation states can be identified via their expression pattern of IFN- γ , TNF- α , IL-2, and GzB. (A): Naïve CD8 cell, (B): Terminal effector CD8 cell, (C): Central CD8 memory cell, (D): Silent CD8 killer cell, (E): Polyfunctional CD8 cell, (F): Stem-cell like CD8 memory cell, T_{SCM} , (G): Dysfunctional CD8 cell, (H): Senescent CD8 cell. The phenotypes shown here correspond to the consensus nomenclature for CD8 T cell phenotypes established in 2015 [14]. Here we focus on the analyte secretion patterns of these subsets. The corresponding cell surface markers that can be established by flow cytometry are summarized in that publication

secrete little to no TNF- α or IL-2 (Fig. 1C) but within 3–4 days after antigen-re-encounter resting memory cells can convert again into terminal effector cells [16].

Recently, a CD8 memory cell type has drawn attention that is called “stem-cell like CD8 memory cells,” T_{SCM} [17]. T_{SCM} secrete low levels of IFN- γ and TNF- α , no GzB, but they produce abundant IL-2 (Fig. 1F) [14]. In the field of adoptive cancer therapy, it was observed that—counter to initial expectations—injections of fully differentiated CD8 terminal effector cells cause less tumor clearance than the infusion of less differentiated CD8 cells. These T_{SCM} have the property, much like naïve CD8 cells, to re-engage in extensive proliferative responses upon re-encounter of the antigen, a property that terminal effector cells increasingly lose with repeated antigen stimulation. Due to their self-renewing potential, and longevity, T_{SCM} enable us to come up with high numbers of terminal effector cells when repeated antigen encounters call for a sustained CD8 cell attack.

There is a lineage of cytotoxic CD8 cells that does not produce IFN- γ (Fig. 1D) [15]. Such effector cells are granzyme B (and

perforin) positive, and their cytolytic activity in a tissue is exerted without causing a local inflammatory reaction [2]. Therefore they are called “Silent CD8 killer cell.” Much attention has been drawn to so-called polyfunctional CD8 cells that co-express IFN- γ with IL-2 and TNF- α (Fig. 1E). While representing a low percentage among CD8 cells, their presence in increased frequencies has been linked to successful immune responses mounted against infectious diseases and cancer [14].

With persistence of a high antigen burden, due to the resulting ongoing immune stimulation, CD8 cells can undergo exhaustion [18]. This occurs in anti-tumor immunity, autoimmunity, and chronic infections. First, “dysfunctional” CD8 cells arise that have lost their self-renewing and cytolytic potential, and display reduced cytokine producing ability (Fig. 1F) [14]. These cells are not completely inactive, as their name might suggest, but exhibit attenuated effector functions as needed to prevent excessive collateral damage on the tissue in which the antigen persists and from which it could not be cleared by the initial vigorous CD8 cell attack. With further antigen persistence, CD8 cells eventually burn out, and become “senescent” (Fig. 1H) Senescent CD8 cells do not secrete IFN- γ , TNF- α , or IL-2 [14]. While void of direct cytolytic or pro-inflammatory cytokine function, senescent cells are still not completely inactive, but secrete IL-6 and IL-8 [14]. The latter cytokines primarily serve tissue repair, but in the case of tumors, this translated to promoting tumor growth [19].

As summarized in Fig. 1, subpopulations of CD8 cells can be identified by their respective profiles of IFN- γ , TNF- α , IL-2, and GzB production. We therefore developed a four color ELISPOT assay in which these analytes can be measured simultaneously, and which will be described in the following. For the accurate identification of the distinct CD8 subpopulations, the following general principles of multicolor ELISPOT analysis need to be considered. First, it is critical that each of the analytes is detected without the cross-bleeding of color. This is accomplished by using combinations of fluorochromes that would cross-bleed if excited broadly, but by selecting narrow excitation wavelengths and emission filters for each color, these can be completely separated, and each color plane analyzed, without the need for compensation, essentially as a single color ELISPOT assay. An example for this type of unambiguous detection of colors is provided in Fig. 1 of a chapter on seven color analysis in this book [20]. In the next step, the images containing the “spots” for the different color planes are superimposed to identify cells that co-express analytes. This cannot be just done by simple matching of centers of masses for the individual spots because T cells migrate from APC to APC during the ELISPOT assay’s capture phase that lasts 24 h, or more. Thus, tolerances need to be accommodated as we describe in detail in a chapter dedicated to the experimental validation of multicolor analysis [21]. If these tolerance

values are set accurately, serial dilution of the T cells will show a linear relationship between analyte co-expressers and cell numbers plated; however, the same percentage of cells will be co-expressers irrespective of the total cell number plated per well. In contrast, the chance for random overlays of single positive spots occurring will drop exponentially as fewer cells are plated. In that chapter [21], these principles have been verified subjecting CD8 cells and B cells to multicolor ELISPOT analysis. With CD8 cells, analyte co-expressers can be expected, while with B cells any multi-positive spot must result from chance overlays of single positive cells as a single B cell can produce only one class or subclass of antibody at a time. Once single vs. multiple analyte producing cells are identified, which is done automatically by the ImmunoSpot® software, the data is converted automatically in flow cytometry standard (FCS format), from where on standard flow cytometry software can be used to analyze the co-expression levels and patterns. The step by step process of multicolor ELISPOT analysis is described in a dedicated chapter in this book [22]. Here we focus on performing the wet lab part of the four color fluorescent ELISPOT assay to measure the antigen-triggered secretion of IFN- γ , TNF- α , IL-2, and GrzB. The results of a representative four color assay in which these analytes have been detected are shown in Fig. 2.

2 Materials

2.1 Instrumentation and Software

1. ImmunoSpot® Series 6 Ultimate Analyzer (CTL, Shaker Heights, OH USA, cat# S6UTM12).
2. ImmunoSpot® 7.0 Software (CTL, cat. IPS01).
3. Cell counting 1.4 software (CTL, cat. LDA01).

2.2 ImmunoSpot Kits

1. IFN γ -IL2-TNF α -GrzB 4 Color Capture kit (CTL, cat. hT4001F).
2. IFN γ Detection kit (CTL, cat. hT02).
3. TNF α Detection kit (CTL, cat. hT13).
4. IL2 Detection kit (CTL, cat. hT56).
5. GrzB Detection kit (CTL, cat. hT59).
6. Live/dead Cell Counting kit (CTL, cat. CTL-LD-100).

2.3 Media

1. Anti-Aggregate (CTL, cat. CTL-AA-005).
2. CTL-TEST serum-free medium (CTL, cat. CCTL-005).
3. L-Glutamine (Gibco, cat. 25030-081).
4. RPMI-1600 (Lonza, cat. BW12167Q).

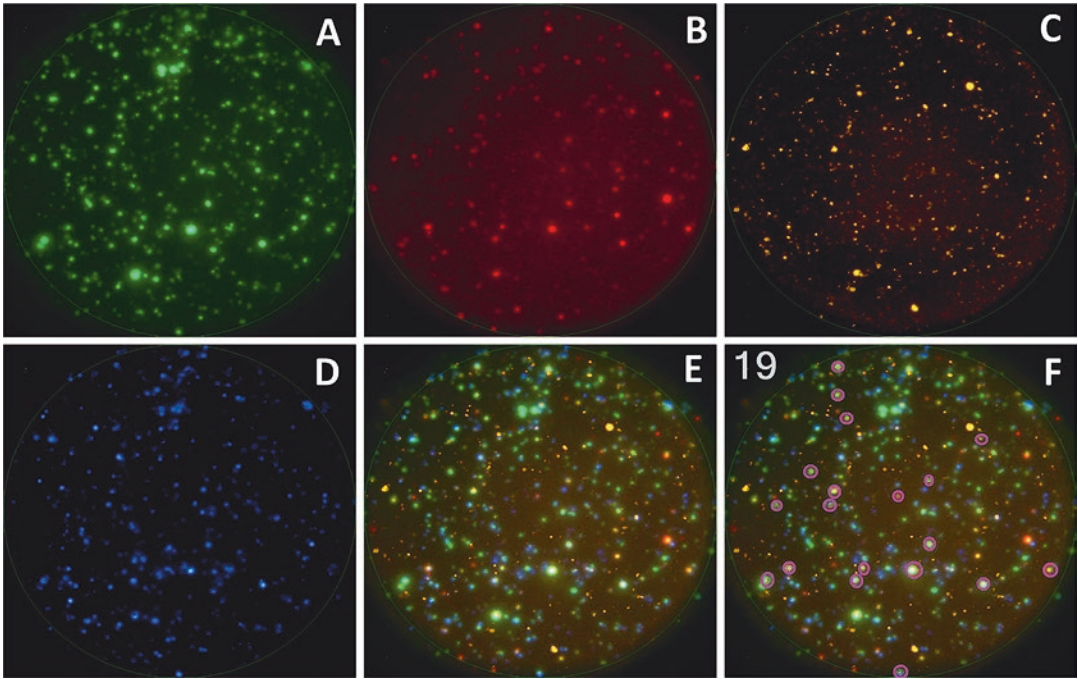


Fig. 2 Four color ImmunoSpot® results measuring IFN- γ , TNF- α , IL-2, and GzB. The four color assay was performed as described here testing CEF-peptide-reactive CD8 cells. For each analyte, a separate channel was established permitting detection of that analyte without cross-bleeding of color. Panel (a) shows the IFN- γ channel in green (b) the IL-2 channel in red, (c) the TNF- α channel in yellow, and (d) the GzB channel in blue. The overlay of the four color planes is shown in (e). Polyfunctional CD8 cells positive for all four analytes have been counted in (f). The fact that these cells are indeed quadruple positive, as opposed to resulting from random overlays was established by serial dilution of the cells

2.4 Miscellaneous

1. CEF plus pool (CTL, cat. CTL-CEF-002).
2. 50 mL conical centrifuge tubes (Falcon, cat. 352070).
3. Sterile gauze (Covidien, cat. 8044).
4. Reservoir (Denville, cat. P8826-ST).
5. Ethyl alcohol, 190 proof (Sigma, cat. 493511).
6. Parafilm “M” (Hach, cat. PM-996).
7. Vacuum Manifold (Millipore, cat. MSVMHTS00).
8. 10 mL syringe Luer Lok tip (BD 309604).
9. 0.1 μ m syringe filter (Millipore, cat. SLVV033RS).
10. Distilled water (Millipore, cat. 6442-85).
11. Tween-20, to make 0.05% Tween-PBS wash buffer (Sigma, cat. P1379).
12. 1 \times Sterile DPBS, Ca-Mg free (Hyclone, cat. SH30028-02).
13. 25 cm² sterile culture flasks (Corning, cat. 430639).

14. Wide-orifice tips for plating cells (Rainin, 17007101).
15. Frozen PBMC (CTL, cat. CTL-CPI) <http://www.immunospot.com/ImmunoSpot-ePBMC>

3 Methods

3.1 Thawing and Stimulation of PBMC

PBMC were used with known IFN- γ reactivity from the ePBMC database. Cells were stored in liquid nitrogen vapor phase until use.

1. Thaw cryopreserved PBMC by warming them in a 37 °C metal bead bath for 15 min. Wipe vial with 70% EtOH to sterile and invert twice to mix cells.
2. Transfer vial contents into a 50 mL conical tube. Rinse the cryovial with 1 mL of warm (37 °C) anti-aggregate solution and add this slowly in a dropwise fashion to the conical tube. Continue to add warm anti-aggregate solution while swirling the cells until the volume reaches 10 mL per vial (*see Note 1*).
3. Centrifuge cells at 300 $\times g$ for 10 min at room temperature with maximum break.
4. Decant the supernatant and resuspend the pellet by flicking the bottom of the conical tube. Add 10 mL of anti-aggregate solution.
5. Take 20 μ L of cell suspension and mix it with 20 μ L of Live/Dead Cell Counting Dye. Load 15 μ L of the mixed solution into a hemocytometer and count the live and dead cells on the ImmunoSpot Series 6 Ultimate Analyzer using the ImmunoSpot® LDA 1.4 Software.
6. Centrifuge cells at 300 $\times g$ for 10 min at room temperature with maximum break.
7. Decant supernatant and resuspend cells at five million live cells/mL in warm (37 °C) CTL-TEST Medium.
8. Add 0.5 μ g/mL of CEF peptide pool plus to the cell suspension.
9. Transfer the contents of the conical tube to a sterile 25 cm² cell culture flask and incubate at 37 °C with 5% CO₂ for 72 h (*see Note 2*).

3.2 Coating of the ELISPOT Plates

Forty eight hours after stimulation of PBMC with CEF peptide pool, a 96 well PVDF membrane plate with low auto-fluorescence needs to be coated for the four color fluorescent ELISPOT assay.

1. Prepare Capture Solution contained in the kit for the four color fluorescent ELISPOT assay according to manufacture protocol.
2. Remove the underdrain carefully and add 15 μ L of freshly prepared 70% ethanol to each well using a multi-channel pipette (*see Note 3*).

3. Immediately add 150 μL of sterile DPBS to each well (*see Note 4*).
4. Decant and wash the plate two more times with 150 μL /well of sterile DPBS.
5. Decant the final wash, tap on sterile gauze to remove excess liquid.
6. Replace the underdrain and quickly add 80 μL Capture Solution to each well. Wrap the plate in parafilm and store at 4 $^{\circ}\text{C}$ overnight.

3.3 Counting PBMC

1. Seventy two hours after the beginning of the stimulation, take cells from cell culture flask and add them to a 50 mL conical tube.
2. Add 40 mL of CTL-TEST Medium, centrifuge at $300 \times g$ for 10 min at room temperature with maximum break.
3. Decant supernatant and resuspend by tapping the bottom of the tube.
4. Add CTL-TEST Medium for cell counting, approximately $5 \times 10^6/\text{mL}$.
5. Take 20 μL of the cell suspension and mix it with 20 μL of Live/Dead Cell Counting Dye. Load 15 μL of the mixed solution into a hemacytometer and establish the number of live and dead cells using an ImmunoSpot[®] Series 6 Ultimate Analyzer using ImmunoSpot[®] LDA 1.4 Software.
6. Centrifuge cells at $300 \times g$ for 10 min at room temperature with maximum break.
7. Decant the supernatant and resuspend the cells according to the live count.

3.4 Plating of Antigen and PBMC

1. Remove the coated ELISPOT plate from 4 $^{\circ}\text{C}$ and unwrap the parafilm.
2. Decant the Capture Solution and wash the plate with 150 μL /well sterile DPBS.
3. Decant the DPBS and tap the plate on sterile gauze to remove any excess liquid.
4. Quickly add 100 μL /well of CEF peptide pool plus solution at 1 $\mu\text{g}/\text{mL}$ in CTL-Test Medium using a multi-channel pipette and sterile reservoirs (*see Note 5*).
5. Add 100 μL of the counted stimulated cells in serial dilution using wide-orifice tips (*see Note 6*).
6. Tap the plate gently before storing it in an incubator at 37 $^{\circ}\text{C}$, 5% CO_2 overnight (*see Note 7*).

3.5 Multicolor ELISPOT Detection

1. Prepare the Detection Solution according to the manufacturer's protocol specified in the kit, and pass through a 0.1 μm syringe filter.
2. After an overnight incubation, remove the plate from the incubator and decant antigen and cells (*see Note 8*).
3. Decant and wash the plate twice with PBS and twice with 0.05% Tween-PBS, 150 μL /well.
4. Tap the plate on paper towels to remove excess liquid.
5. Add 80 μL of Detection Antibody Solution and incubate the plate for 2 h at room temperature, protected from light.
6. Prepare the Tertiary Solution according to the protocol and pass through a 0.1 μm syringe filter.
7. Decant the plate, wash 3 \times with 150 μL /well 0.05% Tween-PBS and tap to remove excess liquid.
8. Add 80 μL /mL of Tertiary Solution and incubate for 1 h at room temperature, protected from light.
9. Decant the plate, wash 3 \times with 150 μL of distilled water. Remove the underdrain and wash the plate upside down on a vacuum manifold with distilled water to remove excess fluorochrome.
10. Let plate dry completely, protected from light under a laminar flow hood, before scanning.

3.6 Data Capture and Analysis

1. The ELISPOT plate is to be scanned using the CTL Series 6 Ultimate Analyzer using fixed exposure. Each filter and corresponding excitation LED was used in order to avoid cross-bleeding of colors (*see Note 9*). The theoretical and practical aspects of accurate multicolor ELISPOT analysis are respectively described in chapters [21, 22].
2. ImmunoSpot 7.0 was used to enumerate spots. Each filter had individual parameters set for accurate counting; gates were set using positive wells and negative wells and the algorithm for pairing was set.
3. Excel spreadsheets are made from every counted plate by ImmunoSpot® Software 7.0 and those were used to make graphs with their corresponding *R* values to calculate linearity, average *R* value 0.98726 (data not shown).
4. Once the linearity was confirmed, the percent analyte of the co-expressing cells was calculated by dividing the number of SFU counted by the number of cells plated; the percent quadruple co-expressing cells was 0.005% (data not shown).

4 Notes

1. Adding warm media slowly helps minimize the osmotic gradient between the interior and exterior of the cells as the concentration of DMSO drops with dilution in the exterior. One can add the content of up to five cryovials of the same PBMC donor to one 50 mL conical tube.
2. CEF-peptide-reactive CD8 cells typically are resting memory cells in healthy donors, and thus have no pre-formed GzB stored. To detect GzB production by CEF-specific CD8 cells, addition to IFN- γ and TNF- α , a 3 day pre-stimulation culture is required during which central memory cells transform into terminal effector cells. If antigen exposure triggers GzB production in CD8 cells *ex vivo* that indicates that *in vivo* the antigen-specific CD8 cells had been in an activated state, being terminal effector cells.
3. For multicolor ELISPOT assays, it is important to activate the PVDF membrane with ethanol pre-wetting in order to increase its binding capacity. It happens instantaneously after the addition of ethanol, and can be seen as the graying of the membrane. Washing afterwards should be done as quickly as possible to avoid leakage of the membrane.
4. Once the plate has been activated with ethanol, for all subsequent steps, the plate membrane should never dry until completion of the assay.
5. We highly recommend the use of CTL Serum-Free Media for freezing, washing, and testing of PBMC. Even brief exposure to mitogenic serum can cause high background while other sera can have suppressive effects.
6. CTL recommends using wide-orifice tips to reduce sheer forces while pipetting cells.
7. Do not stack plates in the incubator and do not allow the incubator or the plates to be disturbed during incubation. Doing so will cause the cells within the wells to roll which will interfere with co-expression analysis.
8. After the incubation of cells on the ELISPOT plate, a large percentage of the cells are still viable and, except for antigen stimulation, largely unaffected by the test procedure. Instead of discarding the cells, they can be used for subsequent analysis, including ELISPOT and flow cytometric analysis.
9. For unambiguous detection of individual analytes using fluorochrome combinations, a setup is needed in which the dyes do not cross-bleed among the detection channels. Each chan-

nel is defined by a narrow excitation wavelength combined with a narrow emission filter and can be selected from an array of channels preinstalled on CTL fluorescent readers. Please consult CTL customer support for the selection of channels suitable for the fluorochrome combinations of choice.

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