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# Virtual Global Transplant Laboratory Standard Operating Protocol for Donor Alloantigen-specific Interferon-gamma ELISPOT Assay

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**Abstract:** The quantification of frequency of IFN- $\gamma$ -producing T cells responding to donor alloantigen using the IFN- $\gamma$  enzyme linked immunosorbent spot (ELISPOT) holds potential for pretransplant and posttransplant immunological risk stratification. The effectiveness of this assay, and the ability to compare results generated by different studies, is dependent on the utilization of a standardized operating procedure (SOP). Key factors in assay standardization include the identification of primary and secondary antibody pairs, and the reading of the ELISPOT plate with a standardized automated algorithm. Here, we describe in detail, an SOP that should provide low coefficient of variation results. For multicenter trials, it is recommended that groups perform the ELISPOT assays locally but use a centralized ELISPOT reading facility, as this has been shown to be beneficial in reducing coefficient of variation between laboratories even when the SOP is strictly adhered to.

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Pretransplant rejection risk stratification is currently determined by assessment of donor-specific antibodies, using the complement dependent cytotoxicity and solid phase (luminex and flow cross match) assays. These assays focus on humoral sensitization to HLA<sup>1</sup> or non-HLA antigens,<sup>2</sup> and have been standardized across laboratories globally through

the availability of commercially validated assay protocols, reagents and equipment.

Although the critical role for T lymphocytes (T cells) in allograft rejection has been long known, T cell sensitization is not routinely measured. A number of shortcomings have limited broader utilization of assays measuring the reactivity of T cells to foreign HLA molecules (allospecific reaction). In particular, these assays have been difficult to standardize between laboratories, are technically, monetarily and timewise challenging to perform, require a source of donor antigens to act as stimulators and have a high intralaboratory and interlaboratory coefficient of variation (CV) in excess of 40% despite centralized counting methods.<sup>3</sup> At present, there is only limited evidence that even the most reproducible assays of T cell reactivity are able to accurately predict acute rejection.

The most basic T cell reactivity measurement assay is a mixed lymphocyte reaction (MLR). In this assay, the pair of responding cells (often a mixture of peripheral blood mononuclear cells [PBMCs] from a transplant recipient) are cultured with cell-cycle arrested, stimulating cells (irradiated B cells or other antigen presenting cells from a transplant donor), and proliferation of the recipient's cells are measured. However, the MLR assay has not been shown to have a predictive value posttransplant.<sup>4,5</sup>

A more sophisticated descendent of the MLR assay is the enzyme-linked immunosorbent spot (ELISPOT) assay that quantifies the frequency of responder cells detected by their secretion a cytokine or other molecule (see Figure 1).<sup>6,7</sup> In transplant immunology, this assay was used initially by Heeger et al<sup>8</sup> in a murine transplantation model to examine the direct and indirect T cell alloresponse in rejection. It was found that when a mouse donor and recipient pair were

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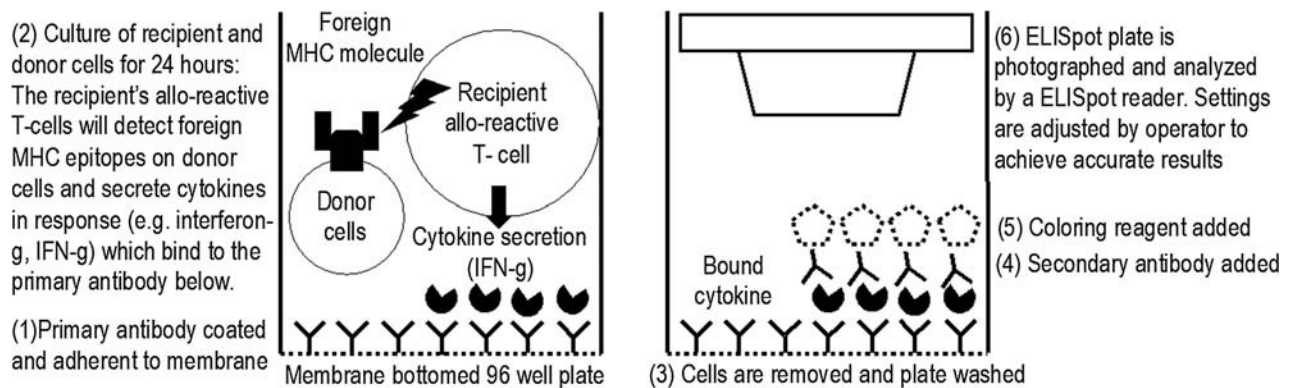
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**FIGURE 1.** ELISPOT protocol: (1) Primary antibody against the target cytokine, IFN- $\gamma$ , adheres to the membrane on the bottom of the ELISPOT plate. (2) Recipient peripheral blood mononuclear cells and donor antigen presenting cells (we recommend expanded and purified B cells) are cultured in the wells for 24 hours during which time period the alloreactive T cells in the culture detect and respond to the foreign MHC antigens. This results in secretion of IFN- $\gamma$  in the region surrounding the T cell, which then binds to the primary antibody. The cells are removed and plate washed (3), with the bound cytokine and antibody pair remaining attached to the bottom of the well. (4) A second antibody (which is usually biotinylated) against a different epitope of the target cytokine is added, adhering to the bound cytokine. (5) Then, a coloring agent is added to visualize the spatially discrete spots representing individual responding T cells. (6) The plate is photographed and counted by an ELISPOT reader with operator adjusting count settings for accurate results.

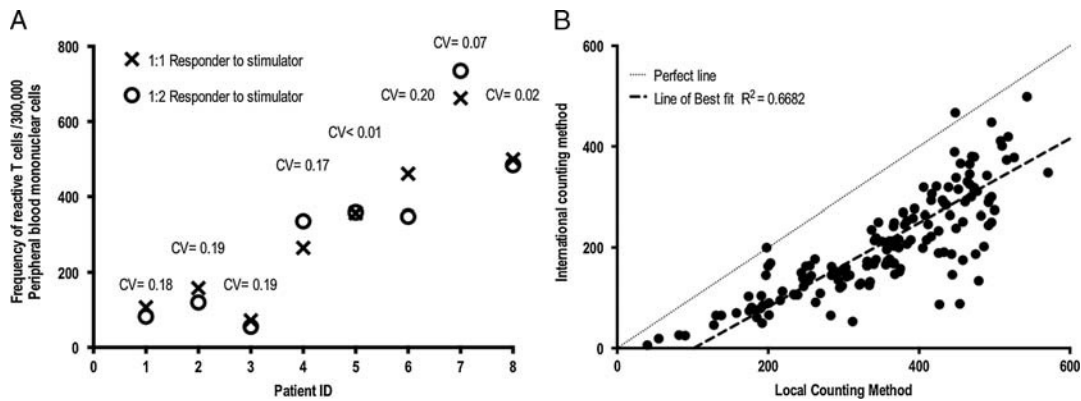
completely MHC mismatched, the direct alloresponse predominated with the recipient's alloreactive T cells to intact MHC molecules on donor cells. In contrast, when stimulator cells were derived from donor  $\times$  recipient F1 mice then alloreactive T cells recognized intact donor MHC as well as donor-derived MHC antigens presented on recipient-matched MHC. Alloreactive T cells were identified and quantified in this assay by their secretion of effector cytokines notably IFN- $\gamma$ . For clinical studies, when using intact fully-HLA mismatched donor cells as stimulators, this assay predominantly measures the frequency of T cells recognizing intact donor MHC molecules and not T cells capable of indirect recognition. However, a recent report by Shiu et al<sup>9</sup> revealed that the use of donor-cell lysates can detect indirect T cell alloreactivity in patients with chronic antibody-mediated rejection. The interest in quantifying T cells that directly and indirectly recognize and respond to donor-MHC stems from the hypothesis that these populations of T cells play distinct roles in early acute rejection versus late, chronic rejection.<sup>10,11</sup>

The development of the ELISPOT assay into a reliable and replicable scientific and clinical tool for measurement of T cell alloresponse has been challenging for multiple reasons. To accurately measure the frequency of alloreactive T cells in recipient's blood specifically against a donor organ, donor cells and tissues have to be preserved at the time of transplantation, and stored using protocols that allow these cells to be recovered many years after transplantation to be used as donor-specific stimulators. Donor-derived PBMC and splenocytes can provide a source of specific donor antigens<sup>11,12</sup> and are likely the most accurate representation of an individual's alloresponse to the graft. If donor-derived PBMC and splenocytes are of limited supply, a renewable source of expanded donor-derived B cells may be used. Indeed, Zand et al<sup>12</sup> reported that *in vitro* expanded B cells stimulated a similar cytokine profile in CD4<sup>+</sup> T cells as unfractionated PBMC. Finally, for patients who have been transplanted many years prior, donor-specific stimulators may be difficult to acquire. This has led to utilization of a panel of third party stimulators (B cells enriched and

expanded from PBMC) with diverse HLA expression for clinical trials.<sup>13,14</sup> The cost and logistics for such an endeavor are significant barriers to the translation of these assays into standard clinical practice, and there is a strong need for more user-friendly sources of donor HLA antigens if this assay is to be used routinely in the clinic.

Although the ELISPOT assay can measure a variety of elaborated cytokines by memory and/or naive T cells, only the IFN- $\gamma$  ELISPOT has undergone extensive international interlaboratory validation in the transplantation setting.<sup>3,11,15</sup> Using this assay, it has been shown that high frequencies of alloreactive T cells prior to transplantation correlates with increased risk of acute rejection.<sup>8,13,16</sup> Bestard and colleagues<sup>11</sup> reported that patients with late acute rejection also had higher frequencies of IFN- $\gamma$ -producing T cells. Finally, this assay has also been reported to predict graft rejection.<sup>10</sup> Conversely low frequency of such cells posttransplantation has been associated with the development of complications of immunosuppression namely infection and malignancy.<sup>14</sup> The CTOT-01 multicenter study recently reported on the utility of pre-transplant IFN- $\gamma$  ELISPOT test as a risk-stratifying biomarker for kidney transplant injury.<sup>15</sup> In the kidney transplant recipients who did not receive T cell-depleting, rabbit antithymocyte globulin induction therapy, higher IFN- $\gamma$  ELISPOT positivity was correlated with lower estimated glomerular filtration rate at 6 and 12 months posttransplantation. These data suggest that the IFN- $\gamma$  ELISPOT assay, when used longitudinally and within the context of an induction therapy without T cell depletion, can identify patients for which maintenance immunosuppression may have to be modified to improve outcomes.

Here, we provide a standard operating protocol (SOP) for IFN- $\gamma$  ELISPOT assay that was consolidated from different sources, namely the CTOT, ITN, and ONE studies, and the human immunology cores of the University of Chicago, University of Pennsylvania and Centre for Clinical and Experimental Transplantation, Adelaide. Comprehensive discussion of the variables affecting the ELISPOT production process has been published elsewhere (see Ashoor et al,<sup>3</sup> Bestard



**FIGURE 2.** Panel A, The performance of the SOP with halved dilution of responder cells on ELISPOT results. Halved concentration of responders provides similar results even when the assay performed on different days, CV less than 0.2. Adjusting the ratio of responders to stimulators may be useful in patient populations known to have high frequency of alloreactive T cells. Panel B: An example of 2 laboratories counting results of the same ELISPOT triplicate wells. The spots were enumerated in 2 laboratories on different ELISPOT readers and with different count protocols. Although there is a good correlation ( $R^2 = 0.6682$ ), the actual values are all significantly higher by local counting method. For multinational trials, it is recommended that a central laboratory experienced in ELISPOT counting perform enumeration to enable accurate comparison between sites.

et al,<sup>11</sup> and Janetzki et al<sup>17</sup>). In this SOP, we have attempted to identify the multiplicity of variables that can affect the production of and the quantification of the IFN- $\gamma$  spots. We note that in populations or patients in whom a high spot count is expected and it is desirable to differentiate the highest responders, it is possible to achieve reproducibly accurate spots counts with dilution methods. By reducing the number of responder cells while keeping the same number of stimulators, hence the antigenic density within the assay remains the same, we found that there was a low degree of variation ( $CV \leq 0.2$ ) between the IFN- $\gamma$  ELISPOT results (see Figure 2A).

Previous validation studies have demonstrated a clear benefit to centralized counting method, and this is the recommended method for counting of produced ELISPOT plates for multinational, multicenter trials.<sup>3</sup> For interim data analysis, local counts strongly correlate with a centralized spot counts despite differences in both operator procedures and ELISPOT reading software (see Figure 2B). However, to enable interlaboratory comparison for multi-national trials, we have relied on a single site counting method. In this SOP, we provide a recommended starting point for counting of the spots based on our laboratories' experiences. We believe with increasing laboratory experience of spot counting, a robust SOP and periodic quality control comparison of local and central counts a good correlation between centers may be achievable.

## Standard Operating Procedure

### Materials

- ELISPOT plate (Multiscreen filter plates (Immobilon-P membrane; Millipore S2EM004M99 or MSIPS4W10)
- Sterile PBS (Mediatech 21-031-CV or Invitrogen 70011)
- RPMI 1640 (Mediatech 10-040-CV)
- Penicillin-Streptomycin (Mediatech 30-002-CI)
- Glutamine (Mediatech 25-015-CI)
- Heat inactivated Human AB Serum (Gem Cell 100-512)
- Heat-inactivated fetal bovine serum (Gemini Bio 100-106)
- cCTL media (Cellular Technology Ltd) (CTLT-005)
- Media filters 0.22  $\mu\text{m}$  filters "Stericup" (EMD-Millipore Stericup SCGPU05RE)
- BSA fraction V (Sigma A8022; 100G)
- DNase- Benzonase 25KU (Sigma E1014)
  - Used from provided concentration: 5  $\mu\text{L}$  benzonase/ 25 mL medium
- PHA (Lectin from *Phaseolus vulgaris*) (Sigma L-1668)
  - Reserved concentration: 2 mg/mL in RPMI
  - 1:100 dilution of PHA in cCTL media
- Coating antibody: anti-IFN- $\gamma$  (Thermo Scientific Pierce Cat M700A)
  - Reserved concentration: 1 mg/mL; diluted concentration for coating of plates: 4  $\mu\text{g}/\text{mL}$
- Secondary detection antibody: anti-IFN- $\gamma$ -biotin (Thermo Scientific Pierce Cat M701B)
  - Reserved concentration: 0.5 mg/mL; diluted concentration: 1  $\mu\text{g}/\text{mL}$
  - Dilute in PBS/5% BSA
- Tertiary Reagent
  - Streptavidin-HRP: BD Biosciences (51-9000209); Supplied at 100 $\times$
- BD Aminoethylcarbazole Substrate Reagent Set (BD Biosciences 555214)
  - Add 200  $\mu\text{L}$  AEC Chromogen to 10 mL of AEC Substrate. Mix gently.
- Wash solution (PBS-Tw): PBS + 0.05% Tween 20 (Sigma P1379)
- Blocking solution: PBS + 1% (w/v) BSA
- Secondary antibody dilution buffer: sterile PBS-Tw + 1% BSA
- Isoprep (Fisher NC9168356)
- B Cell Enrichment kit
  - EasySep B cell enrichment kit (Stemcell Tech, 19054)
  - Rosette Sep B cell enrichment kit (Stemcell Tech, 15064 or 15024)
- Cells
  - Responder cells: PBMC density gradient separated and thawed from frozen or fresh may be used. Freezing and thawing can be performed as per previously published protocols.<sup>18</sup> Frozen cells do not require resting, however frozen cells tend to produce a lower level of reactivity compared to fresh samples.
  - Donor cells: multiple sources of stimulator cells have been described in the literature. Depending on availability, practicality, costs and local preference, the choice is generally between donor PBMC, donor splenocytes, third-party PBMC (MHC mismatched), or specific

B cell lines expressing a variety of MHC antigens. When donor cells is limiting, B cells may be negatively enriched from PBMC or splenocytes using a variety of commercially available kits, expanded as described in previous protocols and frozen for future use.<sup>3,12</sup> Confirmatory expression of both MHC class I and II antigens with flow-cytometry is recommended in all cases. Enriched B cell populations do not require irradiation.

## Assay Protocol

### 1. Initial preparation

- (a) Design a plate layout for your experiment based on the number of stimulators and antigens to be tested. You must determine the amount of reagents, stimulator cells and responder cells that are needed. The assay is best performed in triplicate, however lymphopenic patients such as those with renal transplants may only yield sufficient lymphocytes for duplicates. We recommend performing the assay in substantial batches to limit the variability between experiments and the cost of the reagents and wares.
- (b) Prepare a sterile work area in a laminar flow hood for the subsequent steps 2 to 5.

### 2. Coating the plate

- (a) In a laminar flow hood, add coating (primary) anti-IFN- $\gamma$  antibody to sterile PBS and mix gently.
- (b) Plate 100  $\mu$ l per well according to the plate layout, using a single channel micropipette, to minimize wasting of antibodies. If there is more than 1 plate, use a multichannel pipette with a sterile tray as a reservoir.
- (c) The primary antibody can be incubated at room temperature for 4 hours or overnight at 4°C. For overnight incubation, the plate should be wrapped in plastic and placed inside a sealed container with a moist paper towel on the bottom to prevent evaporation.

### 3. Addition of a blocking solution to prevent secondary antibody binding to membrane.

- (a) In a sterile laminar flow hood, empty the coating antibody from the wells by firmly flicking the inverted plate over a catch basin.
- (b) Block the plate with a sterile PBS + 1% BSA, 150  $\mu$ l/well, at room temperature for 60 minutes.
- (c) After inversion, forcefully tap the plate to remove majority of solution from the wells and wash the plate 3 times with sterile PBS, 200  $\mu$ l/well per wash.
- (d) Leave the last wash in the well and keep the plate at room temperature in a sterile hood until antigens and responder cells are ready to be added to the wells.

### 4. Cell Preparation: fresh or frozen PBMC may be used. Refer to global virtual laboratory standard operating procedures for blood collection, PBMC isolation and storage, PBMC counting on hemocytometer and PBMC thawing.<sup>18</sup>

- (a) When thawing frozen cells, add 5  $\mu$ l of benzonase to 25 mL of 1% Human AB serum in RPMI (containing glutamine and penicillin-streptomycin). Cells can be used immediately, however it has been reported that overnight culture of thawed cells improves the quality of the ELISPOT response.<sup>19</sup>
- (b) Resuspend responder cells in cCTL media to  $3 \times 10^6$  cells/mL (300,000 cells/well); other cell concentrations could be explored in pilot assays.
- (c) Resuspend stimulator cells in cCTL media to a concentration of  $1 \times 10^6$ /mL (100,000 cells/well)

### 5. Plating Cells

- (a) Invert the plate after the final wash. Mix cells thoroughly by gently pipetting, and add cells to the appropriate wells. We recommend performing the assay in triplicate: background (responder cells alone), alloresponse (stimulator

and responding cells) and positive control (PHA + responder cells). We have observed no response from well prepared stimulators, in the IFN- $\gamma$  assay and thus a control well is not required. However, for other cytokines it may be necessary to investigate background spots for stimulators.

- (i) Use large orifice tips (Fisher Scientific, 02707134) to add 100  $\mu$ l of responders to the plate (300,000 cells/well), and then 100  $\mu$ l of stimulators (100,000 cells/well).
  - (ii) Be sure to add 100  $\mu$ l of media or PHA to control wells. Media-only controls are necessary to assess background cytokine production, whereas the PHA wells confirm the viability of the responder cells.
  - (b) Check to make sure that all the wells have 200  $\mu$ l media. Holding the plate in one hand, gently tap the sides of on all 4 sides of the plates to redistribute the cells. This is important because otherwise the spots tend to cluster around the edges of the wells and making it harder to count.
  - (c) Incubate the plate at 37°C in the CO<sub>2</sub> humidified incubator for 24 hours. It is critical that the plate is not disturbed during this incubation as this may lead to the blurring of the spots. Finally, do not stack the plates to ensure that the temperature of the plates is uniform.
- ### 6. Washing plate, preparing and adding detection anti-IFN- $\gamma$
- (a) Mix gently the required amount of the secondary anti-IFN- $\gamma$  PBS-Tw + 1% BSA.
  - (b) Invert and flick the plate over a sink to remove cultured cells and wash the wells 3 $\times$  with PBS and then 4 $\times$  with PBS-Tw. A squirt bottle may be used.
  - (c) After each wash, firmly flick the inverted plate onto a pile of clean towels to remove as much liquid as possible. Allow the last wash to sit on the plate for 5 minutes to ensure that the PBS-Tw lyses any residual cells left in the wells.
  - (d) Discard the last wash, flicking the plate on fresh paper towels as above to dry and add the secondary anti-IFN- $\gamma$  at 100  $\mu$ l/well.
  - (e) Incubate overnight at 4°C or for 2 hours at room temperature. For the 4°C incubation, the plate should be wrapped in plastic and placed inside a sealed container with a moist paper towel.
- ### 7. Tertiary reagent
- (a) Prepare the Streptavidin-HRP solution as per manufacturer's instruction by diluting 1:100 in 10% fetal bovine serum in PBS.
  - (b) Discard secondary antibody from the plate and wash the plate 4 times with PBS-Tw. A squirt bottle may be used.
  - (c) Add the prepared Streptavidin-HRP reagent at 100  $\mu$ l/well.
  - (d) Replace lid and allow the reagent to incubate at room temperature for 1 to 4 hours at 4°C.
- ### 8. Development of plate
- (a) Prepare the AEC substrate solution just before use, by adding 200  $\mu$ l of AEC chromagen to 10 mL of AEC substrate in a 50-mL test tube. Wrap the tube in aluminium foil to prevent deterioration of the reagents.
  - (b) Wash the plate 4 times with 200  $\mu$ l/well PBS; soak for 1 to 2 minutes for the first 3 washes and incubate the plate for 5 minutes in PBS during the final wash.
  - (c) Discard the final wash and add 100  $\mu$ l of AEC substrate solution to each well and monitor carefully for the development of spots—approximately 10 to 15 minutes for IFN- $\gamma$ .
  - (d) Stop reaction by washing both sides of the membrane 3 $\times$  with distilled water.
- ### 9. Cover with foil and allow to dry overnight.
- (a) Air-dry plate at room temperature under a loose foil tent to protect from light. The spots are light sensitive and will fade over time if exposed to light.

- (b) The plates can be stored essentially indefinitely at room temperature if maintained wrapped in aluminium foil.
- (c) When the plates are fully dried, they can be analyzed using a computer-assisted image analyzer.
10. Anticipated results and data acquisition with an ELISpot plate reader
- (a) The spots appear brownish when horseradish peroxidase (with AEC) is used. They are granular in appearance and tend to be darker at the center than at the periphery.
- (b) The number of spots per well can be read with an automated ELISPOT plate reader. There are 2 main providers, namely, Cellular Technology Limited (CTL) and AID Diagnostika GmbH. Depending on the reader and the algorithm (settings) used to automatically read plates, there can be major differences in results.
- (c) Settings used for AID readers in the published literature include:
- (i) Size minimum 50, intensity minimum 10, gradient 0
- (ii) Size minimum 63, intensity minimum 15, gradient 1 to 90
- (d) Depending on the responding subject (heavily immunosuppressed or not) and the types of stimulators used, the expected spot count for allospecific IFN- $\gamma$  response varies from 0 to 200 spots.
- (e) The PHA or positive controls are expected to generate >400 spots.
- (f) Alloresponses which generate greater than 400 spots will need to be repeated with diluted responder cells as the number of spots can no longer be accurately quantified. (Figure 2A)
- (g) Sample Setting for CTL ELISPOT reader

#### Test wells

Sample sensitivity	180	(0-255)
Diffuse processing	Normal	(Small, normal, large, largest)
Spot separation	1	(0-25)
% Counted area	90 (normalize counts)	
Background balance	338	(0-400)
Minimum spot size	0.001 mm <sup>2</sup>	(0-10)
Maximum spot size	10 mm <sup>2</sup>	(0-10)
Positive control and viral wells		
Sample sensitivity	166	(0-255)
Diffuse processing	Normal	(Small, normal, large, largest)
Spot separation	3	(0-25)
% Counted area	90 (normalize counts)	
Background balance	400	(0-400)
Minimum spot size	0.001 mm <sup>2</sup>	(0-10)
Maximum spot size	10 mm <sup>2</sup>	(0-10)

#### Considerations and Alternatives

- (1) This assay can be used to detect other cytokines. Antibodies for detecting cytokines of potential relevance to transplantation are listed below.

#### Primary (Coating) Antibody

Antibody	Source	Catalog	Clone	Purchased Concentration	Final Concentration	Culture, h
IFN- $\gamma$	Pierce	M700A	2G1	1 mg/mL	4 $\mu$ g/mL	18-24
Granzyme B	Holzel	Gr8-11	Gr8-11	2 mg/mL	3 $\mu$ g/mL	18-24
IL-2	R&D	MAB202	5334	0.5 mg/mL	6 $\mu$ g/mL	18-24
IL-4	eBioscience	14-7049-85	8D4-8	0.5 mg/mL	TBD	36-48
IL-5	eBioscience	14-7052-85	TRFK5	0.5 mg/mL	TBD	36-48
IL-10	eBioscience	14-7108-85	JES3-9D7	0.5 mg/mL	TBD	18-24

#### Secondary (Detection) Antibody

Antibody (Biotin)	Source	Catalog	Clone	Purchased Concentration	Final Concentration	Assay Development (m)
IFN- $\gamma$	Pierce	M701B	XMG1.2	0.5 mg/mL	1 $\mu$ g/mL	10-15
Granzyme-B	Holzel	GrB-	GrB-10	1.2 mg/mL	2 $\mu$ g/mL	20
IL-2	Pierce	M-600B	BG2	0.5 mg/mL	0.5 $\mu$ g/mL	80
IL-4	eBioscience	13-7048-85	MP4-25D2	0.5 mg/mL	TBD	60
IL-5	eBioscience	13-7059-85	JES1-5A10	0.5 mg/mL	TBD	60
IL-10	eBioscience	13-7109-85	JES3-12G8	0.5 mg/mL	TBD	80

- (2) Protocol for making the AEC Solution Note that AEC is very toxic, so wear gloves, mask and lab coat while weighing reagent and use a chemical fume hood. When bottle of working solution is empty, rinse with acetone to clean. Empty the acetone into hazardous waste container stored in fume hood.
- (a) AEC Buffer (0.1 M Sodium acetate, pH 5.0)
- (i) 148 mL of 0.2 M acetic acid (11.55 mL glacial acetic acid per liter of Q-water. add acid to water slowly)
- (ii) 352 mL of 0.2 M sodium acetate (16.4 g sodium acetate per liter of Q-water. If reagent precipitates over time, make new.)
- (iii) Bring up to 1 liter with Q-water.
- (iv) Adjust pH to 5.0.
- (b) AEC Reagent
- (i) 10 mg AEC/ mL dimethyl formamide
- (ii) Wrap bottle and stopper in aluminum foil. Store at room temperature.
- (c) Working Solution
- (i) An entire plate requires 800  $\mu$ L of AEC to be added to 24 mL of AEC buffer, and then the solution is filtered using a 0.45- $\mu$ m filter.
- (ii) Add 12  $\mu$ L H<sub>2</sub>O<sub>2</sub> to each well; this must be added last.
- (3) If stimulators are NOT B cell enriched, they must be irradiated for 12 minutes at 3000 rads/min. Irradiated cells must be centrifuged again at 7 minutes, 1200 rpm, brake on and then the cell concentration adjusted to 3 x 10<sup>6</sup>/mL using cRPMI + 1% human AB serum.
- (4) Prewetting might help ensure coating efficiency in some instances. Prewet plates with 70% ethanol, wash well with PBS 3 times afterward, and then follow the recommended protocol.

- (5) Other IFN- $\gamma$  antibody pairs:
  - (i) Mabtech Inc: anti-human IFN- $\gamma$  mab 1-D1k and biotinylated antihuman IFN- $\gamma$  mab 7-B6-1
- (6) Other sources of PBMC separation reagent:
  - (i) Lymphoprep (Fisher NC9182056)

### Critical aspects of the SOP

1. It is critical that when pipetting solutions into the 96 well plate, the pipette tip does not touch the delicate membrane well bottom and a slow pipetting technique should be used for adding cells and solutions to the wells.
  2. A major factor in successful frequency analysis for cytokines using ELISPOT is the availability of high-affinity purified anticytokine antibodies that are directed against different epitopes on the cytokine molecule.
  3. Because IFN- $\gamma$  (and other cytokines) is not constitutively expressed, it is important to determine the optimal type, strength, and duration of stimulus that induces each cytokine. A control set of unstimulated cultures is necessary to determine the rate of spontaneous IFN- $\gamma$  production.
  4. Careful timing of both cell incubation and signal development is crucial for maximizing the signal to noise ratio.
  5. T cells can recognize HLA alloantigen directly or indirectly. Direct allorecognition is measured by coculture with intact donor cells. Indirect allorecognition is much more challenging to measure, and some groups have successfully used donor-cell lysates.<sup>9</sup>
  6. Titration (1:2) of responding cells will allow a more accurate determination, especially when the frequency of IFN- $\gamma$ -producing cells is very high.
  7. Thorough blocking, washing and draining of the plates are necessary to reduce background.
  8. Although not part of the cellular assay per se—the settings on the ELISPOT reader and the type of ELISPOT reader can have profound effect on the results of these assays—consider a centralized plate-reading strategy for multicenter trials.
  9. Time considerations
    - (a) Although washing the plates takes only a few minutes, plates should be incubated with antibody  $\geq 4$  hr at room temperature or 18 to 24 hours at 4°C for optimum sensitivity.
    - (b) Once the assay is completed, the spots can be counted at the convenience of the investigator. Although spots develop in a few minutes, they become more distinct after 24 hours and tend not to fade over time if the plates are wrapped in foil and kept at room temperature.
  10. Patient populations
    - (a) Longitudinal changes in ELISPOT results is affected by the use of depleting or nondepleting antibodies as part of the induction process.<sup>13,15,16</sup>
    - (b) Most published data with this assay pertain to first time transplant recipients and relatively non-sensitized populations. Expect to see much higher levels of spot formation in those on low-dose immunosuppression greater than 2 years posttransplant and in those who have had 2 or more transplants.
    - (c) Although the SOP states the readout as spots per 300,000 peripheral blood mononuclear cells there can be large changes in the number of PBMC in patients undergoing induction and conventional immunosuppression.<sup>15,20</sup> Thus, some SOP normalize to CD4 counts although these have yet to be used to determine posttransplant outcomes.
2. Two types of background problems are seen in an ELISPOT assay. The first is a diffuse darkening of the membrane. This is most commonly observed when large numbers of cytokine-secreting cells are present in the well resulting in the accumulation of secreted cytokine in the culture supernatant and its subsequent binding to the membrane (see point 10). A diffuse background can be reduced by decreasing the number of cells per well or the duration of the cell incubation. The second type of background problem is the appearance of very small, dark spots that do not reflect secreted cytokine. A trained observer can usually discriminate between real ELISPOTs and these artifacts; real spots have dark centers and light rims (the latter formed by the diffusion of cytokine outward from the secreting cell). Artefactual spots are smaller and of uniform intensity. They may appear when the anticytokine antibodies aggregate, but more frequently are caused by inadequate removal of cells from the plate. Some cells have endogenous phosphatase activity, or nonspecifically bind biotin-labeled antibodies, leading to the formation of these artifacts. This problem can be prevented by allowing the last PBS-Tw wash to sit on the plate for 5 minutes to allow for complete lysis of the cells.
    3. To avoid streaks and fuzzy spots, do not move or disturb the plates in the incubator during the cell culture period.
    4. To ensure even temperature, do not stack ELISPOT plates during cell incubation.
    5. Optimization for reading the ELISPOT plates. The way the plate is read can introduce major variability. Settings on the ELISPOT reader can make corrections to the automated results after well images have been analyzed, if the image contains artefacts. Artefacts caused by unequal cell distribution, damaged membranes or overdevelopment can lead to inconclusive results. Fortunately, most software will allow manual correction and below is a list of how to manage common problems.
      - (a) Overdeveloped background: trace the areas to be excised and the machine will compute a new spot count value normalizing count over the area that has been excised.
      - (b) Missed spots: Increase sensitivity
      - (c) Counting too many spots: Decrease sensitivity
      - (d) Not counting faint spots: Increase background balance
      - (e) If large/diffuse spots: Change 'Diffuse processing' to "large"
      - (f) Adjacent spots counted as single: Lower spot separation level
      - (g) Spot clustering: Set spot size to maximum
      - (h) Well is tainted with minute threads/human hairs or other contaminants: Place a checkmark on the 'Fiber Removal' box in count parameters
      - (i) Artefacts around the perimeter of the well: Set the counted area to 90% or lower to avoid counting excessive artefacts around the perimeter. Choose 'normalization' for an accurate count estimate of the excluded area.
      - (j) It is recommended that plates or digital images of the plate be compared at a national or internationally laboratory to determine whether the laboratory ELISPOT reader settings need to be altered to reduce CV between the testing lab and the centralized review laboratory.

### SUMMARY

This SOP for interferon gamma ELISPOT is standardized across international laboratories. When followed, it should produce results with acceptable CV. Importantly, a major source of CV variability arises in the process of automated reading of the ELISPOT plate, and we have provided a method to standardize the ELISPOT reading process. For multicenter trials and global standardization, we recommend

### Troubleshooting

1. If an assay suddenly stops working, the substrate solutions or the enzyme-labeled developing antibody may have lost activity and need to be replaced.

reading of plates or their digital images at a centralized laboratory to ensure reproducible and comparable results.

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