ELISPOT Standard Operating Procedure (SOP)

1.0	Title				
	Performing Alloreactive ELISPOT assays to Assess Interferon-gamma Production				
2.0	Purpose				
	To detect and enumerate individual cells which secrete interferon-gamma in vitro.				
3.0	Equipment and Reagents				
	3.1	Equipment			
		3.1.1	Biological Safety Cabinet		
		3.1.2	Centrifuge, Sorvall Legend RT		
		3.1.3	Conical tubes, 50 ml, 15 ml		
		3.1.4	Large orifice pipette tips. (200 uL)		
		3.1.5	Gloves		
		3.1.6	Hemacytometer		
		3.1.7	Micropipettors		
		3.1.8	Pipete-Aids		
		3.1.9	Serological Pipettes, 5 ml, 10 ml.		
		3.1.10	Light Microscope		
		3.1.11	Various tube racks		
		3.1.12	Refrigerator		
		3.1.13	-80 C Freezer		
		3.1.14	Liquid Nitrogen		
		3.1.15	Liquid Nitrogen Storage System		
		3.1.16	Incubator, 37^0C/ 5% CO_2		
		3.1.17	Immunospot Analyzer		
		3.1.18	Immunospot Software		
		3.1.19	Microfuge tubes, 1.5 ml		
		3.1.20	Multi-channel pipette, 20-200		
		3.1.21	Multiscreen filter plates, Immobilon-P membrane	Millipore	S2EM004M99 or MSIPS4W10
		3.1.22	Solution Basins, Sterile & non-sterile		
	3.2	Reagents			Cat No.
		3.2.1	Sterile and non sterile 1X PBS	Mediatech, Inc	21-031-CV
		3.2.2	Human AB serum, heat - inactivated	Gem Cell	100-512
		3.2.3	Fetal Bovine Serum (FBS)	Gemini Bio	100-106
		3.2.4	Penicillin-Streptomycin	Mediatech, Inc	30-002-CI
		3.2.5	RPMI 1640	Lonza	12-702F
		3.2.6	BD^{TM} AEC Substrate reagent	BD Biosciences	551951

		3.2.7	Anti-human IFNγ	Thermo Scientific - Pierce	M700A
		3.2.8	Anti-human IFNγ-biotin	Thermo Scientific - Pierce	M701B
		3.2.9	PHA	Sigma	L-1668
		3.2.10	Tween-20	Sigma	P-2287
		3.2.11	Streptavidin-HRP	BD Biosciences	51-9000209
		3.2.12	BSA, fraction V	Sigma	A8022
		3.2.13	CTL Test Medium	CTL	CTLT-005
4.0	Procedures (All work needs to be performed under biological safety cabinet observing biosafety regulations using sterile techniques). Please refer to Appendix 1 for information on buffers and reagents used in assay protocol.				
Day -1	4.1	Preparation of reagents – see Appendix A.			
	4.2	Coating Plates with primary antibodies			
		4.2.1	Under a biological safety cabinet coat plates with anti-human IFNγ primary antibody diluted in sterile PBS (see Appendix A). Plate 100 µL per well.		
		4.2.2	Tap the plate gently to spread uniformly all over the well.		
		4.2.3	Store plate at 4º C overnight in a humidified chamber (snap-lid plastic container with moist paper towels on bottom)		
	4.3	Suggested Controls for Alloreactive responses			
		4.3.1	Responder cells should be tested against medium (negative control), B cells (experimental wells), PHA (positive control). For wells containing both responder and B cells, 300,000 responder and 100,000 B cells are added.		
Day 0	4.2	Preparation of templates and labels			
		4.2.1	Design an experiment map		
		4.2.2	Retrieve experiment samples from liquid nitrogen tank		
		4.2.3	Prepare complete medium and buffers.		
	4.3	Block plate			
		4.3.1	In a sterile biological safety cabinet, empty the coating antibody from the wells by firmly shaking the inverted plate over a catch basin.		
		4.3.2	Block the plate with a sterile solution of PBS+1% BSA (see Appendix A), 150 µl/well. Allow the blocking solution to sit on the plate for 60 minutes.		
		4.3.3	Proceed with defrosting of cells.		
	4.4	Defrosting of Cells			
		4.4.1	Retrieve sample vials from liquid nitrogen, placing in styrofoam rack to keep cold while transporting to biological safety cabinet. Take vials out and place in the hood.		
		4.4.2	Pipette 25 ml of RPMI/1% huAB into each of the the previously labeled tubes. Add 5ul of DNAse (25U/ul) to the medium and mix well by inversion.		

		4.4.3	As the cells are starting to defrost, take a 5 ml pipette and take up 5 ml of media from the tube you want that vial's cells to go into. Pipette the media in and out of the cryovial until the cells are defrosted or the frozen mass has been loosened. Wash the vial a few times and put the cap on the 50 mL tube. Continue repeating this process until all samples are done.
		4.4.4	Mix the cells well by inverting the tube then spin in a room temperature centrifuge for 7 minutes, 1200RPM (330g), with the brake on. After the spin, discard the supernatant, but retain the pellet. Resuspend the cells by tapping the pellet to break it up and add 10 ml of RPMI/1% huAB (no DNAse) in each tube.
		4.4.5	Spin in a room temperature centrifuge for 7 minutes, 1200RPM (330g), with the brake on. After the spin, discard the supernatant, but retain the pellet. Resuspend the cells by tapping the pellet to break it up and add 10 ml of RPMI/1% huAB (no DNAse) in each tube.
	4.5	Cell Counting & Resuspension at Proper Concentration	
		4.5.1	For each cell line, determine cell concentration via trypan blue staining and counting on hemacytometer.
		4.5.2	Using obtained count of viable cells/ml and total volume of sample, calculate total number of cells in sample. Calculate final volume necessary for Responder cell samples to contain 300,000 cells/ml and for Stimulator cells to contain 100,000 cells/ml.
		4.5.3	Centrifuge cells (330g, 24 ^{o}C, brake on). As soon as the centrifuge stops, aspirate the supernatant and resuspended (by tapping the tube). Add appropriate mls of CTL-T using volumes obtained in 4.5.2. Now the sample tube will be marked with the letter **x** to indicate that this sample has been counted and media is adjusted to the final volume for plating
	4.6	Plating the cells	
		4.6.1	Discard Blocking solution.
		4.6.2	Wash plate 3 times with sterile PBS, 200 μL per well, rotating the plate 180^{0} between washes.
		4.6.3	Leave the last PBS wash in the well and keep the plate at room temperature (in the sterile hood) until the cells are ready to be added to the wells.
		4.6.4	Add 100 μL of negative control (medium) to appropriate wells.
		4.6.5	Using large orifice tips add 100 μL of cell suspension in CTL-T media to each well according to the experiment map. Make sure the plate is labeled according to the map on the inside/ outside.
		4.6.6	With large orifice tips add 100 μL of Antigen (stimulator cells) to appropriate wells.
		4.6.7	Add 100 μL of positive control (PHA in CTL-T media) to appropriate wells.
		4.6.8	Check the plate for completeness, even volumes and proper labeling. Each experimental well should have 200 μL (100 μL cells + 100 μL

			experimental condition).
		4.6.9	Gently tap the sides of the plates to re-distribute the cells. This prevents clustering of spots along the edge of the wells.
		4.6.10	Incubate 24 hours at 37º C in a 5% CO_2 humidified incubator.
Day 1	4.7	Addition of Detection Antibody (From now onward may continue the experiment under non-sterile conditions)	
		4.7.1	Remove the plate from the incubator and indicate on the cover the wells that have cells.
		4.7.2	Dump liquid from plate. Wash wells x 3 with PBS, 200 µL per well, rotating 180º between each wash and blotting the plate on paper towel between washes. Allow to soak for 1-2 minutes at each wash step.
		4.7.3	Wash plate x3 with Wash Buffer 1 (PBS/Tween), 200 µL/ well. Discard Wash Buffer.
		4.7.4	In PBS-BSA-Tween (see Appendix A) the appropriate dilution of the secondary biotin-labeled Ab (see Appendix A) is prepared. After flicking the plates empty and tapping vigorously on absorbent paper towels, 100µl/well is added.
		4.7.5	Store plate at 4º C in a humidified chamber (with a moist paper towel inside) for 18 hours.
Day 2	4.8	Addition of Tertiary Reagent	
		4.8.1	Late in the afternoon, plates are washed 4 times with PBS-Tween using squirt bottle and tapping vigorously between washes on absorbent paper towels
		4.8.2	Dilute tertiary reagent (Streptavidin-HRP) in dilution buffer (PBS-Tw-1%BSA) and add 100 µL per well.
		4.7.3	Replace lid; incubate for 4 hour at 4ºC.
	4.9	Development	
		4.9.1	Discard tertiary reagent solution. Wash wells 4x with 200 µL/ well wash buffer I (PBS/Tween). Allow to soak 1-2 minutes at each wash step.
		4.9.2	Wash wells 2x with 200 µL/well wash buffer II (1xPBS) letting last wash remain on plate for 5 minutes
		4.9.3	Add 100 µL of AEC substrate solution to each well and monitor for the development of spots for **10 minutes**.
		4.9.4	Stop reaction by washing 3x with DI water.
		4.9.5	Air dry the plate at room temperature overnight or until dry under loose foil to protect from light. Removal of the plastic tray under plate will facilitate drying. Store plate in a sealed plastic bag in the dark until it is analyzed.
		4.9.6	Enumerate spots using an ELISPOT plate reader.

Appendix A: Buffers and Reagents

1) Coating Buffer: 1x PBS (sterile)

2) Blocking Solution: PBS+1% BSA

3) Wash Buffer I: 1x PBS containing 0.05% Tween-20 (0.5 ml Tween-20 per 1 L PBS)

4) Wash Buffer II: 1x PBS

5) Dilution Buffer: sterile PBS-Tw+1%BSA

6) Development Substrate Solution: BD™ AEC Substrate Reagent Set

Add 200uL BD™ AEC Chromogen to 10 mL of AEC Substrate. Mix gently.

7) Coating Primary Antibody @ 4μg/mL (Anti-Human IFNγ):

Thermo Scientific Pierce Cat#M700A
Add 48ul anti-IFNγ antibody (1mg/mL) to 12ml sterile PBS for each plate to be coated. Mix gently.

8) Detection Secondary Antibody @ 1μg/mL (Anti-Human IFNγ-Biotin)

Thermo Scientific Pierce Cat#M701B
Add 24ul biotin-conjugated anti-IFNγ antibody (0.5mg/mL) to 12ml sterile PBS-Tw+1%BSA for each plate. Mix gently.

9) Streptavidin-HRP

BD Biosciences Material No. 51-9000209 Batch No. 29332
Concentration 100X
For use: dilute 1:300 in dilution buffer prior to use.