CATEGORY:	Laboratory Work Instructions	**SOP Number**:	ELISPOT 001
TITLE:	**Performing Alloreactive ELISPOT assays**		

AUTHORS: Dr. Peter Heeger	**Signed:**	
	Title of Authors:	Director
	Date:	

APPROVED BY: Dr. Kasia Bourcier	**Signed:**	
	Title:	Associate Director, Assay Development
	Date:	

APPROVED BY: Marco Morelli	**Signed:**	
	Title:	Assay Specialist
	Date:	

EFFECTIVE DATE: November 2004

REVISION HISTORY:

Number	Section	Pages	Initials/Dates
001	5.7.3, 5.8.2	Changed spleen donor cell isolation procedure	JMM/9.10.04
002	5.8.1	Changed volume of T cell depletion reagent	JMM/10.22.04
003	Appendix C	Added plate layout	MM / 03.16.05
004	Core revision (see outdated version with tracked version)	See tracked changes in 03.16.05 version	MC/08.23.05

1.0	**Title**
	Performing Alloreactive ELISPOT Assays

2.0	**Purpose**
	Analysis of cytokines secreted by PBMCs in response to alloantigen/antigen stimulation using ELISPOT technology.

3.0	**Definitions and Abbreviations**	
	AEC	3-amino-9-ethy carbazole
	Ab	Antibody
	Ag	Antigen
	APC	Antigen presenting cell
	BSA	Bovine serum albumin
	DMF	N, N-Dimethyl-formamide
	RPMI	RPMI 1640 Culture Medium
	PBS	Phosphate Buffer Saline, Dulbecco's
	H_2O_2	Hydrogen Peroxide, 30%
	HBSS	Hanks Balanced Salt Solution
	PBMCs	Peripheral Blood mononuclear cells
	PHA	Phytohemagglutinin
	HuAB	Human AB Serum
	Sample puller	Person who takes the frozen vials from dry ice and places them into racks for the defroster.
	Defroster	Person who defrosts the frozen samples by convective airflow.
	Jumper	Person who collects defrosted tubes and hands them to the thawing station. The jumper also operates the centrifuge.
	Centrifuge operator	Operates the centrifuge.
	Cell plater	Person responsible for plating the cells according to the plate layout.

4.0	**Equipment and Reagents**				
	4.1	Equipment			
		4.1.1	-70 ºC Freezer	Forma Scientific Inc.	VWR 55703-430
		4.1.2	Refrigerator	Danby	Model DCR122W
		4.1.3	Incubator	Forma Scientific	Model 320

		4.1.4	Biological Safety Cabinet	Forma Scientific	Model 1286
		4.1.5	Centrifuge, Allegra 6R	Beckman Rotor Gh3.8A	ALR6
		4.1.6	Water bath 6.7x14.6 Liter	Lab-Line	Fisher# 22-2997-36
		4.1.7	Convective warm-air flow	Remington	
		4.1.8	Plate washer	BioTek Instruments Inc.	Model Elx405R
		4.1.9	ImmunoSpot Analyzer	CTL	Series 1, 2001120 & 2001240 (International) Series 2, 2002120 & 2002240 (International)
		4.1.10	Dry-ice container, Cooler		
		4.1.11	Ice buckets		
		4.1.12	Conical tubes, 50ml	Falcon	Fisher 14-432-22
		4.1.13	Solution Basin, Sterile	Fisher	NC9490002
		4.1.14	Gloves	Fisher	19-048-575A(Medium) &19-048-575B(Large)
		4.1.15	0.2 µm filter unit, 1000ml	Nalgene	Fisher# 09-740-3A
		4.1.16	0.2µm filter unit, 250ml	Nalgene	Fisher# 09-740-2A
		4.1.17	Liquid nitrogen		
		4.1.18	Pipette Aids	Drummond 4-000-100	Fisher#13-681-19
		4.1.19	Pipettes	Fisher	13-678-11B(1 mL) 13-678-11C(2 mL) 13-678-11D(5 mL) 13-678-11E(10ml) 13-678-11(25 mL)
		4.1.20	Multi-channel pipetter. 50-300µL	Labsystem	Ser.# N50391
		4.1.21	Variety Micropipettor 0.5-10µL	Eppendorf	Ser.# CTL7005A
		4.1.22	Variety Micropipettor 0.5-10µL	Eppendorf	Ser.# CTL7006A
		4.1.23	Variety Micropipettor 0.5-10µL	Eppendorf	Ser.# 469067

		4.1.24	Variety Micropipettor 5-20μL	Eppendorf	Ser.#CTL7004A
		4.1.25	Variety Micropipettor 2-20μL	Eppendorf	Ser.# 488141
		4.1.26	Variety Micropipettor 2-20μL	VWR	Ser.# 41430235
		4.1.27	Variety Micropipettor 2-20μL	Gilson	Ser.# K13986B
		4.1.28	Variety Micropipettor 10-100μL	Eppendorf	Ser.# 499708
		4.1.29	Variety Micropipettor 10-100μL	Eppendorf	Ser.# 447870
		4.1.30	Variety Micropipettor 50-200μL	Eppendorf	Ser.3 CTL7002A
		4.1.31	Variety Micropipettor 50-200μL	Eppendorf	Ser.# CTL7003A
		4.1.32	Variety Micropipettor 20-200μL	Gilson	Ser.# 045186N
		4.1.33	Variety Micropipettor 20-200μL	VWR	Ser.# 968450668
		4.1.34	Variety Micropipettor 100-1000μl	Eppendorf	CTL7001A
		4.1.35	Variety Micropipettor 100-1000μL	VWR	Ser.# 841820499
		4.1.36	Variety Micropipettor 100-1000μL	Gilson	K18347K
		4.1.37	Single Chan. 1000μL	Beta-Pette	Ser.# 146564670
		4.1.38	Single Chan. 1000μL	Beta-Pette	Ser.# 146564600
		4.1.39.	Single Chan. 200μL	Beta-Pette	Ser.# 146550695
		4.1.40	Single Chan. 200μL	Beta-Pette	Ser.# 146550678
		4.1.41	Single Chan. 20μL	Beta-Pette	Ser.# 146530559
		4.1.42	Pipette Tips1-200μL, large orifice	VWR	53503-616
		4.1.43	Pipette Tips1-200μL small orifice	VWR	53509-009
		4.1.44	Pipette Tips 101-1000μL,blue	Fisher	21-197-8A
		4.1.45	Pipette Tips 5-300μL,racked, sterile	Lab.Prod.Sale	L110803
		4.1.46	ImmunoSpot M200 plates	CTL	M200/10 M200/50
		4.1.47	ImmunoSpot Software	CTL	Part#2001500
		4.1.48	15ml Conical Tubes	Falcon	14-959-70C
		4.1.49	Gloves Ltx. Ambi pf.Ultra1 M	Fisher	11-387-56C

		4.1.50	Gloves Ltx. Pt.shldmstr Md.	Fisher	19-050-246C
		4.1.51	Repeater Syringe Dispenser	Oxford	S/N 0Z00J
		4.1.52	Repetitive Syringe	VWR	53498-974 (1.5ml) 53519-458 (3ml) 53498-976 (6ml)
		4.1.53	Various racks		
		4.1.54	Stainless steel screen (#80 mesh)		
		4.1.55	30-60 cc sterile syringe		
	4.2	Reagents			
		4.2.1	Acetic acid glacial	Fisher	A38S-500
		4.2.2	BSA, fraction 5, RIA grade	Sigma-Aldrich	A-7888
		4.2.3	DMF	Sigma-Aldrich	D8654
		4.2.4	Sodium Acetate	Fisher	S210500
		4.2.5	Glutamine	Gibco	25030-081
		4.2.6	RPMI 1640	Bio-Whittaker	Fisher 12-167Q
		4.2.7	1x PBS	Cellgro	VWR 45000-432
		4.2.8	10x PBS 500ml	Cellgro	VWR 45000-428
		4.2.9	Freezing Medium A, Tissue culture tested, heat inactivated, filtered, HuAB serum	CTL	2002001
		4.2.10	Ficoll, Isoprep	Robbins Scientific	1070-01-0
		4.2.11	T-cell depletion Cocktail	StemCell Technologies	15621
		4.2.13	HBSS		

5.0	**Procedures** **All work needs to be performed under biological safety cabinet observing biosafety regulations using sterile techniques.**	
Day 0	5.1	Preparation of reagents - see Appendix A.
	5.2	Coating Plates with primary antibodies

		5.2.1	Under a biological safety cabinet, coat plates with anti-cytokine antibodies (these are the primary antibodies) diluted in sterile PBS. Plate 100µL /well. (Concentration of Ab was previously determined by validation experiments.)
		5.2.2	Tap the plate gently to spread 100 µL uniformly all over the well.
		5.2.3	Leave the plates at 4ºC overnight in a humid chamber (this is the minimum amount of time required; plates may be stored no longer than 48 hours
	5.3	Suggested Controls for Alloreactive responses	
		5.3.1	Responder cells should be tested against medium (negative control), donor cells depleted of T cells (experimental wells), PHA (positive control), a typical responsive antigen (i.e. Mumps), and donor derived peptides if available. For wells containing both responder and donor cells, 300,000 cells of each are added.
		5.3.2	T cell-depleted APCs should be tested against medium alone and PHA to confirm their inability to produce cytokines.
Day 1	5.4	Preparation of templates and labels	
		5.4.1	Three copies are made of the ImmunoSpot Manager plate design (see Appendix C).
		5.4.2	Printed labels are placed on 50ml tubes identifying each sample with internal ID (if sample size is 3ml or less, 15ml conical tubes are used). The top of the tube is also marked with the internal ID. Internal IDs are created for each experiment to make subject/sample identification easier. For data reporting purposes we use the original subject IDs.
		5.4.3	Wash media (see Appendix A) and media are warmed up in incubator with loosened caps. Wash media and media can also be warmed to room temperature by using the water bath (bottles are closed tightly). After the wash media has reached room temperature it is distributed to work station and counting station. The media container should be wrapped in aluminum foil to protect it from light.
	5.5	Preparation of antigen	
		5.5.1	Antigens for the assay are prepared in the biological safety cabinet by diluting them with cold complete media (see Appendix A; RPMI-based media) in labeled tubes according to the specifications of the project leader.
	5.6	Plating of Antigen	

		5.6.1	100 μL/well of antigen is plated (according to ImmunoSpot Manager plate design) from filled reservoirs with multi-channel pipetters or filled from dilution tube with a repeat pipetter. Leftover antigen is stored at 4°C in the refrigerator until the end of the experiment (to be discarded the following day). Once all antigens are plated, plates are placed in 4°C refrigerator in a humid chamber, while cells for the test are prepared. About 15 minutes before cells are ready to be plated, Ag-coated plates are moved from the humid chamber to the 37°C incubator to warm up.
	5.7	Preparing a single cell suspension from donor spleen sections	
		5.7.1	Because irradiation or mitomycin C treatment of donor cells is NOT sufficient to prevent cytokine production, the best way to perform a "one way" responder against donor assay is to T cell deplete the donor cells. *Note: additional depletion of donor NK cells may be desirable and is currently being tested.
		5.7.2	Cut off a ~1 inch cube size of a spleen sample and place it onto the Sieve # 80 (Sieve should be placed on top of the receiving container)
		5.7.3	Process spleen by crushing it with the back of the 60ml syringe so it easily goes through the Sieve # 80
		5.7.4	Wash the sieve with no more than 40 ml of 5% ABO solution in RPMI media. For the recipe refer to the Appendix A.
		5.7.5	Take drained matter & put into a sterile 150-250 ml container/receiver. Let sit for 5 minutes.
		5.7.6	Pour entire contents of the container back onto the sieve and press white matter through again. Rinse with a small amount of RPMI + 5% ABO to ensure that cells are through the screen and into the basin
		5.7.7	The material that has come through the sieve will be aliquoted into 10ml portions and each placed in a 50 ml Falcon tube
	5.8	T cell depletion from spleen cells	
		5.8.1	Into each tube add 50 μl of T cell depletion cocktail for every 1 ml of the sample Example: 500 μl of the cocktail for each 10 ml sample
		5.8.2	Let the mixture incubate at room temperature for 20 minutes, mixing well, to make sure antibody contact with target molecules.
	5.9	PBMC Isolation using Ficoll Isoprep	

		5.9.1	After the incubation is complete, underlay 10ml Ficoll Isoprep into each tube. Since you aliquoted 10 ml into each tube in Step 5.7.7 you will now have a 1:1 proportion of reagent volume to the sample volume
		5.9.2	Centrifugate for 20 minutes at 2,300 RPM with the brake off
		5.9.3	Using a sterile serological pipette aspirate the interface layer of cells along with the upper layer of serum and place into a fresh 50 ml Falcon Tube
		5.9.4	Centrifugate this mixture for 7 minutes at 1,200 RPM with the brake on
		5.9.5	Discard the supernatant and resuspend the cells in 10ml of 10% FCS solution (prepared in PBS, see Appendix A)
	5.10	Isolation of Donor APCs from whole blood	
		5.10.1	To isolate APCs from whole blood of a living donor, 50uL of the T-cell depletion antibody cocktail is added per 1 mL of heparinized blood and incubated at room temperature for 20 minutes. Then follow steps 5.9.1 to 5.9.5 as above.
	5.11	Cell counting preparation	
		5.11.1	Guava cell counter is turned on and calibrated. UV microscope is turned on. Hemocytometer chambers are prepared: see references for “Guava cell counter operation and maintenance” SOP#20713 and “Hemocytometer counting“ SOP#20714.
	5.12	Centrifuging & Cell Counting	
		5.12.1	Cells are recentrifuged at room temperature at 330g for 10 minutes with a brake. Resuspend pellet in 5-10mL RPMI +5% human serum for counting.
		5.12.2	From each sample tube 20μl of cell solution is taken and added to a prepared Guava tube (380μl Guava Viacount Reagent) for counting. Or, cells are stained with acridine/orange ethidium/bromide and counted on hemocytometers under the UV microscope. See SOP#20713 #20714. Each Guava tube is labeled with the appropriate sample number (same as on the sample tube).
		5.12.3	The jumper transfers the sample tubes to the incubator with loosened caps until the next step. Guava tubes are transferred for counting to the Guava counting station by the person operating the Guava counter.

		5.12.4	After total cell counts have been determined, the cells are centrifuged again (330g, 24 ^{o}C, brake on). As soon as the centrifuge stops, the tubes are decanted and resuspended (by tapping the tube) with media (see Appendix A). The amount of media depends on the cell count and the cell concentration needed for plating. 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 (see Section 5.13 on plating cells to determine number of cells added per well). Marked sample tubes are returned to the incubator with loosened caps until the next step. Extra cells which are not needed for the assay are frozen down following SOP#20700.
	5.13	Plating the cells	
		5.13.1	Typically, 300,000 responder PBMCs and 300,000 donor T cell-depleted APCs are added together per well. Based on the plate design: the jumper supplies the cell platers with one Ag coated plate and the appropriate number of marked sample tubes from the incubator. The jumper writes the sample numbers according to plate design on the lid of the Ag-coated plate.
		5.13.2	According to this plate design the cell plater pipettes 100μl of sample cell suspension into the ELISPOT plate. This is done by pouring the cell suspension into a sterile reservoir and then pipetting with a multichannel pipetter or a repeater pipetter using large orifice tips. The cell plater writes the plate number on the plate itself. The finished plate is handed back to the jumper. If the cell numbers are not enough to plate all wells as planned then the PI will provide an alternative plan (previously agreed on).
		5.13.3	The jumper checks the plate for completeness, even volumes, and proper labeling. The plate is then placed in the CO_2 incubator where it is left undisturbed for 24 or 48 hrs (depending on the cytokine measured).
Day 2	5.14	Addition of Secondary Antibody NOTE: Incubation time varies from 24h-48h, depending on the cytokine being measured.	
		5.14.1	20-24 hr (example: IL-2, IFN-γ) or 44-48 hr (example: IL-4, IL-5 and IL-10) after incubation the plates are removed from the incubator in groups of maximum 5. Plate condition is assessed by two individuals. Unusual color, low volume and empty wells are recorded on form #8. See Appendix C.
		5.14.2	With the BioTek Plate Washer, the plates are washed 3x with PBS and 3x with PBS-Tween (200μl/well) (see Appendix A).

		5.14.3	In PBS-BSA-Tween the appropriate dilution of the secondary biotin-labeled Ab (based on previous titration-see SOP#20716) is prepared. After flicking the plates empty, 100μl/well is added.
		5.14.4	Incubate at 4°C overnight in a humid chamber.
Day 3 or 4	5.15	Addition of Tertiary Reagent	
		5.15.1	Plates are washed 4 times with PBS-Tween (200μl/well) using the BioTek plate washer.
		5.15.2	Add 100 μL/well of diluted tertiary reagent in PBS-BSA (1%). Use streptavidin-HRP (DAKO) as tertiary reagent.
		5.15.3	Incubate at room temperature for 1.5-2 hours.
	5.16	Development	
		5.16.1	Wash plates 3 times with PBS with the BioTek plate washer (200μl/well).
		5.16.2	Prepare a fresh developing solution: see Appendix A.
		5.16.3	Add 200μl/well of freshly made developing solution.
		5.16.4	Incubate at room temperature for 3-45 minutes depending on cytokine measured until obvious spot development is observed. During this time, plate should be closely monitored to for spot development.
		5.16.5	Stop reaction by flushing plate 3 times with distilled water. Flick plate dry.
		5.16.6	Dry plates for 24 hr with the plates in upright position, protecting from light.
6.0	Scanning of ELISPOT plates SOP#20705.		
7.0	ELISPOT counting SOP#20706.		
8.0	QC of ELISPOT results SOP#20707.		
9.0	ELISPOT data storage and recording SOP#20708.		

6.0	**References**	
	6.1	Reagent preparation (Appendix A).
	6.2	List of cytokines specific antibodies (Appendix B).
	6.3	ImmunoSpot Manager plate design (Appendix C).
	6.5	Guava cell counter operation and maintenance SOP#20713.
	6.6	Hemacytometer counting SOP#20714.

	6.7	Scanning of ELISPOT plates SOP#20705.
	6.8	ELISPOT counting SOP#20706.
	6.9	QC of ELISPOT results SOP#20707.
	6.10	ELISPOT data storage and recording SOP#20708.
	6.11	Separation and Freezing Technique for T cell-depleted Donor Cells from spleen (Peter Heeger SOP) SOP # TCD_Spleen

APPENDIX A

Reagent Preparation:

Sterile PBS

- Fill a beaker with 900 mL distilled H_2O.
- Add 100 mL 10x PBS.
- Mix well.
- Filter through (0.2um) and store in 1L bottle at room temperature for up to 1 year.

Non-Sterile PBS

- Fill a beaker with 900 mL distilled H_2O.
- Add 100 mL 10x PBS.
- Mix well.
- Store in 1L bottle at 4ºC for up to 1 month.

PBS-Tween (0.05%)

- Fill a beaker with 895 mL distilled H_2O.
- Add 100 mL 10x PBS to beaker.
- Add 5ml 10%Tween 20 to the solution.
- Mix solution together.
- Pour in 1L bottle and store at 4ºC for up to 1 month.

PBS-BSA (1%)

- 1L of PBS
- 10g of BSA
- Pour PBS into beaker and add BSA on top.
- Do not mix. Let solution sit for approximately 30 minutes/until completely dissolved.
- Once completely dissolved, filter (0.2um) reagent.
- Store at 4°C for up to 6 months.

PBS-Tween (0.05%)-BSA (1%)

- Fill beaker with 895ml of distilled H_2O.
- Add 100ml of 10X PBS.

- Measure out 10g of BSA and add on top of mixture.
- Let solution sit for XXX minutes at least, to allow BSA to dissolve completely.
- Add 5ml of 10%Tween 20.
- Filter (0.2um), pour in 1L bottle and store in 4°C up to 6 months.

AEC Buffer (0.1M Acetate)

- Place 900ml of distilled H_2O in a beaker.
- Stir in 1.702 mL of glacial acetic acid (17.4N).
- Add 5.775g of Na acetate (FW 82.03).
- Mix well.
- pH to 5.0 .
- Bring the solution up to a total of 1 L.
- Store in 4°C up to 6 months.

AEC Solution

Note: Chemical hazard!

- Prepare with a glass tube, in a fume hood while wearing proper glove and lab coat.
- 100mg AEC (3-amino-9-ethyl carbazole).
- 10ml DMF (N, N, Dimethylformamide).
- Store at room temperature, away from light up to a year.

Developing Solution

- Prepare within 10min of use.
- Measure out 24 mL AEC buffer and place in 50ml tube.
- Measure out 800 µL AEC solution, in glass pipette, and add to 24ml of AEC buffer; mix.
- Filter solution through a 0.45µm filter.
- Add 12µl H_2O_2. (30%).
- This amount is enough for one plate.
- Use immediately.

Media

For human PMBC

- 940ml of 1640 RPMI (w/o glutamine).
- Add 50ml heat inactivated HuAB (Freezing Media A, equivalent to 5%).
- Add 10ml glutamine (equivalent to 1%) for a final 2mM solution.
- Filter through 0.2um filter and store in 4°C for up to a month wrapped in aluminum foil to protect it from light.
- Add glutamine again before usage if the medium has been stored for more than a week.

Wash Media

for human PMBC

- 940ml of 1640 RPMI (w/o glutamine).
- Add 50ml heat inactivated HuAB (Freezing Media A, equivalent to 5%).
- Add 10ml glutamine (equivalent to 1%) for a final 2mM solution.
- Filter through 0.2um filter and store in 4°C for up to a month wrapped in aluminum foil to protect it from light.
- Re supplement glutamine before usage if it had been stored for more than a week.

Benzonase is added to wash media: 50 unit/mL of media.

10% Tween 20

- Measure out 55g of Tween 20 to beaker.
- Use a total of 450ml of distilled H_2O to dissolve and rinse out the beaker. Pour the solution from the beaker into a bottle.
- Store the total solution of 500ml at 4C.

Note: Tween 20 has a specific gravity of 1.1 Divide 55g by 1.1=50ml.

1 L of RPMI with 5% ABO

950 ml RPMI Medium 1640

50 ml Human ABO Serum

10 ml of 10% FCS solution in PBS

1.0 ml of FCS

9.0 ml of PBS

APPENDIX B

Cytokine-specific antibodies

Coating Ab:	Catalogue #	Concentration
hu-IFN-γ	Endogen (M-700A, 2G1)	4µg/mL
hu-IL-2	R & D Systems (5334.21)	2µg/mL
hu-IL-4	Ebioscience (13-7049-85, clone 8D4-8)	4µg/mL
hu-IL-5	eBioscience (14-7052-85, TRFK5)	4µg/mL
hu-IL-10	eBioscience (14-7108-85, JES3-9D7)	4µg/mL
Secondary Ab:		
hu-IFN-γ-biotin	Endogen (M-701, B133.5)	2µg/mL
hu-IL-2	Endogen (BG5)	0.5µg/mL
hu-IL-4-biotin	eBioscience (13-7048-85, MP4-25D2)	2µg/mL
hu-IL-5-biotin	eBioscience (13-7059-85, JES1-5A10)	2µg/mL
hu-IL-10-biotin	eBioscience (13-7109-85, JES3-12G8)	2µg/mL
Tertiary Reagent:		
Streptavidin-HRP	Dako	1:2000

All concentrations given above are estimates. Due to batch-to-batch variations, each new batch is tested at CTL to establish the ideal concentration of a new reagent batch. Refer to Antibody testing SOP#20716

APPENDIX C

Add a positive control well (responder vs PHA) in H4

Plate condition form (Newell):