STANDARD OPERATING PROCEDURE

Pre-clinical Consortium on Combination Therapies for Type I Diabetes

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Applicable to:	ITN Project 5P	**Category:**	Mechanistic Analysis

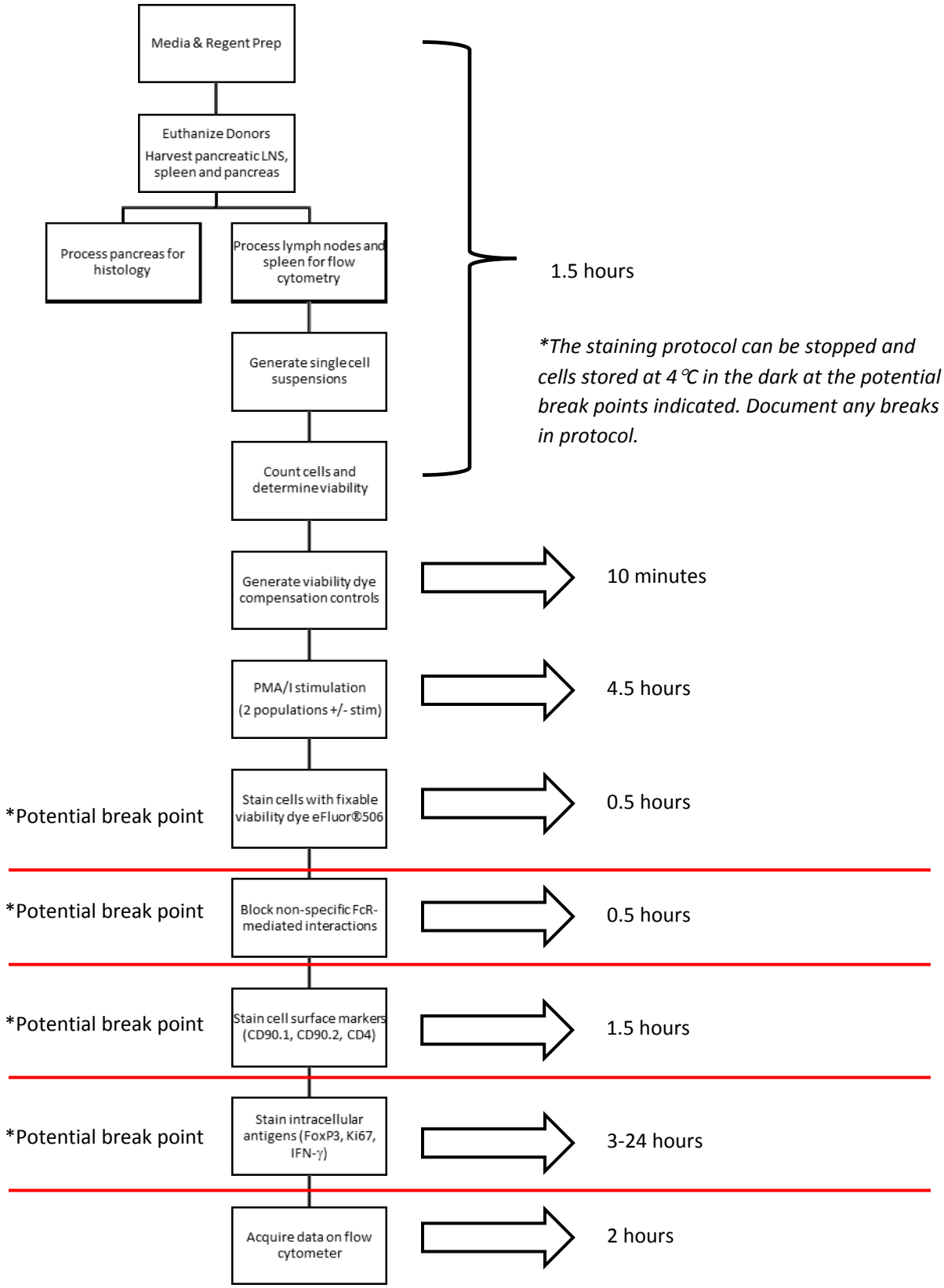
Title: Analysis of Treg and Teffector Cell Populations by Flow Cytometry

INTRODUCTION/PURPOSE

The pilot study described in this protocol is a short-term dosing study to determine, based on mechanistic analyses, the optimal dose of IL-2/anti-IL-2 complex that will be combined with one of two BDC 2.5 Treg doses in a therapeutic study. In this study, NOD mice at disease onset (day 1) will be dosed with 3×10^6 expanded BDC2.5 Thy1.1+ Tregs in the presence of IL-2 or IL-2/anti-IL-2 complexes. Spleen and pancreatic lymph nodes will be evaluated for Treg expansion, activation state and survival of the transferred and endogenous Tregs at 1 week and 1 month. Additional analyses will assess pathogenic immunity, including overall changes in effector T cells (Teff) versus naïve T cells (Tnaive) by flow cytometry. Not all studies will be performed at each site depending on capabilities.

The primary endpoint of this study is to determine whether IL-2 treatment enables the ex vivo expanded Tregs to persist in a recipient. Histologic and flow cytometric analyses will seek to determine whether the Tregs are still there, whether they have expanded, and whether they have retained their Treg (FoxP3+) phenotype.

WORKFLOW AND TIMELINE FOR FLOW CYTOMETRY EXPERIMENTS



METHODS

See Appendix A for ordering information on antibodies, reagents and materials. Refer to Appendix B for Solution Recipes. Refer to Appendix C for Supplemental Methods.

Table 1: Specimens

Sample Type	Source	Gender	Time Point	Group
Pancreatic Lymph Node Cells	Treated NOD	Female	7 or 28 days post-onset	Test Group
Splenocytes	Treated NOD	Female	7 or 28 days post-onset	Test Group
Cryopreserved Lymphocytes	NOD-Thy1.1 (Jax # 004483)	Female	10-26 weeks-old	CD90.1 Positive Control

Expected cell yields:

- Diabetic NOD: 3 x 10^6 pancreatic LN cells & 100 x 10^6 splenocytes
- Expect fewer cells from non-diabetic controls.

Experiment Set-up

29-33 samples total = 8 compensation controls + 1 unstained control + 7 FMO controls (PMI stimulated only) + 13-17 test samples

Compensation Controls (8 samples):

1. UltraComp eBeads eFluor 506+ autofluorescence control
2. UltraComp eBeads + APC-eFluor 780 CD90.1
3. UltraComp eBeads + FITC CD90.2
4. UltraComp eBeads + BV650 CD4
5. UltraComp eBeads + PE FoxP3
6. UltraComp eBeads + eFluor 660 Ki67
7. UltraComp eBeads + BV421 IFN-g
8. Dead cell viability dye compensation control

UltraComp eBeads should be subjected to all of the same staining procedures as the cells because different buffers affect fluorochrome performance.

Fluorescence minus One (FMO) Controls (8 samples; PMI stimulated conditions only):

- Splenocyte unstained control
- Splenocyte FMO controls (see Table 2) = 7 samples

Note: The spleen of one animal should contain enough cells for FMO controls and test samples.

Table 2: Antibody Combinations of the 7-color Panel and Fluorescence minus One (FMO) Controls

Sample Type		IFN-γ BD Horizon™ BV421	Viability Dye eFluor® 506	CD4 BD Horizon™ BV650	CD90.2 FITC	FoxP3 PE	Ki67 eFluor® 660	CD90.1 APC-eFluor® 780
Autofluorescence		-	-	-	-	-	-	-
7-color Panel		+	+	+	+	+	+	+
FMO Controls	IFN-γ BD Horizon™ BV421	-	+	+	+	+	+	+
	Viability Dye eFluor® 506	+	-	+	+	+	+	+
	CD4 BD Horizon™ BV650	+	+	-	+	+	+	+
	CD90.2 FITC	+	+	+	-	+	+	+
	FoxP3 PE	+	+	+	+	-	+	+
	Ki67 eFluor® 660	+	+	+	+	+	-	+
	CD90.1 APC-eFluor® 780	+	+	+	+	+	+	-

Table 3: Test Samples (13 to 17 samples)

Source	Culture Conditions	Stain
Pancreatic LN cells	BFA Media	None
Pancreatic LN cells	BFA Media	7-color panel (3 replicates if possible)
Pancreatic LN cells	PMA/I Media	None
Pancreatic LN cells	PMA/I Media	7-color panel (3 replicates if possible)
Pancreatic LN cells	PMA/I Media	Isotype Controls: replace PE FoxP3, BV421 IFN-γ, eFluor660 Ki67
Splenocytes	BFA Media	None
Splenocytes	BFA Media	7-color panel (3 replicates if possible)
Splenocytes	PMA/I Media	None
Splenocytes	PMA/I Media	7-color panel (3 replicates if possible)
Splenocytes	PMA/I Media	Isotype Controls: replace PE FoxP3, BV421 IFN-γ, eFluor660 Ki67
CD90.1+ Lymphocytes	PMA/I Media	None
CD90.1+ Lymphocytes	PMA/I Media	7-color panel
CD90.1+ Lymphocytes	PMA/I Media	Isotype Controls: replace PE FoxP3, BV421 IFN-γ, eFluor660 Ki67

Solution Preparation

Refer to Appendix B for Solution Recipes. You will need to prepare PMA/I restimulation base media, 10 mg/mL Brefeldin A stock solution, 400 μg/mL PMA stock solution, 500 μg/mL ionomycin stock solution, 2X BFA media, 2X PMA/I restimulation media, and Fc Blocking Buffer.

Allow vial of Fixable Viability Dye (FVD) to equilibrate to room temperature before opening.

FoxP3 buffers should be made up immediately prior to use.

Cell Preparation from Lymphoid Tissue

1) Euthanize the donor(s) according to the method described in the IACUC-approved animal protocol specific to your site.

2) Carefully remove the pancreatic lymph nodes and spleen from your test animal so as to preserve the structure of the pancreas for histology. Refer to **SOP-ITN5P-004: Collection and Freezing of Fresh NOD Pancreas Tissue in OCT for Cryostat Sectioning** for details on how to handle the pancreas sample for histology. Remove the spleen from the NOD-Thy1.1 (CD90.1+) control.

3) Place the harvested tissue into tissue culture dishes. Mechanical disruption of lymphoid tissue is generally sufficient to release cells to a single cell suspension. Dissociate organs to generate single cell suspensions using the method preferred by your site. Document this method. Some options are:
   a) Mash tissue between two frosted microscope slides using 10 mL of 1X PBS.

b) Tease apart into a single cell suspension by pressing tissue with the plunger of a 3 mL syringe through a 70 μm cell strainer.

4) Collect cells in 10 mL of 1X PBS and pass cell suspension through a 70 μm cell strainer to eliminate clumps and debris. Collect cell suspension in a 50 ml conical tube.

5) Centrifuge cell suspension 4 minutes at 400xg at 4°C, discard supernatant.

6) Remove Red Blood Cells (RBCs) from spleen samples. It is not necessary to remove RBCs from pancreatic LN samples because there are so few. Document the method used. One option for RBC removal is:
   a) Red Blood Cell Lysing Buffer Method:
      i) Add 1 mL of Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma R-7757) to the cell pellet (cell pellet =1 spleen or 100-200 million cells).
      ii) Gently mix for 1 minute.
      iii) Dilute the buffer with 15-20 mL of 1X PBS.
      iv) Centrifuge at 500xg for 7 minutes and decant the supernatant.

7) Resuspend the cell pellet and perform a cell count and viability analysis.
   a) If your site has an automated cell counter that you plan to use, please retain a copy of your cell count and viability protocol in your notes for later submission to ITN.
   b) If you plan to determine cell counts and viability manually using a hemocytometer, please use the Sigma Aldrich protocol located in Appendix C.
   c) Document the method used to count and determine the viability of the cells. Document the cell counts and viability.

8) Thaw one 5 x 10^6 vial of cryopreserved NOD-Thy1.1 lymphocytes using the procedure described in **SOP-ITN-5P-002: Thawing Mouse Treg Cell Stocks** with the following change: cells may be washed in 1X PBS instead of warmed wash medium.

9) At this point you should have the following samples:
   a) Test animal pancreatic lymph node cells
   b) Test animal splenocytes
   c) Control NOD-Thy1.1 lymphocytes

10) Centrifuge cell suspensions for 4 minutes at 400xg and 4°C, discard supernatant and resuspend cells in an appropriate volume of **PMA/I restimulation base media** so that the final cell concentration of pancreatic LN cells and control NOD-Thy1.1 lymphocytes is 1 x 10^7 cells/mL. Test splenocytes should be brought to a final cell concentration of 5 x 10^7cells/mL. Maintain cells on ice.

Allot Cells for Viability Dye Compensation Controls

For compensation of the viability dye, generate a sample of live and dead cells as follows.

11) Remove two 5 x 10^6 cell aliquots (100 μl) of splenocytes. This will provide you with extra cells to determine compensation and gates. Reserve one cell sample and set aside on ice.

    Note: Viability Dye Compensation Control only needs to be generated for one sample. DIVA will get confused if you try to run more than one compensation control for eFluor506.

12) Heat one aliquot of cells at 65°C for 3 minutes then immediately place on ice for 3 minutes.

13) After this treatment, combine the heat-killed cells 1:1 with the reserved live cells. You should now have one 10 x 10^6 cell sample of splenocytes to be used as viability dye compensation controls. This sample is referred to as a Dead Cell Control in the plate maps below.

14) Centrifuge the live/dead cell suspension for 4 minutes at 400xg and 4°C, discard supernatant and resuspend cells in 100 µl of **PMA/I restimulation base media** so that the final cell concentration is 1x10^8 cells/mL. Maintain cells on ice.

PMA/Ionomycin Restimulation for Detection of Cytokines

15) Label a 96-well flat-bottom tissue culture plate:

NOTE: If you were unable to obtain sufficient numbers of pancreatic LN cells to plate 5 wells at 10^6 cells/well, eliminate the unstimulated controls cultured in 2X BFA Media (without PMA/I) and only plate the three PMA/I wells.

	1	2	3	4	5	6	7	8	9	10	11	12	
A									Test SCs Unstained Control PMA/I	Test SCs FMO no eFluor506 PMA/I			A
B		Test pLN e506 only BFA	Test SCs e506 only BFA			Test pLN e506 only PMA/I	Test SCs e506 only PMA/I	CD90.1+ SCs e506 only PMA/I	Test SCs Dead Cell Control e506 only PMA/I				B
C			Test SCs Isotype BFA				Test SCs Isotype PMA/I	CD90.1+ SCs Isotype PMA/I	Test SCs FMO no CD90.1 PMA/I	Test SCs FMO no FoxP3 PMA/I			C
D		Test pLN 7-color BFA	Test SCs 7-color BFA			Test pLN 7-color PMA/I	Test SCs 7-color PMA/I	CD90.1+ SCs 7-color PMA/I	Test SCs FMO no CD90.2 PMA/I	Test SCs FMO no KI-67 PMA/I			D
E			Test SCs 7-color BFA				Test SCs 7-color PMA/I		Test SCs FMO no CD4 PMA/I	Test SCs FMO no IFN-g PMA/I			E
F			Test SCs 7-color BFA				Test SCs 7-color PMA/I						F
G													G
H													H
	1	2	3	4	5	6	7	8	9	10	11	12	

2X BFA Media (without PMA/I) — 2X PMA/Ionomycin Restimulation Medium

16) At this point, your cells should be at a concentration of 1 x 10^7 cells/mL in base medium with the exception of the Dead Cell Control which is at 1x10^8 cells/mL. Place the entire 100 μl Dead Cell Control in the appropriate well.

17) Dispense 100 μl of 1 x 10^7 cells/mL cells suspensions (10^6 cells per well) into a 96-well flat-bottom tissue culture plate. Cell yield from pancreatic LNs is expected to be 2-3 x 10^6 cells per donor, consequently, there may not be enough pLN cells to dispense 10^6 cells per well.

18) To a volume of 100 μl of cells in base medium, add 100 μl of 2X PMA/Ionomycin Restimulation Medium to stimulated wells or 2X BFA Media (without PMA/I) to control wells. Mix gently.

19) Incubate plate for 4 hours at 37°C and 10% CO_2.

20) Following restimulation, transfer cells from the flat-bottom plate to a 96-well V-bottom plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A									Test SCs Unstained Control PMA/I	Test SCs FMO no eFluor506 PMA/I	No eFluor 506 – these 2 wells		**A**
B		Test pLN e506 BFA	Test SCs e506 BFA			Test pLN e506 PMA/I	Test SCs e506 PMA/I	CD90.1+ SCs e506 PMA/I	Test SCs Dead Cell Control e506 PMA/I				**B**
C			Test SCs Isotype BFA				Test SCs Isotype PMA/I	CD90.1+ SCs Isotype PMA/I	Test SCs FMO no CD90.1 PMA/I	Test SCs FMO no FoxP3 PMA/I			**C**
D		Test pLN 7-color BFA	Test SCs 7-color BFA			Test pLN 7-color PMA/I	Test SCs 7-color PMA/I	CD90.1+ SCs 7-color PMA/I	Test SCs FMO no CD90.2 PMA/I	Test SCs FMO no KI-67 PMA/I			**D**
E			Test SCs 7-color BFA				Test SCs 7-color PMA/I		Test SCs FMO no CD4 PMA/I	Test SCs FMO no IFN-g PMA/I			**E**
F			Test SCs 7-color BFA				Test SCs 7-color PMA/I						**F**
G													**G**
H	Ultra Comp eBeads Unstained	Ultra Comp eBeads + APC-e780 CD90.1 1:200	Ultra Comp eBeads + FITC CD90.2 1:500	Ultra Comp eBeads + BV650 CD4 1:200	Ultra Comp eBeads + PE FoxP3 1:200	Ultra Comp eBeads + eFluor 660 KI-67 1:400	Ultra Comp eBeads + BV421 IFN-g 1:200						**H**
	1	2	3	4	5	6	7	8	9	10	11	12	

21) Set-up compensation controls. Mix UltraComp eBeads vigorously by inverting the tube at least 10 times or pulse-vortexing. UltraComp eBead controls are good for 7 days from staining, so these controls can be used on any other assays set-up during the next 7 days.

22) Add 1 drop (50 µl) of UltraComp eBeads to the 7 wells designated as compensation controls.

23) Centrifuge plate for 4 minutes 400xg at 4°C.

24) Discard the supernatant, resuspend the cells in 200 µl of 1X PBS (must be azide-free and serum/protein-free) and centrifuge plate for 4 minutes 400xg at 4°C.

25) Repeat this spin and wash step at least 2 more times to remove all protein prior to staining with the Fixable Viability Dye. Completely decant supernatant.

Fixable Viability Dye Cell Staining in 96-well Plates

Allow vial of Fixable Viability Dye (FVD) to equilibrate to room temperature before opening. Staining with Fixable Viability Dye *must* be done in azide-free and serum/protein-free PBS.

Note: UltraComp eBeads should be subjected to all of the same staining procedures as the cells because different buffers affect fluorochrome performance.

At this point, each sample well in a 96-well V-bottom plate should contain approximately 10^6 cells except the viability control well, which should contain 10 x 10^6 cells.

26) Prepare a working stock solution of the Fixable Viability Dye by diluting it 1:1000, in azide- and serum/protein-free PBS. Make enough for 100 µL/well.

ATTENTION: Stain the UltraComp eBead wells and all sample wells EXCEPT the unstained control well and the eFluor 506 FMO well with the Fixable Viability Dye, since this dye alters the autofluorescence of the cells. Do NOT add the Fixable Viability Dye to the unstained control well or the eFluor506 FMO wells.

27) Add 100 µL of the working stock solution of the Fixable Viability Dye to each well and mix immediately by pipetting or gentle vortexing.

28) Add 1X PBS to wells not being stained with eFluor 506, so that they do not dry out.

29) Incubate for 10 min at 37°C, protect from light.

30) Wash cells 2 times with flow staining buffer or equivalent. Centrifuge plate for 4 minutes 400xg at 4°C. Completely decant supernatant.

POTENTIAL STOPPING POINT. You can store samples 2-8°C protected from light overnight and resume the protocol the next day.

Block Non-specific Fc-mediated Interactions

31) To make Fc Block Solution, add 50 µl of Anti-Mouse CD16/CD32 Purified (eBioscience 14-0161 or equivalent) to 4.95 mL of Flow Cytometry Staining Buffer (eBioscience 00-4222 or equivalent). Store any unused buffer for future use at 2-8°C.

32) Add 100 µl of Fc Block Solution to each well including eBead wells.

POTENTIAL STOPPING POINT. You can store samples 2-8°C protected from light overnight and resume the protocol the next day.

33) Incubate for 10-20 minutes at 2-8°C while diluting the antibodies for staining.

Staining Cell Surface Antigens for Flow Cytometry

Notes:

- *APC-eFluor® 780 is a tandem dye that is sensitive to photo-induced oxidation. Please protect this vial and stained samples from light.*
- *UltraComp eBeads should be subjected to all of the same staining procedures as the cells because different buffers affect fluorochrome performance.*

The antibodies to be used in this study have been titrated for optimal performance. The following dilutions are recommended for this study. Antibody dilutions should be made up in Flow Cytometry Staining Buffer.

Antibody Specificity & Fluorochrome	Vendor & Product #	Dilution
Rat Anti-Mouse CD4 BD Horizon™ BV650	BD Biosciences 563747	1:200
Anti-Mouse/Rat CD90.2 (Thy-1.2) FITC	eBioscience 11-0903	1:500
Anti-Mouse/Rat CD90.1 (Thy-1.1) APC-eFluor® 780	eBioscience 47-0900	1:200

ATTENTION: Do NOT stain the unstained control wells! Remember to set-up single color stains for compensation controls and FMO stains for FMO controls.

34) Wash cells with flow staining buffer. Centrifuge plate for 4 minutes 400xg at 4°C. Completely decant supernatant.

35) Combine the recommended quantity of each primary antibody in an appropriate volume of Flow Cytometry Staining Buffer so that the final staining volume is 50 µL and add to cells. Pulse vortex gently to mix. Antibody preparations:
    a) Cocktail #1 for 7-color panel samples, isotype control samples and no eFluor506 & intracellular stain FMOs (16 samples x 50 µL/sample = 800 µL): CD4 BD Horizon™ BV650, CD90.2 FITC, and CD90.1 APC-eFluor® 780
    b) Single color compensation controls for CD4 BD Horizon™ BV650, CD90.2 FITC, and CD90.1 APC-eFluor® 780
    c) FMO controls:
        i) CD90.1 APC-eFluor® 780 + CD90.2 FITC (no CD4 BD Horizon™ BV650)
        ii) CD90.1 APC-eFluor® 780 + CD4 BD Horizon™ BV650 (no CD90.2 FITC)
        iii) CD90.2 FITC + CD4 BD Horizon™ BV650 (no CD90.1 APC-eFluor® 780)

36) Incubate for 30 minutes at 2-8°C or on ice. Protect from light.

37) Wash the cells by adding cold Flow Cytometry Staining Buffer. Use 150 µL/well for 96-well plates.

38) Pellet the cells by centrifugation at 600 xg for 5 minutes at room temperature.

39) Repeat for a total of two washes using 200 µL/well cold Flow Cytometry Staining Buffer, discarding supernatant between washes.

POTENTIAL STOPPING POINT. You can store samples 2-8°C protected from light overnight and resume the protocol the next day. Wash one more time when you resume the staining protocol.

Staining Intracellular Antigens for Flow Cytometry

The antibodies to be used in this study have been titrated for optimal performance. Isotype controls should be used at the same concentration as the antibodies of interest. The following dilutions are recommended for this study. Antibody dilutions should be made up in 1X Permeabilization Buffer.

Antibody Specificity & Fluorochrome	Vendor & Product #	Dilution
Rat Anti-Mouse IFN-γ BD Horizon™ BV421	BD Biosciences 563376	1:200
Rat IgG1, κ Isotype Control BD Horizon™ BV421	BD Biosciences 562868	1:200
Anti-Mouse/Rat Foxp3 PE	eBioscience 50-5698	1:200
Rat IgG2a, κ Isotype Control PE	eBioscience 50-4321	1:200
Anti-Mouse/Rat Ki-67 eFluor® 660	eBioscience eFluor® 660	1:400
Rat IgG2a K Isotype Control eFluor® 660	eBioscience eFluor® 660	1:400

ATTENTION: Do NOT stain the unstained control wells! Remember to set-up single color stains for compensation controls and FMO stains for FMO controls.

Notes:

- *It is critical to use the Foxp3 Staining Buffer Set (cat. 00-5523). The buffer set is included with all Foxp3 Staining Sets.*
- *UltraComp eBeads should be subjected to all of the same staining procedures as the cells because different buffers affect fluorochrome performance.*

Buffers and Solution Preparation

40) Prepare fresh Foxp3 Fixation/Permeabilization working solution by diluting Foxp3 Fixation/Permeabilization Concentrate (1 part) with Foxp3 Fixation/Permeabilization Diluent (3 parts). You will need 0.25 mL of the Fixation/Permeabilization working solution for each sample. This buffer should not be stored for more than 1 day.

Caution: This solution contains Paraformaldehyde, which is toxic and a suspected carcinogen. Contact with eyes, skin and mucous membranes should be avoided. Wear proper protective clothing and gloves.

41) Prepare a 1X working solution of Permeabilization Buffer by diluting the 10X concentrate with distilled water prior to use. You will need 1.0 mL of Permeabilization Buffer for each sample. This buffer should not be stored for more than 1 day.

Note: The 10X Permeabilization Buffer has a natural tendency to precipitate, however, its function is not affected by this. To clarify, the solution can be filtered after dilution to 1X working solution.

Staining Procedure

42) After the last wash (step 39), discard the supernatant and pulse vortex the sample to completely dissociate the pellet.

43) Add 200 µL of freshly prepared Foxp3 Fixation/Permeabilization working solution to each well. Fully resuspend the cells in solution by pipetting.

44) Incubate at 4°C for 30 minutes in the dark.

Note: Comparable results, using the same donor, are obtained when the sample is incubated in the Fixation/Permeabilization Solution for varying times between 30 minutes and 18 hours. If necessary, the protocol can be stopped here and resumed the next day.

45) Centrifuge samples at 400xg at room temperature for 5 minutes, then discard the supernatant.

46) Add 200 µL 1X Permeabilization Buffer to each well.

47) Centrifuge samples at 400xg at room temperature for 5 minutes, then discard the supernatant.

48) Repeat steps 46-47.

49) Resuspend pellet in residual volume.

ATTENTION: Do NOT stain the unstained control wells! Unstained control wells should be incubated in 1X Permabilization Buffer without antibody. Remember to set-up single color stains for compensation controls and FMO stains for FMO controls.

50) Add the antibody cocktail in 1X Permeabilization Buffer to the appropriate wells and incubate at 4°C for 30 minutes in the dark.
    a) Cocktail 1 for 7-color panel samples and no eFluor 506/surface stain FMOs (12 samples x 50 µL/sample = 600 µL): FoxP3 PE, IFN-γ BV421, and Ki67 eFluor® 660
    b) Cocktail 2 for isotype control samples: PE-, BV421-, and eFluor® 660-labeled isotype controls
    c) Single color compensation controls for FoxP3 PE, IFN-γ BV421, and Ki67 eFluor® 660
    d) FMO controls:
        i) FoxP3 PE + IFN-γ BV421 (no Ki67 eFluor® 660)
        ii) FoxP3 PE + Ki67 eFluor® 660 (no IFN-γ BV421)
        iii) Ki67 eFluor® 660 + IFN-γ BV421 (no FoxP3 PE)

51) Add 200 µL of 1X Permeabilization Buffer to each well.

52) Centrifuge samples at 400xg at room temperature for 5 minutes, then discard the supernatant.

53) Add 200 µL of 1X Permeabilization Buffer or Flow Cytometry Staining Buffer to each well.

54) Centrifuge samples at 400xg at room temperature for 5 minutes, then discard the supernatant.

55) Resuspend cells in an appropriate volume of Flow Cytometry Staining Buffer and analyze on a flow cytometer.

Note: Due to the fixation and permeabilization procedure, the forward scatter and side scatter distribution of the cells will be significantly different than live cells. Therefore, the gates and voltages will need to be adjusted.

DATA ACQUISITION

Recommended Instrument Configuration

Wavelength (nm)	Detector	Dichroic Mirror (nm)	Recommended Bandpass Filter (nm)	Fluorochrome	Ex Max (nm)	Em Max (nm)
405		None	450/50	BD Horizon™ BV421	407	421
		505	510/50	eFluor® 506	?	506
		630	660/20	BD Horizon™ BV650	405	650
488		505	530/30	FITC	488	518
		550	575/26	PE	488	519
633		None	660/20	eFluor® 660	633	660
		735	780/60	APC-eFluor® 780	633	780

Gating Strategy

Transferred Tregs: FSC/SSC Lymphocytes → Live cells → Singlets → CD90.1+ → CD4+ → FoxP3+

Endogenous Tregs: FSC/SSC Lymphocytes → Live cells → Singlets → CD90.2+ → CD4+ → FoxP3+

Events

Collect as many events as possible or a minimum of 1000 events in the FSC/SSC Lymphocytes → Live cells → Singlets → CD90.1 → CD4+ → FoxP3+ gate.

APPENDIX A: ANTIBODIES, REAGENTS AND MATERIALS

Antibodies & Dyes

Antibody Specificity	Clone	Isotype	Stock Concentration	Fluorochrome	Vendor	Product Number
Fixable Viability Dye	--	--		eFluor®506	eBioscience	65-0866
Anti-Mouse/Rat CD90.1 (Thy-1.1)	HIS51	Mouse IgG2a, κ	0.2 mg/mL	APC-eFluor® 780	eBioscience	47-0900
Anti-Mouse/Rat CD90.2 (Thy-1.2)	30-H12	Rat IgG2b, κ	0.2 mg/mL	FITC	eBioscience	11-0903
Rat Anti-Mouse CD4	RM4-5	Rat (DA) IgG2a, κ	0.2 mg/mL	BD Horizon™ BV650	BD Biosciences	563747
Anti-Mouse/Rat Foxp3	FJK-16s	Rat IgG2a, κ	0.2 mg/mL	PE	eBioscience	72-5775
Rat IgG2a, κ Isotype Control	eBR2a	Rat IgG2a, κ	0.2 mg/mL	PE	eBioscience	12-4321
Anti-Mouse/Rat Ki-67	SolA15	Rat IgG2a, κ	0.2 mg/mL	eFluor® 660	eBioscience	50-5698
Rat IgG2a K Isotype Control	eBR2a	Rat IgG2a, κ	0.2 mg/mL	eFluor® 660	eBioscience	50-4321
Rat Anti-Mouse IFN-γ	XMG1.2	Rat IgG1, κ	0.2 mg/mL	BD Horizon™ BV421	BD Biosciences	563376
Rat IgG1, κ Isotype Control	R3-34	Rat IgG1, κ	0.2 mg/mL	BD Horizon™ BV421	BD Biosciences	562868

Reagents and Materials

Protocol Segment	Product Description	Vendor	Product Number
Cell Prep	1X PBS	Life Technologies	10010-02*
Cell Prep	Tissue culture dish		
Cell Prep	Frosted microscope slides		
Cell Prep	3 mL syringe	Fisher Scientific	
Cell Prep	70 μm cell strainer	Fisher Scientific	223-63-548*
Cell Prep	Red Blood Cell Lysing Buffer Hybri-Max™	Sigma-Aldrich	R-77567*
Cell Prep	15 mL and 50 mL conical centrifuge tubes		
PMA/I Restim	Base Medium	See recipe in Appendix B	
PMA/I Restim	Brefeldin A	Sigma Aldrich	B-7651-5mg
PMA/I Restim	Ethanol	Sigma Aldrich	E7023-500ML*

APPENDIX A: ANTIBODIES, REAGENTS AND MATERIALS (continued)

Reagents and Materials (continued)

Protocol Segment	Product Description	Vendor	Product Number
PMA/I Restim	Brefeldin A Stock Solution	See recipe in Appendix B	
PMA/I Restim	Phorbol-12-Myristate 13-Acetate (PMA)	Sigma Aldrich	P8139-1MG
PMA/I Restim	PMA Stock Solution	See recipe in Appendix B	
PMA/I Restim	Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D2650-5X5M
PMA/I Restim	Ionomycin calcium salt	Sigma Aldrich	I0634-1MG
PMA/I Restim	Ionomycin Stock Solution	See recipe in Appendix B	
PMA/I Restim	2X BFA Media	See recipe in Appendix B	
PMA/I Restim	2X Restimulation Media	See recipe in Appendix B	
PMA/I Restim	96-well flat bottom plates Costar 3596*		
Viability Staining	96-well V-bottom plates Costar 3897*		
Viability Staining	UltraComp eBeads	eBioscience	01-2222
Viability Staining	Fixable Viability Dye eFluor® 506	eBioscience	65-0866
Fc Blocking	Flow Cytometry Staining Buffer*	eBioscience	00-4222*
Fc Blocking	Anti-Mouse CD16/CD32 Purified (Fc Receptor Block)	eBioscience	14-0161
Intracellular Staining	Foxp3 Staining Buffer Set	eBioscience	00-5523
Flow Cytometry	Round-bottom Polystyrene Tubes	Fisher Scientific	14-959-5*

*or equivalent from your preferred vendor

APPENDIX B: SOLUTIONS AND RECIPES

100 mL PMA/Ionomycin Restimulation Base Media

	Component	Vendor	Product Number
85.0 mL	Minimal Essential Medium (MEM)	Life Technologies	11090
10.0 mL	Fetal Bovine Serum		
2.0 mL	1 M stock HEPES (20 mM final concentration)	Life Technologies	11344
1.0 mL	Penicillin/Streptomycin/Neomycin	Life Technologies	15640
1.0 mL	100X MEM Non-essential amino acids	Life Technologies	11140
1.0 mL	100 X GlutaMAX	Life Technologies	35050
0.1 mL	0.1 M 2-Mercaptoethanol	Sigma Aldrich	M3148

10 mg/ml Brefeldin A Stock Solution

Brefeldin A is a protein transport inhibitor that prevents transport from the endoplasmic reticulum to the Golgi apparatus.

Amount	Component	Vendor	Product Number
5 mg	Brefeldin A	Sigma Aldrich	B-7561
0.5 mL	Ethanol	Sigma Aldrich	E7023-500ML*
*or equivalent from your preferred vendor Dispense into 20 µl aliquots. Store at -20°C in the dark.			

400 µg/mL PMA Stock Solution

Phorbol-12-Myristate 13-acetate (PMA) activates Protein Kinase C.

Amount	Component	Vendor	Product Number
1 mg	Phorbol-12-Myristate 13-Acetate (PMA)	Sigma Aldrich	P8139-1MG
2.5 mL	Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D2650-5X5M*
*or equivalent from your preferred vendor Dispense into 20 µl aliquots. Store at -20°C. May be freeze-thawed a maximum of 5 times.			

500 µg/mL Ionomycin Stock Solution

The calcium salt of ionomycin is an effective mobile Ca2+ carrier. It facilitates calcium flux which leads to downstream effects, such as up-regulation of CD7 in T cells (signal of activation), or the hydrolysis of phosphoinositides and activation of Protein Kinase C in T cells.

Amount	Component	Vendor	Product Number

1 mg	Ionomycin calcium salt	Sigma Aldrich	I0634-1MG
2 mL	Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D2650-5X5M*
*or equivalent from your preferred vendor Dispense into 20 µl aliquots. Store at -20°C. Do NOT re-freeze.			

2X BFA Media

Amount	Component	Final Concentration
1 µl	10 mg/mL Brefeldin A Stock Solution	2 µg/mL
5 mL	PMA/Ionomycin Restimulation Base Media	
Store at 4-8°C. Use same day.		

2X PMA/Ionomycin Restimulation Media

Amount	Component	Final Concentration
5 mL	PMA/Ionomycin Restimulation Base Media	
1 µl	10 mg/mL Brefeldin A Stock Solution	2 µg/mL
1 µl	400 µg/mL PMA Stock Solution	0.08 µg/mL
10 µl	500 µg/mL Ionomycin Stock Solution	1 µg/mL
Store at 4-8°C. Use same day.		

Fc Blocking Buffer

Amount	Component	Final Concentration
100 µl	0.5 mg/mL anti-Mouse CD16/CD32 Purified (eBioscience 14-0161 or equivalent)	50 µg/mL
9.9 mL	Flow Cytometry Staining Buffer (eBioscience 00-4222 or equivalent)	
Store at 4-8°C.		

APPENDIX C: Trypan Blue Protocol

ProductInformation

Trypan Blue

Product Nos. T 8154, T 6146 and Z 35,962-9 (H7901)

USE OF TRYPAN BLUE STAIN AND THE HEMOCYTOMETER TO DETERMINE TOTAL CELL COUNTS AND VIABLE CELL NUMBER

Trypan Blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of cell morphology.

NOTE: Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted and resuspended in protein-free medium or salt solution prior to counting.

1) Prepare a cell suspension in a balanced salt solution (e.g., Hanks' Balanced Salts [HBSS], Product No. H 2513).
2) Transfer 0.5 ml of 0.4% Trypan Blue solution (w/v) to a test tube. Add 0.3 ml of HBSS and 0.2 ml of the cell suspension (dilution factor = 5) and mix thoroughly. Allow to stand for 5 to 15 minutes.

NOTE: If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

3) With the cover-slip in place, use a Pasteur pipette or other suitable device to transfer a small amount of Trypan Blue-cell suspension mixture to both chambers of the hemocytometer. Carefully touch the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers.
4) Starting with chamber 1 of the hemocytometer, count all the cells in the 1 mm center square and four 1 mm corner squares (see **Diagram I**). Non- viable cells will stain blue. Keep a separate count of viable and non-viable cells.

NOTE: Count cells on top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at bottom and right sides (see **Diagram II**).

5) Repeat this procedure for chamber 2.

NOTE: If greater than 10% of the cells appear clustered, repeat entire procedure making sure the cells are dispersed by vigorous pipetting in the original cell suspension as well as the Trypan Blue-cell suspension mixture. If less than 200 or greater than 500 cells (i.e., 20-50 cells/square) are observed in the 10 squares, repeat the procedure adjusting to an appropriate dilution factor.

6) Withdraw a second sample and repeat count procedure to ensure accuracy.

7) **CELL COUNTS** — Each square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations:

CELLS PER ml = the average count per square $\times$ dilution factor $\times$ 10^4 (count 10 squares)

Ex: If the average count per square is 45 cells $\times$ 5 $\times$ 10^4 = 2.25 $\times$ 10^6 cells/ml.

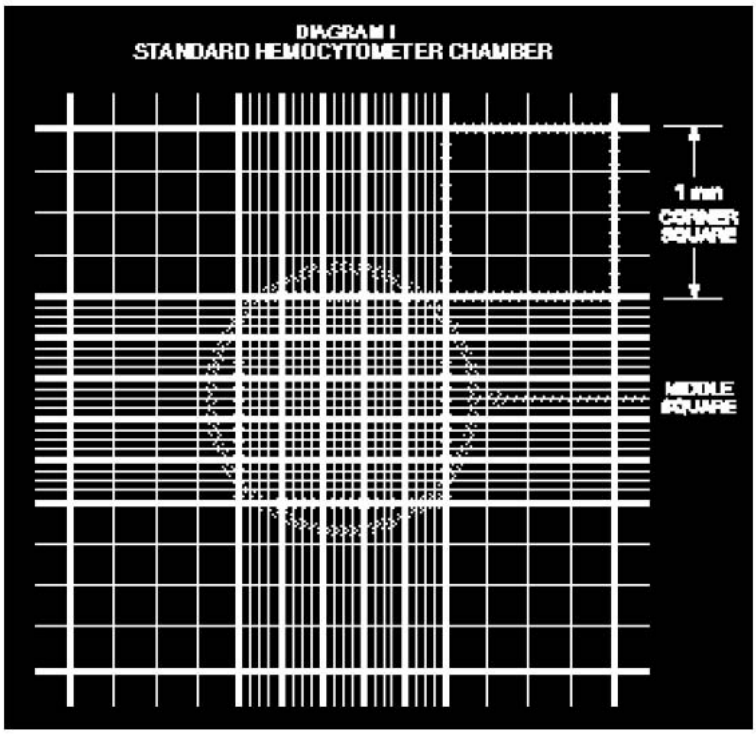
TOTAL CELLS = cells per ml $\times$ the original volume of fluid from which cell sample was removed.

Ex: 2.25 $\times$ 10^6 (cells/ml) $\times$ 10 ml (original volume) = 2.25 $\times$ 10^7 total cells.

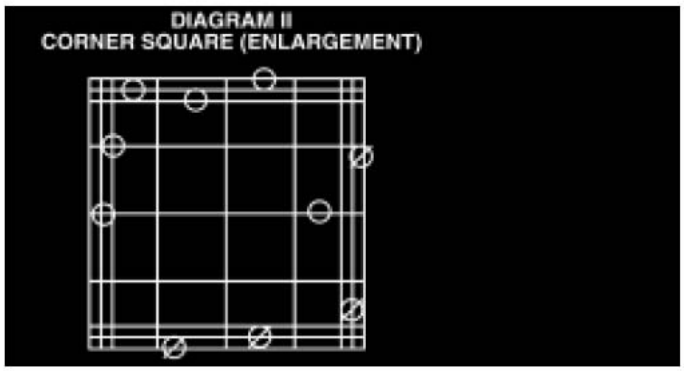
8) **CELL VIABILITY (%)** = total viable cells (unstained) $\div$ total cells (stained and unstained) $\times$ 100.

Ex: If the average count per square of unstained (viable) cells is 37.5, the total viable cells = [37.5 $\times$ 5 $\times$ 10^4] viable cells/ml $\times$ 10 ml (original volume) = 1.875 $\times$ 10^7 viable cells. Cell viability (%) = 1.875 $\times$ 10^7 (viable cells) $\div$ 2.25 $\times$ 10^7 (total cells) $\times$ 100 = 83% viability.

Trypan Blue continued



The circle indicates the
approximate area covered at 100× microscope magnification (10× ocular and 10× objective). Include cells on top and left touching middle line (○). Do not count cells touching middle line at bottom and right (⌀). Count 4 corner squares and middle square in both chambers (one chamber represented here).



Count cells on top and left touching middle line (○). Do not count cells touching middle line at bottom and right (⌀).

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Red Blood Cell Lysing Buffer. Product Information Sheet. Sigma, R-7757.

REVISION HISTORY

Effective Date	Revision Version	Author	Description of Changes
14-Aug-2015	Version 1	T Kupfer	Original Version