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FoxP3 Staining for Flow Cytometry

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1.0 Title

Foxp3 Staining for Flow Cytometry

2.0 Purpose

To stain whole blood or ficolled cells for expression of Foxp3.

Background:

Expression of Foxp3 on CD3+, CD4+ and CD25+ cells is associated with T regulatory (Tregs) and some activated T cells. Regulatory T cells/Tregs (also known as suppressor T cells) are a specialized subpopulation of T cells that act to suppress immune responses thereby maintaining homeostasis and self tolerance. Intense investigation has tried to determine exclusive protein markers for Tregs. It has been proposed that the "natural" regulatory T cells originally recognized by their constitutive expression of CD4 and CD25 are further defined by expression of the transcription factor Foxp3. Additionally Foxp3 has also been characterized in CD8+CD28- suppressor T cells.

3.0 Definitions and Abbreviations

BSA	Bovine Cytometry Media
DNAse	Deoxyribonuclease I
EDTA	Ethylene Diamine tetra acetic acid
FCM	Flow Cytomety Media
FCS	Fetal Calf Serum
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
PBMC(s)	Peripheral Blood Mononuclear Cell(s)
PBS	Phosphate buffer saline

4.0 Equipment and Reagents

4.1 Equipment

	Type	Supplier	Catalog or Vendor Number
4.1.1	Rack	VWR	
4.1.2	Pipettors and Tips	Pipettors-Gilson Tips- USA Scientific	Pipet tips- yellow- 1111-0816 20ul and 200ul Blue- 1111-2831 1000ul
4.1.3	Timer	VWR	
4.1.4	12 x 75 mm Flow Tubes	BD Falcon	352052
4.1.5	Waste Container and Blotting Cloth	Waste container at RPCI center Blot cloth- Kimberly-Clark	Blot cloth- 05812
4.1.6	Centrifuge	Kendro	

4.1.7	15ml and 50ml Centrifuge tubes for buffers	15ml- Biologix 50ml- Falcon	15ml-BCT-P15RS 50ml-352098
4.1.8	BD Canto or BD LSRII flow cytometer	BD	LSRII- product number-340551

4.2 Reagents

	Type	**Supplier**	**Catalog or Vendor Number**
4.2.1	PBS	Invitrogen	# 21300-017
4.2.2	Bovine Serum Albumin	Sigma	A4503
4.2.3	Sodium azide	Fisher	# 5227
4.2.4	Na_2EDTA	Sigma	We have tetra it is ED4SS
4.2.5	Ammonium Chloride	Sigma	A-5666
4.2.6	Potassium Bicarbonate	Sigma	P-4913
4.2.7	Tetra Sodium EDTA	Sigma	ED4SS
4.2.8	FCM Buffer (see Appendix)		
4.2.9	Surface Antibodies (see Appendix)		
4.2.10	Fix/Perm Concentrate*	eBioscience	# 00-5123-43
4.2.11	Fix/Perm Diluent*	eBioscience	# 00-5223-56
4.2.12	10X Permeabilization Buffer*	EBioscience	# 00-8333-56
4.2.13	Alexa Fluor 488 Mouse IgG1 Isotype (at the appropriate titer)	Biolegend	# 400134
4.2.14	Alexa Fluor 488 Anti-human Foxp3 (at the appropriate titer)	Biolegend	# 320112
4.2.15	Human IgG Block at 12 mg/ml	Sigma	G-4386
4.2.16	Methanol Free Formalin	Polysciences	# 08379
4.2.17	Deoxyribonuclease I	Sigma	DN25
4.2.18	Complete Media (See Appendix)		
4.2.19	HEPES	Made in Media center at RPCI	
4.2.20	L-glutamine	Cellgro	

4.2.21	Na Pyruvate	Made in Media center at RPCI	
4.2.22	Gentamicin sulfate	Cellgro	30-005-CR
4.2.23	2-units Heparin	Abraxis	NDC 63323-276-02
4.2.24	FCS	Cellgro	35-010-CV
4.2.25	Nonessential Amino Acids	Cellgro	25-025-CI
4.2.26	2% Formalin	Polysciences	04018

Notes:
*We have found that Fix/Perm reagents from either eBioscience or BioLegend yield comparable results. The investigator should choose these reagents at their own discretion.

*This SOP details staining methods for cells from whole washed blood or from cells separated by Ficoll gradient.

5.0 Procedures

5.1 Prepare reagents

5.1.1 Prepare Lysing Reagent (see Appendix).

5.1.2 Prepare FCM Buffer (see Appendix).

5.1.3 Prepare Fix/Perm Buffer as a 1:4 dilution using the Fix/Perm Diluent (see Appendix).

5.1.4 Prepare Permeabilization Buffer as a 1:10 dilution in dH_2O.

5.1.5 Prepare Complete Media (see Appendix).

5.2 Thaw frozen cells (If using whole blood, proceed with step 5.3.1 or 5.4)

5.2.1 When cells are ready to be thawed place freezing vials into the 37°C water bath, and remove vial(s) right before contents are completely thawed.

5.2.2 Wash outside of vial with 70% Alcohol.

5.2.3 Remove the cell suspension from the vial using a pipette and place into a 15 ml conical tube containing 10 ml complete media.

5.2.4 Next, wash the inside of cells cryovial with 1 ml of the complete media/cell suspension from the 15 ml tube, and then add the rest of cell suspension to the 15 ml conical tube of cells.

5.2.5 Add 12.5 µg/ml of DNAse to the cells (use 10 µL of DNAse I @ 20,000 U/ml for every 10 ml of complete media).

5.2.6 Gently mix cell suspension by inverting 3-5 times.

5.2.7 Incubate at 37°C for 30 minutes. Centrifuge at 330*g* for 5 minutes, aspirate, and then re-suspend the pellet by flicking with a finger or tapping lightly on the bench.

5.2.8 Add 5 ml of FCM Buffer, and centrifuge at 330*g* for 5 minutes. Aspirate, and then re-suspend the pellet by flicking with a finger or tapping lightly on the bench or very LIGHTLY vortexing.

5.2.9 Repeat step 5.2.8.

5.2.10 Resuspend cells up to a volume of 5 ml using complete media.

5.2.11 Proceed with step 5.3.2.

5.3 Prepare frozen cells/cells separated by Ficoll gradient technique.

5.3.1 Ficoll cells (follow your own SOP for this step); resuspend in 5 ml of complete media. Proceed with step 5.3.2.

5.3.2 Centrifuge the cells at 330*g* for 5 minutes.

5.3.3 Aspirate off as much of the media without disturbing the pellet.

5.3.4 Measure the remaining volume and count cells (refer to your own counting SOP).

5.3.5 Add FCM Buffer (see Appendix) so that each tube will have a total volume of 100 µl.

5.3.6 Add 10 µl per $1x10^6$ cells of Human IgG to block the FC receptors on the cell surface. Vortex tube.

5.3.7 Place tube on ice for 10 minutes.

5.3.8 Continue with step 5.5.

5.4 Prepare whole blood – using washed blood lysis technique.

5.4.1 Collect blood in a glass sodium heparin tube. Place 2 - 3 ml of whole blood in a 15 ml centrifuge tube.

5.4.2 Add 10 - 12 ml of cold PBS containing 10 U/ml heparin, to the tube to wash and vortex.

5.4.3 Centrifuge at 330*g* for 5 minutes.

5.4.4 Aspirate off the clear supernatant as close as possible to the darker, cell containing layer without disturbing the layer, typically within 500µl of the layer.

5.4.5 After brief agitation by vortexing, add 15 ml of PBS (no heparin) and centrifuge the cells a second time and aspirate the clear layer.

5.4.6 Resuspend the washed blood in FCM buffer to a volume of 1.5 ml with human IgG to a final concentration of 2 mg/ml.

5.4.7 Centrifuge the tube at 330*g* for 5 minutes. Aspirate off the FCM Buffer so there is 2 ml left.

5.4.8 Place on ice for 10 minutes.

5.4.9 Continue with step 5.5.

5.5 Label, lyse, and permeabilize cells.

NOTE: The following part of the procedure is the same for either cells separated by Ficoll gradient or whole (washed) blood

5.5.1 Resuspend cells in FCM so that you have $5x10^6$ - $1x10^7$ cells/ml. Aliquot 100 µl ($5x10^5$ – $1x10^6$) to each of two 12 x 75 mm flow tubes. For this protocol, there are two tubes.

5.5.2 Add the surface antibody panels (see Appendix) (volume varies from 10-30 µl, dependent upon the antibody titer) to each tube and vortex/mix. Place in ice bath in the dark for a 1 hour incubation.

Surface antibodies:
Tube 1: CD127 PE / CD4 PcP / CD3 PECy7 / CD25 APC
Tube 2: CD127 PE / CD4 PcP / CD3 PECy7 / CD25 APC

5.5.3 Gently vortex the tubes and add 3.5 ml of Lysing Reagent to each (see Appendix). Cover the tubes and invert 3 times to mix completely and let sit at room temperature (25°C) for 5 minutes.

5.5.4 Centrifuge at 330*g* for 5 minutes. Decant the liquid and blot the tubes 4 times and vortex.

5.5.5 Add 3 ml of FCM Buffer. Centrifuge at 330*g* for 5 minutes. Decant the liquid and blot 4 times and vortex.

5.5.6 Repeat step 5.5.5

5.5.7 Resuspend each tube in 1 ml of the 1:4 dilution of the Fix/Perm Buffer. Incubate at room temperature in the dark for 30 minutes.

5.5.8 Centrifuge at 330*g* for 5 minutes. Decant, blot, and vortex the tubes.

5.5.9 Resuspend each tube in 2 ml of the 1:10 dilution of Perm Buffer. Centrifuge at 330*g* for 5 minutes, decant, blot, (do **not** vortex until after the last wash). Repeat step 5.5.9. There should be less than 50 µl of residual Perm Buffer left in the tubes after the final wash.

5.5.10 Add 10 µl of Human IgG to each tube and let them sit on ice for 10 minutes.

5.5.11 Add the appropriate volume (volume varies from 10-30 µl, dependent upon the antibody titer) of the Isotype Control or Foxp3 antibody (see Appendix) to the appropriate tube.

Intracellular antibodies:
Tube 1: Foxp3 Ax488
Tube 2: mIgG1 Ax488

Vortex and incubate at room temperature for 1 hour in the dark.

5.5.12 Add 1 ml of the 1:10 dilution of Perm Buffer to each tube. Centrifuge at 330*g* for 5 minutes. Decant and blot the tubes. Do **not** vortex.

5.5.13 Add 3 ml of FCM Buffer to each tube. Centrifuge at 330*g* for 5 minutes. Decant, blot, and vortex the tubes.

5.5.14 Add between 300 µl and 500 µl of 2% Formalin to fix the cells.

5.5.15 Place the tubes in the refrigerator overnight and run samples on the BD Canto or BD LSRII flow cytometer the next day.

Appendix A

SPECIMEN REQUIREMENTS:
Ficolled cells or whole blood

SPECIMEN STABILITY:
Ficolled cells stored in RPMI are stable for several days; check viability.
Whole blood collected in EDTA is stable for 24 hours.
Whole blood collected in Heparin is stable for 48 hours.

CITERIA FOR SPECIMEN REJECTION:

1. Any unlabeled specimen will be rejected. All specimens must be labeled with the patient name or number and the date drawn.
2. The requisition slip must have the time drawn; if it does not, the specimen will not be processed. Rather, we will call the appropriate individual for a time of draw. Once this is accomplished, the specimen will be processed.
3. For the complete list of rejection criteria see Criteria for Sample Rejection page.
4. Viability of <85%

Appendix B

REAGENTS AND PREPARATION:

1. Lysing Reagent

PREPARE FRESH SOLUTION DAILY!

It is recommend weighing the reagents and storing them as packets. The dry reagents are dissolved in water as required.

- 4.13 g Ammonium Chloride (NH_4Cl) – Sigma A-5666
- 0.5 g Potassium Bicarbonate ($KHCO_3$) – Sigma P-4913
- 0.0185 g Tetra Sodium EDTA – Sigma ED4SS

(All above chemical must be less than on year old and stored tightly sealed)
Measure the above chemicals carefully. Pour solution into a flask (must be at lease 500 ml flask) to which a stir bar has been added. Add 500 ml double distilled water. Place on stir plate and stir until the powder is dissolved.
Do not pH. Scale quantities for daily use: must be made fresh daily. Use at room temperature or warm to 27°C.

2. FCM Buffer Reagent

- Dissolve in 1 liter of 1X PBS, 10 g BSA, 0.04 g Na_2EDTA and 1 g sodium azide.
- Stored at 4-8˚C, this solution is stable for 6 months.

3. Complete Media (CM)

- Combine with one 250 ml bottle of RPMI 1640 (Roswell Media Resource (RMR), cat: 1640) the following:
  - FCS, 25 ml
  - 25 mM HEPES, 12. 5 ml of 1 M Stock
  - 2.0 mM L-glutamine, 5 ml of 100X stock
  - 1.0 mM Na Pyruvate, 5 ml of 100X stock
  - 0.1 mM nonessential Amino Acids, 5 ml of 100X stock
  - Gentamicin sulfate, 0.5 ml of 50 mg stock
  - $5x10^{-5}$ (final concentration) of $5x10^{-2}$ 2-ME stock, diluted 1:1000 in Complete media

4. Fix/Perm Concentrate

- 1:4 dilution with the Fix/Perm Diluent.

Add 1 ml of the concentrated Fix/Perm concentrate to 3 ml of the Fix/Perm diluent. Prepare enough for each tube (each tube needs 1ml).

5. 10X Permeabilization Buffer (Perm Buffer)

- 1:10 dilution in dH_2O

Add 1 ml of the Perm Buffer to 9 ml of dH_2O. Prepare enough for each tube (each tube needs 5 ml).

6. Human IgG Block at 12 mg/ml
Block IgG:

- 12 mg/ml human IgG Cohn fraction II and III globulins.
- Dilute in PBS.
- Store frozen. Thaw when needed and store in the refrigerator. Keep this sterile.

7. Methanol Free Formalin

- 2% EM grade 10% buffered formalin dilute in PBS**.**

8. Antibodies

a. Anti-Foxp3-Alexa 488 (BioLegend, clone 206D, Cat#320111)
b. Anti-mouse IgG1-Alexa 488 (Biolegend, clone MOPC-21, Cat #400133)
c. Anti-CD3-PECy7 (BD BioSciences, clone SK7, cat # 341091)
d. Anti-CD4-PerCP (BD BioSciences, clone SK3, cat # 347324)
e. Anti-CD25-APC (BD BioSciences, clone 2A3, cat # 340938)
f. Anti-CD127-PE (BD BioSciences, clone hIL-7R-M21, cat # 557938)

Surface staining panels:

1: CD127 PE / CD4 PcP / CD3 PECy7 / CD25 APC
2: CD127 PE / CD4 PcP / CD3 PECy7 / CD25 APC

Intracellular staining panels:
1: FoxP3 Ax488
2: mIgG1 Ax488

Complete panels:

Panel	Alexa 488	PE	PerCP	PECY7	APC
1	Foxp3	CD127	CD4	CD25	CD25
2	IgG1	CD127	CD4	CD25	CD25

Appendix B

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3. Fontenot, J. D., Gavin, M. A., and Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol, 4: 330-6, 2003.
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5. Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A., Kapranov, P., Gingeras, T. R., Fazekas de St Groth, B., Clayberger, C., Soper, D. M., Ziegler, S. F., and Bluestone, J. A. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med, 203: 1701-11, 2006.

REVISION HISTORY:			
Rev	**Section**	**Type**	**Initials/Dates**
1	All	First edited version.	JMG / 11.5.07
2	All	Revised for website	JMG / 3.18.09