RPCI 001 v.003

In vitro Intracellular Cytokine Staining With and Without Stimulation

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1.0 Title

In vitro Intracellular Cytokine Staining at the Flow Core With and Without Stimulation

2.0 Purpose

To stain whole blood with antibodies to intracellular cytokines and surface markers for flow cytometric analysis

3.0 Definitions and Abbreviations

FCS	Fetal calf serum
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
BFA	Brefeldin A
CM	Complete Media

4.0 Equipment and Reagents

4.1 Equipment

	Equipment	Vendor	Catalog #
4.1.1	FACs tubes, 5ml, 12x75mm, capped	Beckton Dickenson	#352052
4.1.2	Eppendorf tubes	Eppendorf	#2260002-8
4.1.3	Centrifuge		
4.1.4	Thermoblock, Eppendorf thermostat.	See Appendix.	

4.2 Reagents

	Reagent	Vendor	Catalog #
4.2.1	Formalin, 2 %	Polyscience EM grade 10% buffered Formalin (Polysciences 08379) dilute to 1% in PBS	

4.2.2	FCS, 100%	Invitrogen	#16000-044
4.2.3	Monoclonal antibodies	BD; See Appendix	
4.2.4	1 M HEPES	Invitrogen, RMR	
4.2.5	100x stock L-glutamine	Invitrogen	#25030-081
4.2.6	Sodium pyruvate, 100x stock	Sigma	#S8636
4.2.7	Non-essential amino acids, 100x stock	Sigma	#M7145
4.2.8	Gentamicin sulfate, 50mg stock	RMR	
4.2.9	2-ME (2-mercapto-ethanol)	Sigma	#M7522
4.2.10	Heparin	American Pharma. Partners	#NDC-63323-276-02
4.2.11	Phosphate buffered saline	Invitrogen	#21300-017
4.2.12	Bovine serum albumin	Sigma	#A-2153
4.2.13	Sodium azide	Sigma	#S-2002
4.2.14	Caltag Fix and Perm Medium B	Caltag	#GAS002S
4.2.15	PMA (phorbal 12-myristate 13-acetate)	Sigma	#P-8139
4.2.16	Ionomycin	Sigma	#I-0634
4.2.17	Brefeldin A (BFA)	Sigma	#B-7651
4.2.18	DMSO	Dimethyl sulfoxide	
4.2.19	Mouse IgG	Caltag	#10400

4.2.20	2% Para-formaldehyde, 10x10 ml, prepared	Electron Microscopy Sciences, 16% Paraformaldehyde, Dilute to 2% in PBS	15710
4.2.21	Ammonium chloride (NH_4Cl)	Sigma	A-5666
4.2.22	Potassium bicarbonate ($KHCO_3$)	Sigma	P-4913
4.2.23	Tetra sodium EDTA	Sigma	ED4SS
4.2.24	Complete Media	See Appendix	

5.0 Procedures

5.1 Draw blood into 10 ml sodium heparin green top tube.

5.2 Prepare reagents for stimulation and control samples.

5.2.1 BFA: dilute 5 mg/ml stock 1/10 in Complete Media (CM) (see Appendix), use 10 µl per sample

5.2.2 Ionomycin: dilute 0.5 mg/ml stock 1/10 in CM, use 10 µl per sample

5.2.3 PMA: dilute 2 mg stock 1:1600 stock in CM, (example: 5µl of PMA stock in 8 ml of CM), use 10 µl per sample

5.3 Stimulation Procedure- **In vitro Assay**

5.3.1 Take 1-2 ml blood and centrifuge at 1500 x g for 3 minutes.

IMPORTANT: If blood is collected in a sodium heparin tube, proceed to step 5.3.2. If blood is collected in an EDTA tube, proceed to step 5.3.3.

5.3.2 **For blood collected in a sodium heparin tube:** aspirate plasma and then re-suspend in PBS to 2x the original volume (in this example that would be 2-4 ml). Proceed to step 5.3.5.

5.3.3 **For blood collected in an EDTA tube:** The blood must be washed to replace the anticoagulant with heparin. Aspirate plasma and then add 10 ml of CM. Mix the blood. Centrifuge at 1500 x g for 3 minutes. Aspirate, leaving the buffy coat/leukocyte layer.

5.3.4 Re-suspend in CM to 2x the original volume (in this example that would be 2-4 ml).

5.3.5 Aliquot 0.50 ml of diluted blood to each of two 1.5 ml Eppendorf tubes. Label one "not stim" for not stimulated and the other "stim" for stimulated.

5.3.6 Add 10 µl diluted BFA to the "not stim" tubes, mix on vortexer.

5.3.7 Add 10 µl diluted BFA, 10 µl diluted Ionomycin and 10 ul diluted PMA to the "stim" tubes. Mix on vortexer.

5.3.8 Place tubes in the Thermoblock. Activate P1 program and press the "Start/Stop" button once. This will incubate samples for 4 hours at 37°C; follow by selecting hold temperature of 4°C on the Thermoblock.

5.4 Labeling procedure

5.4.1 Add 33.3 µl of 3.0 mg/ml mouse IgG to each tube at 4°C and wait 10 minutes.

5.4.2 Dispense 20µL of antibodies from Group A (surface antibodies) into two new 12x75mm tubes. Label one "stim" and one "non stim."

5.4.3 Transfer 100 µl of the stimulated blood tube to the tubes to which surface antibodies (Group A) have been added. Repeat with the unstimulated blood, so that the tubes now contain either 100µl of simulated or unstimulated blood. Mix on vortexer. Incubate at 4°C for 20 minutes.

5.4.4 Lyse the red blood cells with 3.5 ml of lysing reagent (see Appendix). Put a cap on each tube (or use parafilm to seal the tubes), invert once and place directly into a centrifuge adaptor. Invert all tubes 1-2 times, and centrifuge at 1500 x g (3200RPM) for 3 minutes.

5.4.5 Remove caps or parafilm, decant the supernatant and blot on a terry cloth towel three times. Rack tubes (mix samples by sliding tube across a test tube rack) or flick tubes with finger to gently mix and place in the adaptor.

5.4.6 Add 3.0 ml 1X PBS.

5.4.7 Centrifuge again at 1500 x g (3200RPM) for 3 minutes.

5.4.8 Decant and carefully blot tubes again.

NOTE: Do NOT leave cells in this state for long. Fix immediately and leave on ice.

Add 100 µl of 2.0% paraformaldehyde. Incubate at room temperature for 20 minutes only!

5.4.9 Wash samples with 3.0 ml staining PAB buffer (see Appendix); centrifuge again at 1500 x g (3200 RPM) for 3 minutes. Very carefully decant and blot tubes.

5.4.10 Add 100 µl of permeabilization buffer (diluted ¼ in PBS; see Appendix). Do not rack to mix, mix gently.

5.4.11 Add 20 µL of intracellular stain antibodies (Group B), mixing gently. Incubate at room temperature for 30 minutes.

5.4.12 Add 3.0 ml 1X PBS. Incubate at room temperature for 15 minutes. Centrifuge sample at 1500 x g for 3 minutes. Decant and blot.

5.5 Analysis

5.5.1 Re-suspend cells in 250 uL of 2.0% paraformaldehyde in PBS. Wait at least 20 minutes before acquisition on the flow cytometer.

Appendix

Reagents

Complete Media

Combine with one 250 ml bottle of RPMI 1640 (Roswell Media Resource (RMR), cat: 1640) the following:

FCS, 250 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
2 units Heparin (from 1000 units/ml Heparin stock)

Monoclonal Antibodies

Surface mAbs (A stain)
Intracellular mAbs (B stain)

Staining buffer (PAB):

1X Phosphate buffered saline
0.5% Bovine Serum Albumin
0.1% Sodium azide

Dissolve in 1 liter of 1X PBS, 5 g BSA, and 1 g sodium azide. Filter through 0.45 µm membrane and store at 4-8˚C, this solution is stable for 6 months.

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 chemicals must be less than one year old and stored tightly sealed)

Measure the above chemicals carefully. Pour the solution into a flask (must be at least a 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% Fixation Buffer for Fix&Perm

Phosphate buffered saline
EM grade 10% buffered Formalin

Prepare 2% formalin fixation buffer by diluting 20 ml of 10% EM grade formalin in 80 ml of 1X PBS (no protein). Store at room temperature, protect from light by wrapping in foil.

Permeabilization Buffer

Caltag Fix & Perm Medium B

Store at room temperature protect from light by preparing in a brown colored glass and store in the dark. Solution is stable for 1 month.

2 mg/ml PMA (Phorbol 12-Myristate 13-Acetate) Stock

Resuspend PMA at 2 mg/ml in DMSO. Store in 20 µl aliquots, frozen at -80°C.

0.5 mg/ml Ionomycin

Ionomycin

Resuspend at 0.5 mg/ml in 200 proof alcohol. Store refrigerated at -80°C.

2 mM Brefeldin A

Brefeldin A (BFA) Dilute to 5.0 mg/ml in DMSO. Store stock at -80 °C.

Block IgG:

3.0 mg/ml

Thermoblock, Eppendorf Thermostat.

Set for 4 hrs 15 minutes at 37°C on program#1 and 4°C for 24 hours on program#2. To program, Turn on instrument, press "Progr" button, toggle to P1 on display. This setup will automatically run both Program#1 and Program#2.

REVISION HISTORY:			
Rev	**Section**	**Type**	**Initials/Dates**
1	All	First edited version.	JMG / 7.5.05
2	All	Edited all sections; elaborated on missing information.	JMG / 9.13.05
3	All	Revised all sections.	JMG/ 10.10.05