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Staining Procedure For all Directly Conjugated Reagents (Whole Blood Method)

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1.0 Title

Staining Procedure for all Directly Conjugated Reagents (Whole Blood Method)

2.0 Purpose

To stain whole blood with antibodies to surface markers for flow cytometric analysis, with optional intracellular staining

3.0 Definitions and Abbreviations

PBS Phosphate buffered saline

4.0 Equipment and Reagents

4.1 Equipment

	Equipment	Vendor	Catalog #
4.1.1	12 x 75 mm tubes		
4.1.2	Centrifuge		
4.1.3	15 ml conical/centrifuge tubes		

4.2 Reagents

	Reagent	Vendor	Catalog #
4.2.1	Monoclonal antibodies	BD	
4.2.2	Heparin	American Pharma. Partners	#NDC-63323-276-02
4.2.3	Phosphate buffered saline	Invitrogen	#21300-017
4.2.4	Mouse IgG	Caltag	#10400
4.2.5	2% Para-formaldehyde, 10x10 ml, prepared	Electron Microscopy Sciences, 16% Paraformaldehyde, Dilute to 2% in PBS	15710
4.2.9	Caltag Fix and Perm Medium B	Caltag	#GAS002S
4.2.6	Ammonium chloride (NH_4Cl)	Sigma	A-5666

4.2.7	Potassium bicarbonate ($KHCO_3$)	Sigma	P-4913
4.2.8	Tetra sodium EDTA	Sigma	ED4SS

5.0 Procedures

5.1 Preparing blood, tubes, and antibodies

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

5.1.2 Prepare lysing buffer for stimulation and control samples (see Appendix).

5.1.3 Label 12 x 75 mm tubes with the donor name, date, and antibody panel (including standards). Put all tubes on ice.

5.1.4 Dispense all antibody combinations into the appropriately labeled tubes, and set aside, keeping them on ice (20 µl of antibody added to each).

5.2 Processing blood sample

5.2.1 Take 1-2 ml (or more, depending on the number of antibody cocktails being used) of blood and put it into a 15 ml centrifuge tube and top up to 15 ml with PBS containing heparin (10 µ/ml or 1 ml/litre).

5.2.2 Centrifuge at 1500 x g for 3 minutes and aspirate carefully. Mix cell suspension well. Repeat wash with PBS.

5.2.3 Add 200 µl of normal mouse IgG at 1 mg/ml (or 67µl of 3 mg/ml) for each ml of washed cell suspension and incubate on ice for 10 minutes.

5.3 Labeling and lysing procedure

5.3.1 Add 50 µl of the above suspension (5.2.3) to each of the appropriately labeled tubes (5.1.4) containing antibodies. 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.

5.3.2 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 (3200 RPM) for 3 minutes.

5.3.3 Remove caps or parafilm, decant the supernatant and blot on 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.
Add 3.5 ml PBS. Centrifuge again at 1500 x g (3200 RPM) for 3 minutes. Decant and carefully blot tubes again.

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

5.3.4 Re-suspend pellets in residual PBS.

5.3.4.1 Fix all samples with 0.5 ml 2.0% paraformaldehyde in PBS. Cells can be run on the flow cytometer after 30 minutes however, it is best to wait 6 hours for the cells to stabilize.

5.4 Optional intracellular staining

5.4.1 After completing step 5.3.4, keep the tubes at room temperature for 15 minutes (in the dark).

5.4.2 Add 50 µl Caltag Fix and Perm Medium B (NOTE: do not rack tube, mix gently).

5.4.3 At the same time add the appropriate antibody combination (intracellular), mixing gently.

5.4.4 Incubate at room temperature for 30 minutes.

5.4.5 Add 3 ml PBS and continue incubation for 10 minutes.

5.4.6 Centrifuge at 1500x g (3200 RPM), decant, blot, and add 0.5 ml of 2% paraformaldehyde. Cells can be run on flow cytometer after 30 minutes.

Appendix

Reagents

Monoclonal Antibodies

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

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 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.

Block IgG:

3.0 mg/ml

REVISION HISTORY:			
Rev	**Section**	**Type**	**Initials/Dates**
1	All	First edited version.	JMG / 8.2.05
2	All	Edited all sections; elaborated on missing information.	JMG / 9.13.05