

RPCI 004

v.002

## 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,	15710
4.2.9	Caltag Fix and Perm Medium B	Dilute to 2% in PBS Caltag	#GAS002S
4.2.6	Ammonium chloride (NH <sub>4</sub> Cl)	Sigma	A-5666

4.2.7	Potassium bicarbonate (KHCO <sub>3</sub> )	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  $\mu$ l 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 ( $\text{NH}_4\text{Cl}$ ) -- Sigma A-5666

0.5 g Potassium Bicarbonate ( $\text{KHCO}_3$ ) -- 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

