COMBINED EXTRACELLULAR AND INTRACELLULAR STAINING PROCEDURE USING ALL DIRECTLY CONJUGATED REAGENTS

1. Prepare lysing reagent :
   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 carefully the above chemicals. Pour 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.
   PREPARE FRESH SOLUTION DAILY!

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

2. Label 12 x 75 mm plastic Falcon #2054 tubes with donor name, date, antibody panel, (including standards); **put all tubes on ice.**

3. Dispense all antibody combinations into appropriately labeled tubes.

4. Put 1.5 - 2.0 ml whole blood into a 15ml centrifuge tube and q. s. to 15 ml with PBS containing heparin (10 u/ml or 1 ml/ litre).

5. Centrifuge @ 1500 x g (3200 rpm*) for 3 min., aspirate supernatant carefully. Mix cell suspension well. Repeat wash with PBS.

6. To block cell suspension add 200 **µg** of normal mouse IgG (Cal Tag 10400) at 1 mg/ml (or 67 µl of 3 mg/ml) for each ml of the washed cell suspension and incubate for 10 minutes on ice, then add 50 µl of this suspension to each of the appropriate tubes. **Rack each tube after adding.
   Incubate another 15 min. on ice.

7. Add 3.5 ml lysing reagent to each tube, put a cap on each tube (or use parafilm over all tubes), invert once and place directly into a centrifuge adaptor. Do not lyse more than 42 tubes at a time. Invert all once or twice and centrifuge @1500 x g (3200 rpm*) for 3 min. Remove caps (or parafilm), decant the supernatant and blot on teri towel three times. Rack tubes and replace in adaptor.

8. Add 3.5 ml PBS, centrifuge as above, decant, blot three times and resuspend pellets in residual PBS. Note: do not leave samples in this state, fix immediately on ice.

9. Fix all samples in 0.5 ml of 2% formaldehyde. (Polysciences, cat. # 04018 Ultrapure, E. M. Grade 10% solution. Dilute to 2% in PBS.) Samples may be run after 30 minutes, however, it is best to wait 6 hrs. for the cells to stabilize.

FOR INTRACELLUALR STAINING

10. After completing the above staining procedure, keep the tube(s) at room temperature (dark) for 15 minutes (minimum time).

11. Add 3ml of PBS, centrifuge, decant and blot. Rack tube(s).

12. Add 50ul of Permeabliization Medium B (Caltag, cat # GAS002S-5; Note: do not rack tube, mix gently)

13. At the same time add the appropriate antibody combination (again, mix gently)

14. Incubate at room temperature for 30 minutes.

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

16. Centrifuge, decant, blot and add 0.5ml of 2% formaldehyde, to fix the samples.

17. Acquire and Analyze.

* This high speed centrifugation sufficiently packs the pellet so cells are not lost during aspiration or decanting. No adverse affects have been found.

** note: for blocking only 50 µl of washed blood : use 10 µl of mIgG @ 1 mg/ml and add antibodies after 10 min. of incubation. Do not wash between adding mIg and antibodies.

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