WHOLE BLOOD PROTOCOL FOR INTRACELLULAR CYTOKINES BY FLOW CYTOMETRY

Stimulation & Staining Protocol - Human Whole Blood

VERY IMPORTANT: blood must be collected into *heparinized* tubes

1. Aliquot 0.5 ml heparinized, whole blood into each of two 5 ml, 12 x 75 mm snap cap tubes. Add 0.5 ml cell culture medium to each tube to bring the volume to 1 ml.
2. To the first tube add 10 μg Brefeldin A (20 μl of a stock solution of Brefeldin A at a concentration of 0.5 mg/ml in 100% EtOH and stored at -20˚C). Mix contents of tube gently. Label this tube as the "Resting" cell population.
3. To the second tube add 10 μg Brefeldin A, 25 ng PMA (2.5 μl of a stock solution of PMA at a concentration of 1 mg/ml in DMSO and diluted 1:100 in cell culture medium and stored at -20˚C), and 1 mg ionomycin (1 μl of a stock solution of ionomycin at a concentration of 1 mg/ml in DMSO and stored at -20˚C). Mix contents of tube gently. Label this tube as the "Activated" cell population.
4. Incubate both tubes for 4 hours at 37˚C in 7.5% humidified CO_2 incubator.
5. At the end of the incubation period mix cells again. For each sample to be analyzed, aliquot 100 μl of the prepared cell suspension into an appropriate 5 ml, 12 x 75 mm snap cap tube.
6. Add the recommended amount of conjugated antibody directed to the cell surface immunophenotyping marker of interest (e.g. CD3 TRI-COLOR® (TC)).
7. Vortex each tube gently to mix and incubate for 15 minutes in the dark at room temperature.
8. Add 100 μl of Reagent A (Fixation Medium) from the FIX & PERM® kit (catalog no. GAS-003 or GAS-004). Mix cells gently and incubate for an additional 15 minutes in the dark at room temperature.
9. Wash twice with wash medium (PBS + 0.1% NaN_3 + 5% FBS). Use caution as the cell pellet becomes less cohesive on the second wash.
10. To the washed cells add 100 μl of Reagent B (Permeabilization Medium) from the FIX & PERM® kit and the recommended amount of anti-cytokine antibody(ies) or the corresponding isotype control(s).
11. Vortex 1-2 seconds and incubate for 20 minutes in the dark at room temperature.
12. Wash twice with wash medium and resuspend the cells in 0.1% paraformaldehyde.
13. Analyze cells by flow cytometry within 18 hours.
    Advisory: Analyze by gating on SSC verses FL3 (or the appropriate TC channel).

Sample staining protocol for CD3 TC, IFN-γ FITC & IL-4 R-PE:

Tube #	Cell	Antibody
1	Resting	None
2	Resting	Mouse IgG2a-TC isotype control (for CD3)
3	Resting	CD3-TC
4	Resting	CD3-TC + Mouse IgG1-FITC + Rat IgG1-PE isotype controls (for anti-cytokine abs)
5	Resting	CD3-TC + anti-IFN-γ-FITC + anti-IL-4-PE
6	Activated	None
7	Activated	Mouse IgG2a-TC
8	Activated	CD3-TC
9	Activated	CD3-TC + Mouse IgG1-FITC + Rat IgG1-PE
10	Activated	CD3-TC + anti-IFN-γ-FITC + anti-IL-4-PE