RPCI FCM Surface Marker and Intracellular Staining

Primary monoclonal antibodies (mAb): usually use 1X or 10X culture supernatant; 1:1000 or greater dilution of ascites; or purified mAb at 1μg/test (1-30 μg/ml final concentration). These are approximate specifications only, each mAb must be tested to determine that the optimal or saturating concentration for any particular application is used.

REAGENTS

Complete Media (CM)

RPMI 1640, 500 ml
FCS (10%), 50 ml
25 mM HEPES, 12,5 ml of 1 M Stock
2.0 mM L-glutamine, 5 ml of 100X stock
0.1 mM Na Pyruvate, 5 ml of 100X stock
0.1 mM nonessential Amino Acids, 5 ml of 100X stock
Gentamicin, 0.5 ml of 50 mg stock

Staining buffer:

Phosphate buffered saline
Heat inactivated fetal calf serum
Sodium azide (Fisher 5227)

Dissolve in 1 liter of 1X PBS, 10 ml FCS, and 1 g sodium azide. Filter through 0.45 μm membrane and store at 4-8˚C, this solution is stable for 6 months.
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4% Fixation Buffer for Fix&Perm

Phosphate buffered saline
EM grade 10% buffered Formalin (Polysciences 08379)

Prepare 4% formalin fixation buffer by diluting 40 ml of 10% EM grade formalin in 60 ml of 1X PBS. Check pH and adjust to 7.4-7.6. Store at 4-8˚C, protected from light.

Permeabilization Buffer

Phosphate buffered saline
Heat inactivated fetal calf serum
Sodium azide (Fisher 5227)
Saponin (Sigma S-7900)

Dissolve in 1 liter of 1X PBS, 10 ml FCS, 1 g sodium azide and 1 g saponin. Check pH and adjust to 7.4-7.6. Filter through 0.45 μm membrane and store at 4-8˚C, this solution is stable for 6 months.

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

PMA (Sigma, P-8139)

Resuspend 1 mg of PMA in 1.0 ml of DMSO. Store in aliquots, frozen at -20ºC

1 mM Ionomycin

Ionomycin (Sigma I-0634, FW=747.1)

Resuspend 1 mg of Ionomycin in 1.34 ml of H_2O. Store refrigerated at 4ºC for less than a month or at -20ºC for longer periods.

2 mM Monensin

Monensin (Sigma M-5273, FW=692.9)
Prepare 2 mM stock by dissolving 1.386 mg/ml in 100% ethanol. Then add 1 μl/ml of 1N NaOH. Store tightly capped and refrigerated.

Block IgG:

12 mg/ml human IgG Cohn fraction II and III globulins (Sigma G-4386)
Dilute in Staining buffer and store frozen. Dilute 1:2 in Staining buffer before use.

For mouse cells mAb 2.4G2 (Pharmingen 01241D) which blocks FcγRII and FcγRIII receptors should be used instead of Block IgG

1% Formalin (for short term storage of cells)

Phosphate buffered saline
EM grade 10% buffered Formalin (Polysciences 08379)

Prepare 1% formalin buffer by diluting 10 ml of 10% EM grade formalin in 90 ml of 1X PBS. Check pH and adjust to 7.4-7.6. Store at 4-8˚C, protected from light. Alternatively dilute 4% solution made above in PBS (protein free) to 1%.

CELL ACTIVATION

1. Divide sample to be assessed into two tubes, one tube for resting and one tube for activated cells. We routinely use Ficol-Hypaque separated PBMC and culture these cells in CM at 2.5 - 5 X 10^6 cells/ml in a 50 ml polyproplene tube.

2. Add 2mM Monensin, 1 μl/ml of cells, for a final concentration 2 μM, to both the resting and activated cells. This inhibits intracellular transport of antigens and cytokines produced during activation. (Alternatively, add Brefeldin A from Sigma B-7551 at 2-10 μg/ml).

3. To activate the cells, add the follwoing:
   a) 20 ng/ml of PMA by diluting stock 1 mg/ml PMA 1:1000 in CM and then adding 20 μl of this for each ml of cells.
   b) 1 μl of 1 mM Ionomycin stock for each ml of cells.

4. Cap tubes loosely, gently mix and incubate at 37ºC, 5% CO_2 for 4 hours.

ANTIBODY STAINING

Surface Antibody Staining

All surface and intracellular staining procedures are done on ice using cold buffers and a chilled centrifuge throughout.

1. Wash cells once in CM to remove activation reagents, count and resuspend at 10 x 10^6 cells /ml in IgG block (diluted 1:2 in Staining buffer, final concentration 6 mg/ml)
2. Label 96 well plate, and add 25 µl of cells to appropriate wells.
3. Incubate on ice 10 minutes.
4. Label surface antigens by adding 25 μl of directly conjugated antibody diluted to approximately 40 μg/ml.
5. Incubate on ice 30 minutes.
6. Wash 2 times with 175 μl changes of Staining Buffer and pellet. (For extracelluar only studies, resuspend in 1% formalin and store at 4°C protected from light.)

Fix and Perm for Intracellular Antibody Staining

1. Thoroughly resuspend cells in 100 μl of 4% Fixation buffer. To avoid aggregation of cells caused by fixation, the cells should be thoroughly de-aggregated by pipeting.
2. Incubate on ice for 20 minutes.
3. Wash cells in 2 times with 175 μl changes of Permeabilization buffer.
4. Resuspend cells in 50 μl of Permeabilization buffer containing the appropriately diluted directly conjugated anti-cytokine antibody (approximately 20 µg/ml). Saponin acts in a reversible manner and must be maintained in these washing and intracellular labeling steps to keep cells permeable.
5. Incubate on ice 30 minutes.
6. Wash 2 times with 175 μl changes of Permeabilization buffer and 1 time with 175 μl of Staining Buffer to seal in anti-cytokine fluorescene. Resuspend in Staining Buffer and analyze immediately or resuspend in 1% formalin and store at 4°C protected from light for later analysis.

Staining Controls

1. Isotype matched controls
2. Blocking control, preincubate Fix & Permeabilized cells with an excess of cold un-labeled antibody from the same clone as the fluorochrome labeled anti-cytokine antibody. 10X the concentration of the fluorochrome labeled anti-cytiokine should be a sufficient amount of cold antibody.