STAINING PROCEDURE FOR CORRELATION OF SURFACE ANTIGEN EXPRESSION SIMULTANEOUSLY WITH DNA CONTENT

MATERIALS:

1. 2% formaldehyde solution (see preparation methods below)

2. 1 X PBS (without sodium azide and serum)

3. 1 X PBS + 2% newborn calf serum + sodium azide (buffer)

4. Polyoxyethylensorbitan monolaureate (Tween 20, e.g., Cat#1379 from Sigma-Aldrich, St. Louis, MO)

5. Human AB serum (HAB, heat-inactivated, e.g., from Innovative Research, MI)

6. Propidium iodide (PI, e.g., Cat# 537059 EMD Millipore, MA)

7. Ribonuclease A (RNase, e.g., Cat# R4875 Sigma-Aldrich, St. Louis, MO)

8. 7-amino-Actinomycin-D (7-AAD, e.g., Cat #129935, EMD Millipore, MA)

9. 37°C waterbath, refrigerated centrifuge.

METHOD:

Staining of surface antigens

1 x 10^6 PBS-washed cells from a single cell suspension are pelleted in a 12 X 75 mm culture tube. Then the staining proceeds as described in A1-3 for antibodies whose fluorochrome emission profile is compatible with PI, e.g., FITC, Alexa488, and in B1-3 for staining with antibodies whose fluorochromes are compatible with 7-AAD, e.g., FITC, Alexa488, PE, APC.

Fixation

The pellet is re-suspended in 0.875 mL of cold PBS and the suspension is mixed gently. Then, 0.125 mL of cold 2% formaldehyde solution is added and the mixture is immediately vortexed briefly. The suspension is incubated for at least 30 min or for up to 1 h at 4°C, centrifuged for 5 min at 250g, then the supernatant is removed.

Permeabilization

The pellet is gently re-suspended in room temperature Tween 20 solution (0.2% in PBS) and the mixture is incubated for 15 min in a 37°C water bath. One ml of buffer is added and the suspension is spun for 5 min at 250g, then the supernatant is removed.

Staining of DNA

Proceeds as described in A5 for PI staining and in B5 for staining with 7-AAD.

A. Procedure for staining of surface antigens with antibodies conjugated to fluorochromes compatible with DNA staining with PI

1. Add 50 µL of HAB to your cell pellet, mix and wait for approximately 1 min.

2. Add 50 µL of buffer and the appropriate amount of antibody, vortex briefly and incubate for 30 min at 4°C in the dark.

3. Wash twice with 1 mL of buffer by centrifugation at 250g for 5 min.

4. Fix and permeabilize as described under METHOD.

5. Resuspend samples in 0.5 mL of buffer containing 10 µg/mL of PI and 11.25 Kunitz units of RNase and incubate for at least 30 min at 4°C in the dark. Keep them in the staining solution until analysis on the flow cytometer.

B. Procedure for staining of surface antigens with antibodies conjugated to fluorochromes compatible with DNA staining with 7-AAD

1. Add 50 µL of HAB to your cell pellet, mix and wait for approximately 1 min.

2. Add 50 µL of buffer and the appropriate amount of antibody, vortex briefly and incubate for 30 min at 4°C in the dark.

3. Wash twice with 1 mL of buffer by centrifugation at 250g for 5 min.

4. Fix and permeabilize as described under METHOD.

5. Resuspend samples in 1 mL of buffer containing 10-25 µg/ml of 7-AAD and incubate for at least 20 min at 4°C in the dark. Keep them in the staining solution until analysis on the flow cytometer.

Note: it is not essential to use 50% HAB for surface staining, but it can prevent problems with Fc binding of antibodies. However, do not use HAB for staining of immunoglobulin chains and when you are using antibodies that are directed against Fc receptors (e.g. CD16).

7-AAD has to be used for DNA staining in combination with PE-labeled antibodies due to the spectral emission overlap between PE and PI.

PREPARATION OF 2% FORMALDEHYDE STOCK SOLUTION (2 METHODS)

METHOD 1:

Formaldehyde preservative – 2% formaldehyde solution in protein-free PBS.

Prepare as follows:

10% formaldehyde*	20 ml
10 x PBS	10 ml
Distilled water	70 ml

* 10% formaldehyde solution (e.g., Polysciences Inc., Warrington, PA, ultrapure, Cat.#04018), depolymerized paraformaldehyde, EM grade, methanol-free solution.

METHOD 2:

Formaldehyde preservative – 2% formaldehyde solution in protein-free phosphate-buffered saline (PBS).

Prepare as follows:

Add 2 g paraformaldehyde powder (e.g., Sigma-Adrich, St. Louis, MO) to 100 mL of 1 X PBS. Heat to 70°C (do not exceed this temperature) in a fume hood until the paraformaldehyde goes into solution (note that this happens quickly as soon as the suspension reaches 70°C). Allow the solution to cool to room temperature. Adjust to pH 7.4 using 0.1 M NaOH or 0.1 M HCl, if needed. Filter and store at 4°C protected from light.