1. Harvest cells by adding a solution of 1 mM EDTA/1 mM EGTA in PBS (calcium- and magnesium-free) accompanied by gentle rocking to remove the cells from the plate. Centrifuge at 1000*g* at 4°C for 5 minutes.

Keep on ice throughout the remainder of the protocol.

This procedure may take longer than normal trypsinization. For cells growing attached as a monolayer, trypsin cannot be used because it may strip receptors from the cell surface.

2. Wash cells in 4 ml of PBS + 0.1% sodium azide and 1% BSA or heat-inactivated serum.

The addition of azide is optional. The chemical can stop internalization of receptors (which can also be prevented by keeping samples very cold on ice throughout the procedure), but it can also be toxic to living cells.

3. Count the cells using a minimum concentration of 1×10^6 cells/ml for each sample.

Volumes can be adjusted depending on the number of available cells and the quantities and cost of antibodies. In most cases, final volume should not be less than 200 µl with a minimum concentration of 2×10^5 cells.

4. Aliquot cells for staining into small sample volumes (50 µl) of PBS containing 0.1% sodium azide and 1% BSA or 1% heat-inactivated serum.

5. Add the proper dilution of primary antibody, using a series of dilutions to determine the appropriate range.

ALL antibodies used for flow cytometry analysis should be carefully titrated to a constant cell number for best results. Dilutions may be quite different from those used for fluorescent microscopy. Start by following the manufacturer's recommended dilution, such as 1:50, and then try 1:10, 1:25, 1:75, and 1:100. It may be necessary to perform a second series of dilutions after the general range is found.

USE OF TWO-COLOR ANALYSIS: If analyzing for two or more proteins, more than one directly labeled antibody can be added in this step

6. Incubate samples on ice for 30 minutes.

Protect samples from light throughout remainder of protocol.

7. Wash cells in 4 ml of PBS containing sodium azide and BSA or heat-inactivated serum.

8. Centrifuge cells at 1000*g* for 5 minutes.

9. Resuspend cells in an appropriate volume of PBS containing 0.1% sodium azide and 1% BSA or heat-inactivated serum to give a final concentration of 1×10^6 to 5×10^6 cells/ml.

Samples are now ready for analysis or can be fixed and stored at this point, if necessary, by the addition of an equal volume of 2% paraformaldehyde in PBS. Remember that this addition changes the total volume of the sample and, therefore, the concentration of cells.