Indirect Staining of Cell Surface Markers using Purified Antibodies or murine Ig containing Fusion Proteins for Analysis by FACS

Rev 1624

Introduction

For this assay, cells in suspension are bound with primary reagent: antibody or recombinant protein; washed and then incubated with a secondary fluorescent reagent which binds to the primary. Ancell catalog # 232-011 Goat anti-Mouse/FITC is a good choice for a secondary reagent to detect binding of Mouse IgG and IgM isotype antibodies, as well as for detection of muIg recombinant fusion proteins [These contain the Fc and Hinge regions of Mouse IgG2a]. Other anti-Mouse Ig polyclonal or monoclonal reagents may be suitable as long as they detect the appropriate species and isotype of the primary reagent and are titered for the application. [Ancell also provides anti-Mouse CD8 alpha fluorescent reagents for detection of our muCD8 recombinant proteins: cat #260-040, 260-050]

There are a number of factors to consider that can affect the results of this assay. These are: target cell type and number; antigen density, amount of antibody or recombinant protein added, and total incubation volume. Other considerations include incubation time and temperature, antibody or binding protein affinity and avidity, non specific binding potential [Fc receptors], and debris in the sample.

This procedure is a general guideline only. For optimal results, all parameters may need to be optimized for a specific application.

Ideally, several amounts of primary reagent should be tested to ensure optimal performance for the cell types being targeted. In some instances, exceeding saturating levels of primary and secondary reagents may result in non specific binding or reduced specific binding [prozone effect].

Materials:

- Appropriate viable, expanded suspension cell cultures, ficoll prepared PBMC, or dissociated adherent cells from culture
- Antibody or Recombinant protein for primary incubation
- Appropriate secondary detection reagent [Goat anti-Mouse/FITC, Goat anti-Mouse/PE, etc]
- FACS buffer: PBS (10mM Sodium Phosphate, 150mM Sodium Chloride pH 7.2-7.5); 1% BSA, 0.05% Sodium Azide; Optional : 1 to 5% Bovine (or Equine) Serum *Store buffer up to 6 weeks at 4^{o}C*
- Centrifuge able to handle cell preparation tubes and 12x 75mm FACS tubes
- Ice bucket
- FACS tubes: 12x75 non sterile (uncapped) polystyrene tubes (Falcon #2008)
- Glass 12 x 75mm test tubes
- P-1000, P-200, P-20 Micro pipettors with appropriate sterile or non sterile tips

Optional Materials:

- 2% formaldehyde in PBS *To fix cells for storage prior to analysis*
- Technical grade Human IgG (Sigma cat.# I8640) at 10 mg/ml. *This is useful to block and adsorb non specific activity.*
- Repeating pipettor with dispensing tips *For dispensing cells in larger experiments*
- Parafilm *To seal tubes for storage prior to analysis*
- Heavy duty aluminum foil *To protect tubes from light during storage prior to analysis*

Procedure

1) ***Prepare Primary incubation using EITHER Step 1a OR Step 1b***

1a) Add concentrated stock directly to cells in suspension

Cell preparation: Count cells or cell lines. Add a suspension of 5 x 10^5 cells per tube in a volume of 100 ul total volume in FACS tubes. [If you choose to use a larger final volume, it may be helpful to increase the incubation time and/or use more reagent]

Optionally add human IgG [Sigma cat.# I8640, 10mg/ml stock concentration] to a final concentration of 60ug/ml to decrease non specific binding for some cell types.

Add antibody or recombinant protein to obtain the suggested concentration indicated on the Product Insert Sheet . [This is often 5 – 10 ug/ml final concentration, requiring the addition of 0.5 – 1 ul of a 1 mg/ml stock of concentrated reagent per tube]

Example: To stain a tube containing **5 x 10^5 cells in 100ul** with anti-CD3 antibody [Ancell Cat# 144-020, 1 mg/ml stock concentration]:
Optionally add 6ul of human IgG
Pre incubate 5 minutes.
Add **0.5ul** of anti-CD3 antibody to the cell suspension for a **5 ug/ml** final concentration.

OR 1b) Add diluted reagent to cell pellet

Prepare reagent dilutions in FACS buffer to obtain the working concentration indicated on the Product Insert Sheet.
This is often 5 – 10 ug/ml final concentration, a 1:200 or 1:100 dilution in FACS buffer.
Make up 100 ul per tube to be analyzed. *80 ul will be added per tube.*

Cell Preparation: dispense 5 x 10^5 cells per tube into FACS tubes. Centrifuge at 500 x G for 5 minutes. Carefully dump tubes into a waste tray, blot on clean, low-lint paper towels.

Optionally add 20ul of human IgG diluted to 300ug/ml in FACS buffer to cell pellets. Vortex briefly. *This step may decrease non specific binding for some cell types.*

Add 80ul of diluted reagent to each tube to be analyzed.

Example: To stain a tube containing **5 x 10^5 pelleted cells** with anti-CD3 antibody [Ancell Cat# 144-020, 1 mg/ml stock concentration]:

Optionally prepare a sub stock of human IgG in FACS buffer at 300ug/ml by adding 1ul of human IgG [from a 10 mg/ml stock] to 32ul FACS buffer. Add 20 ul of this sub stock to pelleted cells. Pre incubate for ~5 minutes at 4^{o}C.

Prepare a sub stock of primary reagent: Add **0.5ul** of anti-CD3 antibody [from 1 mg/ml stock] to **99.5 ul** FACS buffer to obtain **100 ul at 5 ug/ml**.
Add **80 ul** of this sub stock to cells in tube.

2) Incubate 45 minutes on ice or at 4^{o}C.

3) Wash (2X)
Add 0.5 ml FACS buffer to tubes, Centrifuge at 500 x G for 5 minutes. Carefully dump tubes into a waste tray, blot on clean low lint paper towels, and repeat this wash once more dumping and blotting.
2 washes total

4) Secondary Incubation
Prepare a sub stock of appropriately diluted solution of secondary detection reagent.

Add appropriate volume of diluted secondary reagent to pelleted cells in each tube.

Example: To stain 2 tubes containing 5 x 10^5 cells each, prepare a sub stock of GAM/FITC [cat #232-010] at a **1:60** dilution: **Add 2 ul** of concentrated stock **to 118ul** of FACS buffer for a final sub stock volume of **120 ul**.

Optionally include 3.6 ul of human IgG [Sigma cat.# I8640, 10mg/ml stock concentration] to adsorb anti-human IgG cross reactivity [300 ug/ml final concentration in sub stock].

Add 50 ul of GAM/FITC sub stock to pelleted cells in each tube.

Incubate 30 minutes on ice.
Take reasonable steps to protect fluorescent reagents from light.

When observing cell types expressing human surface immunoglobulin, it may be helpful to adsorb anti-human cross reactive activity from Polyclonal anti-Mouse Ig secondary reagent. This can easily be done by adding human IgG to a final concentration of 50 – 100ug/ml to the diluted secondary reagent sub stock, and pre incubating the sub stock > 5 minutes on ice before adding to cells.

5) Wash (2X)
Wash 2 (or optionally 3) times as in step 3. Resuspend cell pellet in 300ul FACS buffer for immediate analysis or 300ul 2% formaldehyde/PBS for storage prior to analysis. Vortex well.
For storage of fixed cells, cover tubes with parafilm and aluminum foil to protect from light. Keep at 4^{o}C until analysis, up to 4 days later