Direct Staining of Cell Surface Markers using Fluorescent Labeled Antibodies or Recombinant Proteins for Analysis by FACS

Rev 1625

Introduction

For this assay, cells in suspension are bound with fluorescent conjugated antibody or recombinant protein; washed and then optionally fixed for storage prior to analysis. Ancell manufactures high quality Fluorescein (FITC), R-Phycoerythrin (R-PE) and Allophycocyanin (APC) conjugated forms of antibody and recombinant protein products.

There are a number of factors to consider that can affect the results of this or any binding 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 conjugated reagent should be tested to ensure optimal performance for the cell types being targeted. In some instances, exceeding saturating levels may result in non specific binding or reduced specific binding due to prozone effect.

Isotype matched controls should be used as a background for comparison to identify non specific binding on some cell types. Ancell offers a complete line of fluorescent-labeled Mouse Immunoglobulin Isotype Controls

Another good option to confirm specific binding of a marker is the use of an unlabeled antibody as a pre block control. Including an excess of unlabeled antibody in the binding reaction effectively competes with conjugated reagent for cell surface sites, reducing or eliminating the fluorescent signal. *Note: Antibody pre blocking may not be effective with large, multi-valent IgM conjugates.*

Materials:

- Appropriate viable, expanded suspension cell cultures, ficoll prepared PBMC, or dissociated adherent cells from culture
- Fluorescent labeled Antibody or Recombinant protein
- 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.*
- Unconjugated specific antibody. *For use as a pre blocking control*
- 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 Incubation using EITHER Step 1a OR Step 1b* *Take reasonable steps to protect fluorescent reagents from light.*

1a) Add concentrated stock of fluorescent conjugate directly to cells in suspension

Cell preparation: Count cells or cell lines. Add a well mixed suspension of 5 x 10^5 cells per tube in a volume of 80 - 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. At this step, a specific blocking control antibody could also be added to a final concentration of 50 – 150 ug/ml.

Add fluorescent conjugate to obtain the suggested concentration indicated on the Product Insert Sheet . This typically requires the addition of **1.6 ul of a 50X** stock of concentrated reagent per tube.

Example: To stain a tube containing **5 x 10^5 cells in 80 ul** with anti-CD3/R-PE conjugate [Ancell Cat# 144-050, 0.5 mg/ml stock concentration]:
Optionally add 6ul of human IgG stock to reduce non specific binding.
Optionally add unlabeled anti-CD3 mAb (cat # 144-020) to a final concentration of 100ug/ml for blocking control tubes.
Pre incubate cells with pre blocks 5 minutes at 4^{o}C.
Add **1.6 ul** of anti-CD3/R-PE to the cell suspension for a **10 ug/ml** final 1X 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 typically a 1:50 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 the supernatant from 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.* At this step, a blocking control antibody could also be added: Prepare an unlabeled antibody sub stock at 0.5 mg/ml, add 20 ul to cell pellet.

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

Example: To stain a tube containing **5 x 10^5 pelleted cells** with anti-CD3/R-PE [Ancell Cat# 144-050, 50X, 0.5 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°C. Optionally prepare and add 20 ul of unlabeled anti-CD3 antibody at 0.5 mg/ml to specific blocking control tubes.

Prepare a sub stock of primary reagent: Add **2 ul** of anti-CD3/R-PE (from 50X 0.5 mg/ml stock) to **98 ul** FACS buffer to obtain **100 ul at 1X** (5 ug/ml).
Add **80 ul** of this sub stock to cell pellet in tube.

2) Incubate Cells and Fluorescent Reagents 45 minutes on ice or at 4°C. *Protect from Light*

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) Resuspend or Fix Stained Cells
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°C until analysis, up to 4 days later