
FITC conjugation of Antibodies

Overview:

Fluorescein (mistakenly abbreviated by its commonly-used reactive isothiocyanate form, FITC) is currently the most commonly-used fluorescent dye for FACS analysis. FITC is a small organic molecule, and is typically conjugated to proteins via primary amines (i.e., lysines). Usually, between 3 and 6 FITC molecules are conjugated to each antibody; higher conjugations can result in solubility problems as well as internal quenching (and reduced brightness). Thus, an antibody will usually be conjugated in several parallel reactions to different amounts of FITC, and the resulting reagents will be compared for brightness (and background stickiness) to choose the optimal conjugation ratio. Fluorescein is typically excited by the 488 nm line of an argon laser, and emission is collected at 530 nm.

Refer to notes about the following procedures used by this protocol:

Column chromatography

Reagent storage

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Conjugation protocol.

The entire conjugation can be performed in about a half-day. In addition to the materials listed below, you will need to have a solution of your antibody at a concentration (optimally) of at least 2 mg/ml. The extent of FITC conjugation to the antibody may depend on the concentration of antibody in solution; for consistent conjugations, use a consistent concentration. You should be familiar with how to use a desalting column and how to take absorbance spectra.

The reactive fluorescein molecule, fluorescein isothiocyanate, is unstable. Once a vial has been cracked and the FITC solubilized, it should be used immediately. Since single vials of FITC contain sufficient material for ~100 mgs of antibody, it is economical to perform multiple FITC conjugations on the same day.

When first conjugating an antibody, a range of FITC to antibody concentrations should be compared. The protocol suggests 40-80 μg per mg of antibody; for a first-time titration of FITC, try a range of 10 to 400 μg FITC per mg of antibody (for instance, 10, 40, 80, 160, 320 μg per mg). Compare each conjugate by staining (you should perform a titration of antibody on cells for each reagent to determine the optimal staining concentration). Select the conjugate with the brightest "positive" cells which still has low background on "negative" cells.

I. Preparation of antibody

Note: it is critical that sodium azide be completely removed from any antibody: it will react with the FITC and prevent conjugation.

Dialyze or exchange over a column the antibody in "Reaction Buffer". Note that the BioRad protein reagent kit reacts spontaneously in "Reaction Buffer"; it is difficult (but not impossible) to determine which column fractions contain the protein by this method... use of a spectrophotometer is preferred. See hints on column separations of nonfluorescent proteins.

Measure the antibody concentration after buffer equilibration. (For IgG, 1 mg/ml has an A(280) of 1.4). If the antibody concentration is less than 1 mg/ml, the conjugation will probably be sub-optimal. If necessary, dilute the antibody to a concentration of 4 mg/ml.

II. Covalent conjugation

FITC is covalently coupled to primary amines (lysines) of the immunoglobulin.

Dissolve 10 mgs (the entire contents of 1 vial; no need to weigh) of FITC in 1 mL anhydrous DMSO immediately before use.

Add FITC to give a ratio of 40-80 μ g per mg of antibody; mix immediately. (See notes above about using different molar ratios of FITC to antibody).

Wrap the tube in foil; incubate and rotate at room temperature for 1 hour.

Remove the unreacted FITC and exchange the antibody into "Storage Buffer" by gel filtration or dialysis.

III. Characterizing the conjugate

Determine F/P and protein concentration by measuring the absorbance at 280 and 495 nm.

IgG: 1 mg/ml has an A(280) of 1.4; mw = 150,000

IgM: 1 mg/ml has an A(280) of 1.2; mw = 900,000

Fluorescein: 1 mM has an A(495) of 68 and an A(280) of 11.8.

F/P values of 3-10 are probably optimal for any particular IgG.

Protein concentration:

IgG (mg/ml) = [A(280) - 0.31 * A(495)] / 1.4

IgM (mg/ml) = [A(280) - 0.31 * A(495)] / 1.2

F/P ratio:

IgG: $3.1 * A(495) / [A(280) - 0.31 * A(495)]$

IgM: $15.9 * A(495) / [A(280) - 0.31 * A(495)]$

Materials, Chemicals, and Buffers

Materials:

For column separations, we often use one of two types of pre-poured columns:

For 1.25ml to 2.5ml sample volumes: PD-10 (Sephadex G-25M).

For <0.5 ml sample volumes: NAP5 columns (Sephadex G-25 DNA grade).

Chemicals:

FITC - fluorescein isothiocyanate

DMSO - anhydrous dimethyl sulfoxide

Note: keep the DMSO absolutely dry at all times. We keep the bottle in a dessicator. Pour out an amount of DMSO sufficient for your need and then pipette that; don't pipetter directly into the bottle.

NaHCO₃ - sodium bicarbonate, mw 84.01

NaCO₃ - sodium carbonate, mw 106

NaCl - Sodium Chloride, mw 58.44

TRIZMA pre-Set crystals 8.0 - Combination of Tris base and TrisHCl, average mw 141.8

NaN₃ – Sodium Azide, mw 65

Buffers:

"Reaction Buffer"
500 mM carbonate, pH 9.5

To make 1 Liter:

17g Na₂CO₃

28g NaHCO₃

pH to 9.5

Note: sodium azide cannot be added to this buffer

"Storage Buffer"

10 mM Tris, 150 mM NaCl, 0.1% NaN₃, pH 8.2

To make 1 Liter:

1.42g TRIZMA 8.0

8.77g NaCl

1g NaN₃

pH to 8.2

See hints on storing buffers.

References and credits:

This protocol is based on an original protocol devised by Aaron Kantor.