

## **FITC conjugation of Annexin V**

### **Overview:**

This protocol serves not only to describe the conjugation of FITC to Annexin V, but to serve as a model for FITC conjugation of any protein. The molecular weight of Annexin V is about 40 kDaltons. When conjugating other proteins, take into account the relative molecular weight. Always perform a titration of FITC to protein ratios when first conjugating a protein; too much FITC can desolubilize the reagent or interfere with its activity, whereas too little may result in undetectable fluorescence.

This protocol is nearly identical to that of conjugation of FITC to immunoglobulins. The only difference arises from the much smaller size of Annexin V compared to IgG (one-fourth the molecular weight), necessitating a difference in the amount of FITC to use. Otherwise, the same procedures and steps are taken, and the protocol below is essentially identical to the Ig conjugation protocol. Annexin V can be purchased from some commercial sources or obtained in collaborative agreements with researchers who make recombinant protein.

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, only a few FITC molecules are conjugated to the protein; higher conjugations may result in solubility problems as well as internal quenching (and reduced brightness). Thus, you should probably try several parallel reactions using different amounts of FITC, and compare the resulting reagents 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 Annexin V at a concentration (optimally) of at least 2 mg/ml. The extent of FITC conjugation to the Annexin V may depend on the concentration of Annexin V 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 Annexin V, a range of FITC to antibody concentrations should be compared. The protocol suggests 10-20  $\mu\text{g}$  per mg of Annexin V; for a first-time titration of FITC, try a range of 2 to 100  $\mu\text{g}$  FITC per mg of Annexin V (for instance, 2, 5, 10, 40, 100  $\mu\text{g}$  per mg). Compare each conjugate by staining (you should perform a titration of Annexin V 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 Annexin V

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

Dialyze or exchange over a column the Annexin V 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 Annexin V concentration after buffer equilibration. If the Annexin V concentration is less than 1 mg/ml, the conjugation will probably be sub-optimal. If necessary, dilute the Annexin V to a concentration of 4 mg/ml.

## II. Covalent conjugation

*FITC is covalently coupled to primary amines (lysines) of the Annexin V.*

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

Add FITC to give a ratio of 10-20  $\mu\text{g}$  per mg of Annexin V; mix immediately. (See notes above about using different molar ratios of FITC to Annexin V).

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

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

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## 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.

NaHCO<sub>3</sub> - sodium bicarbonate, mw 84.01

NaCO<sub>3</sub> - 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

NaN<sub>3</sub> – Sodium Azide, mw 65

### **Buffers:**

"Reaction Buffer"

500 mM carbonate, pH 9.5

To make 1 Liter:

17g Na<sub>2</sub>CO<sub>3</sub>

28g NaHCO<sub>3</sub>

pH to 9.5

*Note: sodium azide cannot be added to this buffer*

"Storage Buffer"

10 mM Tris, 150 mM NaCl, 0.1% NaN<sub>3</sub>, pH 8.2

To make 1 Liter:

1.42g TRIZMA 8.0

8.77g NaCl

1g NaN<sub>3</sub>

pH to 8.2

See hints on storing buffers.

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### **References and credits:**

This protocol was developed and tested by Peter Katsikis and Mario Roederer, based on our standard FITC conjugation protocol.