Cascade Blue conjugation of Annexin V

Overview:

This protocol serves not only to describe the conjugation of Cascade Blue to Annexin V, but to serve as a model for Cascade Blue 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 Cascade Blue to protein ratios when first conjugating a protein; too much Cascade Blue 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 Cascade Blue 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 Cascade Blue to use. Otherwise, the same procedures and steps are taken, and the protocol below is essentially identical to the Ig conguation protocol. Annexin V can be purchased from some commercial sources or obtained in collaborative agreements with researchers who make recombinant protein.

Cascade Blue is a UV-excitable dye that can be used for immunofluorescence labeling. When used with the 351/361 nm excitation lines of an Argon laser, it is not very bright; usually only extremely high density antigens can be well-resolved by Cascade Blue. However, when used with the 405 nm excitation line of a Krypton laser, it becomes a useful dye with a brightness approaching that of fluorescein. Emission is collected at 440 nm.

Refer to notes about the following procedures used by this protocol:
Column chromatography
Reagent storage

Contents:

Conjugation protocol

I. Preparation of antibody
II. Covalent conjugation

Materials, chemicals, and buffers
References

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 Annexin V at a concentration (optimally) of at least 2 mg/ml. The extent of Cascade Blue conjugation to the Annexin V 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 Cascade Blue molecule is unstable. Once the Cascade Blue is solubilized, it should be used immediately.

When first conjugating Annexin V, a range of Cascade Blue to Annexin V concentrations should be compared. The protocol suggests 50 µg per mg of Annexin V; for a first-time titration of Cascade Blue, try a range of 10 to 200 µg Cascade Blue per mg of antibody (for instance, 10, 20, 40, 100, 200 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 Annexin V

Note: it is critical that sodium azide be completely removed from any Annexin V.

Dialyze or exchange over a column the Annexin V in "B Reaction Buffer".

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

Cascade Blue is covalently coupled to primary amines (lysines) of the Annexin V.

Dissolve 5 mgs of Cascade Blue in 500 µl anhydrous DMSO immediately before use. This is tedious and takes a bit of vortexing and time.

Add Cascade Blue to give a ratio of 50 µg per mg of Annexin V; mix immediately. (See notes above about using different molar rations of Cascade Blue to Annexin V).

Wrap the tube in foil; incubate and rotate at room temperature for 4 hours.

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

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:

Cascade Blue acetyl azide, trisodium salt, mw 607.42

SMCC - succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, mw 334.42

NEM – N-Ethylmaleimide, mw 125.1

DMSO - anyhydrous 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.*

$NaHCO_3$ - sodium bicarbonate, mw 84.01

$NaCO_3$ - 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_3 – Sodium Azide, mw 65

Buffers:

"B Reaction Buffer"
100 mM carbonate, pH 8.4

To make 1 Liter:
84g $NaHCO_3$
pH to 8.4
Note: sodium azide cannot be added to this buffer

"Storage Buffer"
10 mM Tris, 150 mM NaCl, 0.1% NaN_3, pH 8.2

To make 1 Liter:
1.42g TRIZMA 8.0
8.77g NaCl
1g NaN_3
pH to 8.2

See hints on storing buffers.

References and credits:
This protocol was developed and tested by Peter Katsikis and Mario Roederer, based on our standard Cascade Blue conjugation protocol.