

APC conjugation of Annexin V

Overview:

This protocol serves not only to describe the conjugation of allophycocyanin (APC) to Annexin V, but to serve as a model for APC 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.

This protocol is similar to that of conjugation of APC to immunoglobulins. One difference arises from the much smaller size of Annexin V compared to IgG (one-fourth the molecular weight). The primary difference is that there is no available sulfhydryl on Annexin V. To introduce one, a heterobifunctional linking reagent is used which reacts with Annexin V lysines with one end of the molecule, and has a sulfhydryl on its other end. This sulfhydryl is reacted with the SMCC-derivatized APC through reductive cross-linking. Annexin V can be purchased from some commercial sources or obtained in collaborative agreements with researchers who make recombinant protein.

APC is emerging as one of the brightest fluorescent probes for FACS analysis. APC is a large protein (approximate molecular weight 80 kd) containing many fluors. Typically, only one APC molecule is conjugated to an antibody. Nonetheless, by virtue of its huge absorption coefficient and almost perfect quantum efficiency, as well as emission in a region of the spectrum with extremely low cellular autofluorescence, it is one of the brightest dyes used today. It emits at about 680 nm, and can be excited by either a dye laser (595-605 nm), or the cheaper HeNe laser (633 nm). Direct APC conjugates are relatively easy to make. Allophycocyanin can be purchased from several vendors, or isolated from the algae directly (be prepared to spend 2+ weeks in the cold room).

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

Reductive cross-linking of antibodies

Column chromatography

Reagent storage

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Conjugation protocol.

The entire conjugation can be performed in a single day. However, dialysis of stored APC prior to conjugation can take 24-48 hours. In addition to the materials listed below, you will need to have a solution of Annexin V at a concentration of at least 2 mg/ml. You should be familiar with how to use desalting columns and how to take absorbance spectra.

The SMCC-APC derivative (the result of steps I and II) is quite stable (at least a few months at 4C in the "Exchange Buffer"). Therefore, it is best to derivatize 10 or more milligrams of APC at the same time, and use it for several antibody conjugations (even over a period of weeks). Steps III and IV together take only a few hours and minimal

preparation; thus, storing the SMCC-derivative is very convenient. It is possible that long-term storage of the SMCC-APC may be best as a saturated ammonium sulfate precipitate--after which extensive dialysis similar to that in Step I below should be performed.

While taking absorbance spectra is not critical to the success of the procedure, it is highly recommended as a quality control, and as a permanent record of the quality of the APC for each conjugation.

Optimization of the Annexin V-APC conjugate requires trying different ratios of 2-IT to Annexin V. The protocol below suggests a ratio of 4; however, higher concentrations may result in brighter reagents. Too high a concentration may result in inactivation of the resulting conjugate. Try ratios of 1, 2, 4, 8, 16, and 32 when attempting a novel conjugation.

I. Preparation of APC

Dialyze or exchange the APC into "Dialysis Buffer". Concentration before derivatization is typically 5-10 mg/ml. Note: APC is most stable as a SAS (sodium ammonium sulfate) precipitate prior to coupling. If the APC is stored as a SAS precipitate, it must be extensively dialyzed prior to use. Dialyze against 2 changes of 1 liter per ml APC of PBS before dialyzing against 1 liter per ml of "Dialysis Buffer".

Use 3-6 mg of APC per mg of IgG to be modified.

To check the APC purity, measure the absorbance at 280, 620 and 655 nm. (1 mg/ml of APC has an OD at 655nm of 5.9). A 655/620 ratio >1.4 indicates adequate removal of contaminating phycocyanin; a 655/280 ratio > 4 indicates adequate removal of all other proteins.

II. Derivatization of APC

The amino groups on the phycoerythrin (APC) react with the succinamide to yield a maleimide-labeled APC.

Prepare a 10 mg/ml stock solution of SMCC in dry DMSO immediately prior to use. Add 6 μ l of SMCC per mg of APC while vortexing. Wrap the reaction tube in aluminum foil and rotate at room temperature for 60 minutes.

Pass the derivatized APC over a gel filtration column pre-equilibrated with "Exchange Buffer". See hints on using columns with fluorescent proteins. The SMCC-derivative is stable and may be stored at 4C for several weeks; a high concentration of APC (> 4 mg/ml) is desirable for such longer-term storage.

Note: for conjugations which fail or are poor, it may help to increase or decrease the amount of SMCC with respect to APC, or to use an alternative heterobifunctional crosslinking reagent.

III. Preparation of Annexin V

A heterobifunctional linking reagent is reacted with Annexin V (via lysines) to provide free sulfhydryl groups.

Dissolve (or dialyze or exchange) the Annexin V into any standard PBS solution (azide-free!); the Annexin V should be at a concentration of greater than 5 mg/ml if possible.

Dissolve 2-IT in the same PBS solution at a concentration of 10 mg/ml. Use immediately: for a molar ratio of 4, add 15.2 μ g of 2-IT per mg of Annexin. See notes above about using different molar ratios of 2-IT to Annexin V.

Incubate and rotate at room temperature for 60 minutes.

Prepare a fresh solution of 1 M DTT (15.4 mg/100 μ l) in distilled water.

Make each Annexin V reaction 20 mM in DTT: add 20 μ l of DTT stock per ml of reaction while mixing. Let stand at room temp for 15 minutes without additional mixing (to minimize reoxidation of cysteines to cystines).

Pass the reduced 2-IT-Annexin V over a filtration column pre-equilibrated with "Exchange Buffer". Collect 0.25 ml fractions off the column; determine the protein concentrations and pool the fractions with the majority of the Annexin V. This can be done either spectrophotometrically or colorimetrically (see hints on using columns for separation of nonfluorescent proteins).

Carry out the APC conjugation as soon as possible after this step.

IV. Covalent conjugation

The APC is covalently coupled to 2-IT-Annexin V through reaction of the maleimide groups with the free sulfhydryl on the 2-IT. Do not delay this step since the 2-IT sulfhydryls will reoxidize.

Add 3-6 mg of SMCC-APC per mg of 2-IT-Annexin V. Wrap the reaction tube in aluminum foil and rotate for 60 minutes at room temp. Note: These molar ratios (~1-2 APC per Annexin V) have worked. For conjugations which fail or are poor, different molar ratios may help.

After 60 minutes, unreacted free sulfhydryls on the 2-IT-Annexin V must be blocked.

Prepare a fresh solution of 10 mg NEM in 1.0 ml dry DMSO.

Add 20 μ g (2.0 μ l) per mg of 2-IT-Annexin V. Wrap and rotate for 20 minutes at room temperature.

The product can be either dialyzed or exchanged over a column into an appropriate buffer (e.g. "Storage Buffer"). It is best to keep the product at high concentration (> 1 mg/ml) for optimal stability. Never freeze the conjugates. It may be useful to spin APC conjugates prior to use in staining, especially if background seems to be a problem (e.g., at 10,000g in a microcentrifuge, at 4C). See also general hints on storing conjugates.

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:

2-IT - 2-iminothiolane * HCl ("Traut's reagent")

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

NEM - N-Ethylmaleimide, mw 125.1

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 pipette directly into the bottle.

DTT - Dithiothreitol

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

Standard PBS (free of sodium azide)

Buffers:

"Dialysis Buffer"

50 mM Sodium phosphate, 1 mM EDTA, pH 7.0

To make 1 Liter:

13.41g Sodium phosphate dibasic (7*H₂O)

0.37 g EDTA

"Exchange Buffer"

50 mM MES, 2 mM EDTA, pH. 6.0

To make 1 Liter:

9.76 g MES

0.74 gm EDTA

pH to 6.0

"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 was devised and tested by Mario Roederer, and is based on the reductive cross-linking protocol used for phycobiliprotein conjugates.

Hardy, RR: Purification and coupling of fluorescent proteins for use in flow cytometry. In: Handbook of Experimental Immunology, 4th ed. DM Weir, LA Herzenberg, C Blackwell, and LA Herzenberg, editors. Blackwell Scientific Publications, Boston, 1986, pp. 31.1-31.12.