
Cy7APC conjugation of Antibodies

Overview:

The resonance energy transfer dye Cy7-Allophycocyanin (Cy7APC) is a relatively new entrant onto the fluorescence field. It provides a bright third color for dye laser (595 - 605 nm excitation) and a second color for HeNe excitation (633 nm), with an emission at almost 800 nm. Cy7APC conjugates of immunoglobulins are relatively straightforward to make, requiring an additional step over the standard APC conjugation: the synthesis of the Cy7APC tandem dye. 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 (long) 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 your antibody 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 first time you make Cy7APC derivatives, you should probably make several different conjugates with varying ratios of Cy7 to APC. Make 1-3 mg of each; conjugate each to a test antibody. Compare the different conjugates for brightness and compensation requirements; select the appropriate ratio of Cy7 to APC. In the protocol below, a molar ratio of 10 is suggested; this ratio resulted in a dye with absorbance ratios measured at 755nm and 565nm of about 1.9. Such a tandem reagent has the good Cy7 fluorescence while not requiring excessive compensation; however, molar ratios of 2 to 30 should be attempted in initial trials. Once the appropriate ratio is selected, the reaction can be scaled up to make the large enough quantities for many antibody conjugations. Conjugate properties are determined by the absorbance ratio of the product, which in turn, under consistent reaction conditions, should reflect input molar ratios of the reactive dyes.

The SMCC-Cy7APC derivative (the result of step III) is quite stable (at least a few months at 4C in the "Exchange Buffer"). As well, each different lot of Cy7APC may have slightly different spectral characteristics (and thus require

different compensations). Therefore, it is best to derivatize 10-30 (or more) milligrams of APC at the same time, and use it for several antibody conjugations (even over a period of weeks). Steps IV and V 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-Cy7APC 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 and Cy7APC for each conjugation.

I. Preparation of APC

Dialyze or exchange the APC into "C Reaction 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 "C Reaction Buffer".

Use 1.7 mg of APC per mg of IgG to be modified; this includes an extra 10% for loss during buffer exchanges.

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. Cy7 conjugation of APC

The amino groups on the APC react with the bis-Cy7 dye to yield a tandem (resonance energy transfer) dye.

Dissolve the appropriate amount of bis-Cy7 in anhydrous DMSO to an effective concentration of 10 mg/ml immediately prior to use. For a molar ratio of 10 (see notes above on using different ratios), add 91.1 nmol Cy7 per mg of APC. Refer to the manufacturer's notes about the effective molecular weight of the preparation of Cy7 you are using.

Incubate and rotate the foil-wrapped tube at room temperature for 60 minutes. Purify the reaction mixture over a gel filtration column pre-equilibrated with "Dialysis Buffer".

Take an absorbance spectrum of the Cy7APC conjugate to determine the degree of Cy7 substitution as well as the concentration. A molar ratio of 10 Cy7 to 1 APC should result in a conjugate which has an A(755):A(655) ratio of approximately 1.9. Significantly different ratios indicate that the reaction proceeded too fast or too slow.

III. SMCC conjugation of Cy7APC

The amino groups on the Cy7APC 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 Cy7APC while vortexing. Wrap the reaction tube in aluminum foil and rotate at room temperature for 60 minutes.

Pass the derivatized Cy7APC 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 Cy7APC (> 4 mg/ml) is desirable for such longer-term storage.

IV. Reduction of IgG

The hinge disulfide bonds are reduced to yield free sulfhydryls.

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

IgG solutions should be at 4 mg/ml or higher for best results. The reduction can be carried out in almost any buffer; MES, phosphate, and TRIS buffers (pH range 6 to 8) have been used successfully. The antibody should be concentrated if less than 2 mg/ml. Include an extra 10% for losses on the buffer exchange column.

Make each IgG solution 20 mM in DTT: add 20 μ l of DTT stock per ml of IgG solution while mixing. Let stand at room temp for 30 minutes without additional mixing (to minimize reoxidation of cysteines to cystines).

Pass the reduced IgG 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 IgG. This can be done either spectrophotometrically or colorimetrically (see hints on using columns for separation of nonfluorescent proteins).

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

Note: for conjugations which are poor or fail, it may help to reduce the DTT concentration.

V. Covalent conjugation

The Cy7APC is covalently coupled to the IgG through reaction of the maleimide groups with the free sulfhydryl on the IgG. Do not delay this step since the IgG sulfhydryls will reoxidize.

Add 1.5 mg of SMCC-Cy7APC per mg of IgG. Wrap the reaction tube in aluminum foil and rotate for 60 minutes at room temp. Note: These molar ratios (~2 PE per IgG) have worked very well. For conjugations which fail or are poor, different molar ratios may help.

After 60 minutes, unreacted free sulfhydryls on the IgG must be blocked.

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

Add 34 μ g (3.4 μ l) per mg of IgG. 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 Cy7APC 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:

Cy7 - Cy7-bis-OSU, N,N'-biscarboxypentyl-5,5'-disulfonatoindotricarbocyanine
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 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:

"C Reaction Buffer"
500 mM carbonate, pH 9.0

To make 1 Liter:

17g Na₂CO₃

28g NaHCO₃

pH to 9.0

Note: sodium azide cannot be added to this buffer

"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:

M Roederer, AB Kantor, DR Parks, and LA Herzenberg: Cy7PE and Cy7APC: Bright new probes for immunofluorescence. *Cytometry*, 24:191-197 (1996).

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.