# Cy5PE conjugation of Antibodies

## Overview:

The resonance energy transfer dye Cy5-Phycoerythrin (Cy5PE) is an extremely bright dye, primarily because of the very low autofluorescence in the region where it fluoresces. It is easily excited at 488 nm, and emits at 680 nm; however, it is also well excited at 633 nm (HeNe laser), and thus using Cy5PE reagents in two laser-system requires interlaser compensation. Cy5PE conjugates of immunoglobulins are relatively straightforward to make, requiring an additional step over the standard PE conjugation: the synthesis of the Cy5PE tandem dye. Phycoerythrin 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 PE 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 Cy5PE derivatives, you should probably make several different conjugates with varying ratios of Cy5 to PE. Make 2-5 mg of each; conjugate each to a test antibody. Compare the different conjugates for brightness and compensation requirements; select the appropriate ratio of Cy5 to PE. In the protocol below, a molar ratio of 12 is suggested; this ratio resulted in a dye with absorbance ratios measured at 650nm and 565nm of about 0.6. Such a tandem reagent has the most Cy5 fluorescence while not requiring excessive compensation; however, molar ratios of 5 to 20 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-Cy5PE 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 Cy5PE may have slightly different spectral characteristics (and thus require

different compensations). Therefore, it is best to derivatize 10-30 (or more) milligrams of PE 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-Cy7PE 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 PE and Cy5PE for each conjugation.

## I. Preparation of PE

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

Use 3.5 mg of R-PE per mg of IgG to be modified; this includes an extra 10% for loss during buffer exchanges.

To check the PE purity and concentration measure the absorbance at 280, 565 and 620 nm. (1 mg/ml of PE has an OD at 565nm of 8.2). A 565/620 ratio > 50 indicates adequate removal of contaminating phycocyanin; a 565/280 ratio > 5 indicates adequate removal of all other proteins.

## II. Cy5 conjugation of PE

*The amino groups on the phycoerythring (PE) react with the bis-Cy5 dye to yield a tandem (resonance energy transfer) dye.*

Dissolve the appropriate amount of bis-Cy5 in anhydrous DMSO to an effective concentration of 10 mg/ml immediately prior to use. For a molar ratio of 12 (see notes above on using different ratios), add 49.8 nmol Cy5 per mg of PE. Refer to the manufacturer's notes about the effective molecular weight of the preparation of Cy5 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 Cy5PE conjugate to determine the degree of Cy5 substitution as well as the concentration. A molar ratio of 12 Cy5 to 1 PE should result in a conjugate which has an A(755):A(565) ratio of approximately 0.6. Significantly different ratios indicate that the reaction proceeded too fast or too slow.

## III. SMCC conjugation of Cy5PE

*The amino groups on the Cy5PE react with the succinamide to yield a maleimide-labeled PE.*

Prepare a 10 mg/ml stock solution of SMCC in dry DMSO immediately prior to use.

Add 11 µl of SMCC per mg of Cy5PE while vortexing. Wrap the reaction tube in aluminum foil and rotate at room temperature for 60 minutes.

Pass the derivatized Cy5PE 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 Cy5PE (> 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 Cy5PE, or to use an alternative heterobifunctional crosslinking reagent.

## 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 µl) 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 µl 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 Cy5PE 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 3.2 mg of SMCC-Cy5PE 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 µg (3.4 µl) 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 congugates. It may be useful to spin Cy5PE 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.

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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:**

Cy5 - Cy5-bis-OSU, N,N'-biscarboxypentyl-5,5'-disulfonatoindodicarbocyanine.

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.*

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 to9.0
*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.

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**References and credits:**
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.