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## PE conjugation of Antibodies

### Overview:

Phycoerythrin (PE) is one of the most commonly-used fluorescent dyes for FACS analysis. PE is a large protein (approximate molecular weight 240 kd) containing 25 fluors. Typically, only one PE molecule is conjugated to an antibody. Nonetheless, by virtue of its huge absorption coefficient and almost perfect quantum efficiency it is one of the brightest dyes used today. It emits at about 570 nm, and can be excited by common Argon laser lines. Direct PE conjugates are relatively easy to make. 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

You can also use the short, less-detailed protocol for reference.

### Contents:

Conjugation protocol

- I. Preparation of PE
- II. Derivatization of PE
- III. Reduction of IgG
- IV. Covalent conjugation

Materials, chemicals, and buffers

References

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### Conjugation protocol.

The entire conjugation can be performed in a single 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 SMCC-PE 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 PE 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-PE 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 for each conjugation.

## I. Preparation of PE

Dialyze or exchange the PE into "Dialysis 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 "Dialysis 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 phycoerythrin; a 565/280 ratio > 5 indicates adequate removal of all other proteins.

## II. Derivatization of PE

*The amino groups on the phycoerythrin (PE) 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  $\mu$ l of SMCC per mg of PE while vortexing. Wrap the reaction tube in aluminum foil and rotate at room temperature for 60 minutes.

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

## III. 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  $\mu$ 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  $\mu$ 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.

## IV. Covalent conjugation

*The PE 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-PE 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 conjugates. It may be useful to spin PE conjugates prior to use in staining, especially if background seems to be a problem (e.g., at 10,000g in a microcentrifuge, at 4°C). 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:

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

NEM – N-Ethylmaleimide  
Sigma, Catalog E-1271, mw 125.1

DMSO - anhydrous dimethyl sulfoxide  
Aldrich, catalog No. 27,685-5.

**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.

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:****"Dialysis Buffer"**

50 mM Sodium phosphate, 1 mM EDTA, pH 7.0

To make 1 Liter:

13.41g Sodium phosphate dibasic (7\*H<sub>2</sub>O)

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% 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 is based on an original protocol devised by Randy Hardy, and modified by Alan Stall and Aaron Kantor.

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.