

Labeling PBD with reactive fluorophores

The dyes tested with this procedure include TAMRA SE, Tetramethyl Rhodamine IAA, Alexa532 and Alexa546 (*Molecular Probes*).

PBD at 2-3 mg/ml or 250-300 μ M was in 50 mM NaP buffer, pH 7.5 for the reaction with NH_2 -reactive forms of the dyes. Reactions with cysteine-reactive dyes were carried out in this buffer at 25 mM or in 20-50 mM Tris. Fresh solution of the dye were prepared in pure DMSO or in dH_2O (for the more water soluble Alexa dyes). The exact concentration of the dye was determined spectrophotometrically. Make a 10,000-50,000 dilution of stock in methanol and use documented extinction coefficients, 78,000 at 528 nm for Alexa532.

A 200-300 μ L aliquot of fresh PBD prep is transferred into an eppendorf tube wrapped in foil (to protect from light). The dye is then added in three-four aliquots to the PBD solution (with gentle stirring or vortexing) to make the final molar dye:protein in the reaction about 7:1 (usually, one needs to have about 2-4 mM final dye concentration). It has been found that using lower dye:PBD ratios (1:3) resulted in low dye:protein ratio in the conjugate, whereas higher ratios (1:15) in the reaction mixture produce excessive amounts of precipitated material. The tube is completely wrapped in foil and put for 2-2.5 hours on a stirring wheel at r.t. In the case of maleimide or iodoacetamide dye derivatives, after the reaction is complete, a small amount of the β -ME (to 5 mM) is added and the tube is incubated another 15 min on the wheel. This assures that there are no reactive species left during the following separation and may remove non-covalently bound dye molecules from the PBD. The tube is centrifuged for **2 min at 14,000 rpm** to sediment insoluble material. The supernatant is then loaded on a small G25 gel filtration column (0.5 cm x 4 – 5 cm) to separate the conjugate from the free dye. The column is equilibrated and eluted with 50 mM NaP buffer, pH 7.5, or any other desired buffer. Approximately 300 μ L per fraction is collected. The first colored band to elute contains PBD-dye conjugate. Fractions are analyzed by 12 or 15% SDS-PAGE to confirm the presence of the modified PBD and the purity of the sample.

The concentration of the protein and dye:protein ratio is determined by taking an absorbance spectrum of the conjugate solution (dilute a small aliquot – 5-10 μ L – in 50mM Tris·HCl, pH 7.5-8.0). In the case of rhodamine derivatives, it is difficult to determine the exact degree of labeling because the dye absorbance spectrum changes upon conjugation.

Concentration of the protein may also be estimated independently by running a Bradford or Coomassie protein assay (*BioRad*) when assay wavelengths do not overlap those of the dye. For many dyes, fairly accurate estimation of the dye:protein is possible using a formula that corrects for dye absorbance at the wavelength used to determine protein concentration. For example, for Alexa532, Molecular Probes provide the following formulas:

$$[\text{Alexa532}] = \text{OD}_{530} / 81,000$$

$$[\text{PBD}] = (\text{OD}_{280} - 0.09 * \text{OD}_{530}) / 8,250$$

Protein solution can be further concentrated using Aquacide powder, as described in the protocol for PBD purification. The solution is aliquoted into 20-30 μL aliquots, flash frozen with dry ice or liquid nitrogen and kept at -80°C until further use. Alternatively, the PBD may be kept without freezing at 4°C for about a week.