

## Labeling with Reactive Fluorophores

For attachment of the dyes to cysteine, CBD-EGFP needs to be in 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.5 at a protein concentration of 100 μM. A fresh solution of dye is prepared in pure DMSO by adding approximately 1 mg of dye into 30-40 μL DMSO. Once dissolved in DMSO, dye cannot be kept more than 12hrs. The exact concentration of the dye is determined spectrophotometrically by diluting the DMSO solution 1:5000 in methanol. The ISO-IAA dye extinction coefficient in methanol at maximum absorption (610 nm) is 125,000 (cm<sup>-1</sup> M<sup>-1</sup>).

$$[\text{ISO-IAA}] \text{ (in mol/L)} = (\text{OD}_{610} * \text{dilution factor}) / 125000 \quad (2)$$

We routinely obtain concentrations of 20~25 mM as the DMSO stock solution.

A 300 μL aliquot of fresh CBD-EGFP protein is transferred into a 2 ml Eppendorf tube wrapped in foil to protect from light. The dye is added in 2-3 aliquots to the CBD-EGFP solution to make the final dye to protein ratio in the reaction to 5:1 or 6:1. The protein-dye mix is vortexed once briefly at highest setting immediately after addition of the dye stock solution. Using higher dye to CBD-EGFP ratios (e. g. 10:1 or 15:1) in the reaction mixture can produce excessive amounts of precipitated material and “over-labeling” (dye to protein ratio in the purified covalent adduct greater than 1.0). The optimal dye to protein ratio depends on the reactivity of the dye, so it can vary from different batches of the dye preparations. The tube containing the reaction mixture is wrapped in foil and gently rotated on the rotating wheel for exactly 1 hour on a wheel at room temperature. Here, the time is critical since the dye is very reactive; therefore longer reaction times result in over-labeling conditions. After the reaction is complete, 1~5 μl β-ME is added to stop the reaction and the tube is incubated for 5-10 min at room temperature on a rotating wheel. The tube is centrifuged at room temperature to pellet insoluble material (i.e, 2 min at 13,000 rpm in an Eppendorf benchtop microcentrifuge). The supernatant is then loaded on a small G15 gel filtration column (0.5 cm x 6-8 cm) to separate the conjugate from free dye. This column is equilibrated and run in 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer at pH

7.5. The first colored band to elute contains the dye-labeled CBD-EGFP (MeroCBD). Fractions of approximately 200  $\mu\text{L}$  each are collected. An aliquot (3-5  $\mu\text{L}$ ) of each fraction is analyzed by 12% SDS-PAGE to confirm the presence and purity of the MeroCBD. Fluorescence visualization of the gel assures labeling with the dye.

Protein concentration can be determined by taking an absorbance spectrum of the conjugate solution. Dilute a small aliquot (5-10  $\mu\text{L}$ ) in 50mM Tris HCl, pH 7.5-8.0 and use OD280 for the calculation using the Equation (1). However, the protein absorbance at 280 overlaps some portions of the dye absorbtion. One can determine the protein concentration independently by comparing a sample of MeroCBD to unlabeled CBD-EGFP using different sample concentrations. Colorimetric assays, limited to those with readouts that do not overlap dye absorbance, have been more variable in our hands.

The dye concentration is determined by taking an absorbance spectrum of MeroCBD. 5~10 $\mu\text{l}$  of MeroCBD is diluted in DMSO. DMSO as a solvent will overwhelm effects of the protein on the dye absorbance to result in a consistent dye extinction coefficient. OD at the dye absorbance maximum of 610nm is used to calculate the dye concentration using the Equation (2) with the extinction coefficient of I-SO-IAA in DMSO ( $145,000 \text{ cm}^{-1} \text{ M}^{-1}$ ).

We routinely recover approximately 40-60% of the CBD-EGFP as purified MeroCBD, due to some precipitation during labeling and loss during gel filtration. The final eluate concentration is usually 50-60  $\mu\text{M}$ . Labeling efficiency under these conditions varies between 0.7-0.9 dye to protein ratio. MeroCBD solution is aliquoted into 15-20  $\mu\text{l}$  and flash frozen with dry ice or liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Alternatively, the MeroCBD may be kept at  $4^{\circ}\text{C}$  for up to one week.