

## Protein Expression and Purification

CBD-EGFP is expressed in the form of C-terminal 6xHis fusion from the prokaryotic expression vector pET23. This vector has a strong T7 promoter, and is designed to work with BL21(DE3) strains of *E.coli* (Stratagene). It was determined experimentally that the highest levels of expression are observed when a plain T7 promoter (not *T7lac*) is used in combination with a BL21(DE3) strain and not BL21(DE3)pLysS, which allows for leaky expression. The protein is induced and expressed at room temperature (26°C), to increase the portion of the correctly folded, soluble CBD-EGFP.

Key points include:

- 1) The bacteria strain is critical. We use BL21(DE3). Do not use BL21(DE3)pLysS.
- 2) Use Talon resin (Co<sup>2+</sup> affinity, Clontech Inc.). Do not use Ni-NTA resin. Use approximately 2 ml Talon resin (dry volume) for 3g of cell pellet.
- 3) Use the suggested buffers in this article.
- 4) Use enough buffer during lysis; i.e., for 3g cell pellet use 35 ml total lysis buffer.

Day 1

1. Competent BL21(DE3) cells are transformed with pET23-CBD-EGFP according to standard protocols {Sambrook, 1989 #5531}, and plated on LB-carb (100 µg/ml carbenicillin) plates. We usually split the 200µl transformation volume over 2 plates.
2. Plates are incubated at 37°C overnight.

Day 2

3. 500 ml of LB-carb (100 µg/ml carbenicillin) are inoculated with the colonies from the plates. 5 ml of media are added on each plate and cells are resuspended into the media.

The cell suspension is transferred into the 500 ml LB-carb and grown in a shaker at 37°C, 225rpm to OD<sub>600</sub> = 0.8-0.9. The culture is briefly chilled on ice to 26°C and put back in the shaking incubator turned down to 26°C.

4. IPTG (1 M stock in water, kept at -20°C) is added to a final concentration of 0.2 mM, and the cultures are allowed to grow for another 6 hours at 26°C at 225rpm. IPTG concentrations of 0.2~0.5mM has been used successfully.
5. Cells are collected by centrifugation (Beckman J-6M rotor, 20 min, 4,000 rpm), and stored as a pellet at -20°C until use. Approximately 2.5-3 g of cells is usually obtained from each liter of culture.

#### Day 3

6. Cells (~3 g) are resuspended in 35 ml of the Lysis buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.6, 300 mM NaCl, 10% glycerol, 5 mM MgCl<sub>2</sub>, 2 mM β-ME, 1 mM PMSF] and lysed by sonication (4 pulses, 30 sec each on ice with 1 min rests).
7. The lysates are centrifuged at 13,000 rpm for 30 min, and the supernatant containing CBD-EGFP is carefully transferred into a 50 ml Falcon tube.
8. While the lysates are being centrifuged, 2 ml of Talon resin (dry volume) is transferred into a 50 ml Falcon tube and centrifuged at 700g in a swinging bucket centrifuge. We use 2 ml resin per 3 g cell pellet.
9. Talon resin is washed twice with 10 volumes of the lysis buffer (no β-ME and PMSF) in a 50 ml Falcon tube. Again, pellet the resin by centrifuging at 700g.
10. The cell lysate is added to the washed Talon resin in the 50 ml falcon tube and inverted gently using an orbit shaker at room temperature for 40 min ~ 1hr, wrapped in foil to

avoid unnecessary exposure of EGFP to light. The resin is then separated by centrifugation at 700g in a swinging bucket centrifuge.

11. The supernatant is removed and saved (unbound fraction). If a large portion of unbound material is present in this fraction, consider increasing the Talon resin volume by preparing 2 tubes of 2ml resin and splitting the lysates into 2 tubes during the binding reaction.
12. The resin is washed twice (5 min each at room temperature, orbit shaker) with 20 ml of fresh buffer, excluding PMSF or  $\beta$ -ME.
13. Final wash is performed with 10 volumes of buffer containing 5 mM imidazole. It is convenient to prepare freshly, 5ml of stock 1M imidazole solution in the same buffer. Always prepare the stock imidazole solution fresh.
14. The elution is performed by adding 5 ml buffer containing 150 mM imidazole to the resin and rotated using the orbit shaker at room temperature for 5 min. Pellet the resin again by centrifugation.
15. The supernatant is removed and saved (eluted fraction).
16. The resulting 5 ml eluate is concentrated with the Ultrafree – 4 Centrifugal Filtration Device (Millipore; 5000Da cut-off) by centrifugation at 4 °C following the manufacturer's directions. Check the concentration process every 20 min to ensure proper filtration. Do not over-concentrate; optimal final concentration should be approximately 120  $\mu$ M. The concentration of CBD-EGFP is measured by taking a small aliquot (5-10  $\mu$ L) and diluting into 50 mM Tris HCl (pH 7.5-8.0). We measure the absorbtion at 280 and use 28260 ( $\text{cm}^{-1} \text{M}^{-1}$ ) as extinction coefficient for the protein, using the following equation:

$$[\text{CBD-EGFP}] \text{ (in mol/L)} = (\text{OD}_{280} * \text{dilution factor}) / 28260 \quad (1)$$

On average, 10-15 mg of CBD-EGFP is obtained per liter culture.

17. If dye-labeling is to be performed the following day, a part of the concentrated protein is dialyzed overnight against 2 L of 50 mM NaH<sub>2</sub>PO<sub>4</sub> (monobasic sodium phosphate) buffer at pH 7.5. Slide-A-Lyzer cassette (PIERCE) with a molecular weight cut-off of 3,500Da is used.
18. For long term storage, the protein is dialyzed over night against 2 L of storage buffer [50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 10% Glycerol] and is flash frozen on dry ice or liquid nitrogen.

### **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  $\mu\text{L}$   $\beta\text{-ME}$  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 G25 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  $\text{NaH}_2\text{PO}_4$  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.