

CBD-EGFP (Reagent for Cdc42 activation assay) 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* (available from 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, not the more stringent BL21(DE3)pLysS, which allows for leaky expression. The protein is expressed at lowered temperature (30°C), to increase the portion of the correctly folded, soluble CBD-EGFP.

We have had calls from people having trouble with this purification. They have succeeded by following this protocol, which provides high yields of clean product. These are points at which changes have made a difference:

- The bacteria strain is critical. We use BL21(DE3). Do not use BL21(DE3)pLysS.
- Use Talon resin (Co²⁺ affinity, Clontech) and not Ni-NTA resin! Don't use too much resin (2ml dry volume of talon resin for 6g of cell pellet).
- Use the suggested buffers.
- Use enough buffer during lysis, e.g. for 6g cell pellet use 50 ml total lysis buffer (divide between two 50 ml tubes).

Summary of prep:

- A) lysis (Tris buffer, pH 7.8, β -ME, PMSF)
- B) separation of pellet (cell debris) and supernatant (protein)
- C) binding to beads
- D) wash (2x Tris buffer alone, followed by 1x Buffer +10 mM imidazole)
- E) beads poured into a column for elution, in last wash solution
- F) elution of the protein with 60 - 62 mM imidazole (collect fractions)
- G) SDS PAGE, combine fractions
- H) overnight dialysis in 25mM NaP buffer (pH7.3)
- I) protein can be stored in a Tris buffer with Glycerol at -80.

Day 1

1. Competent BL21(DE3) cells are transformed with pET23-CBD-EGFP according to standard protocol (Maniatis), and plated on LBamp plates. We usually split the transformation volume over 3 plates.
2. The plates are incubated at 37°C overnight.

Day 2

3. The next morning the plates should have colonies colored GFP green.
4. 500 ml (up to 700 ml) of LB-*carb* (100 μ g/ml carbenicillin) are inoculated with the colonies from the plates. For that, 5 ml of media are added on each plate and cells are resuspended with that media. The cell suspension is transferred into the 500 ml LB-*carb*. and grown in a shaker at 37°C to $OD_{600} = 0.8-0.9$. The culture is briefly chilled on ice to 30°C, then put back in the shaking incubator turned down to 30°C. Cells do not degrade carbenicillin as quickly as ampicillin. Therefore a higher percentage of cells retain the vector at the culture density appropriate for induction.
5. 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 5 hours at 30°C (shaker). In the case of CBD-EGFP one can use different concentrations of IPTG ranging from 0.2-0.5 mM.

6. The cells are collected by centrifugation (***Beckman J-6M rotor, 20 min, 4000 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

7. Purification of CBD-EGFP-6xHis is performed essentially as described in the Clontech manual for Talon affinity resin.
8. The cells (3-5 g) are thawed in 20-30 ml of the Lysis buffer [50 mM NaH_2PO_4 , pH 7.6, 300 mM NaCl, 10% glycerol, 5 mM MgCl_2 , 2 mM β -ME, 1 mM PMSF], homogenized by pipetting up and down and sonicated (4 pulses, 20 sec each).
9. The cells are then centrifuged at ***13000 rpm for 20-30 min***, and the supernatant containing CBD-EGFP is carefully transferred into a 50 ml Falcon tube.
10. While the cells are spinning, 2 ml of Talon resin is transferred into a 50 ml Falcon tube and centrifuged with 700xg. We use 1ml resin per 2.5 g cell pellet. Depending on the expression level of your protein you can increase or decrease the amount of resin. ***To pellet the resin we use a swinging bucket centrifuge at a setting of "3", 2-3 minutes.***
11. The 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 ***for how long?***
12. The cell lysate is added to the 2 ml of washed Talon resin in the 50 ml falcon tube and inverted gently using an orbit shaker at r. t. for 40 min (Wrap it with foil to avoid unnecessary bleaching of the EGFP). The resin is then separated by centrifugation (700g ***for how long?***) in a swinging bucket centrifuge.
13. The supernatant is removed and saved (unbound fraction).
14. The resin is washed twice (5 min each, r. t., orbit shaker) with 20 ml of the lysis buffer, without PMSF and β -ME.
15. A third (final) wash is performed with 10 ml lysis buffer + 5 mM imidazole (add 1 M stock in water, kept at r.t.).
16. The elution is performed by adding 10 ml Lysis buffer + 100 mM imidazole to the resin and rotation (orbit shaker) at r. t. for 5 min. Pellet the resin again by centrifugation.

17. The supernatant is removed and saved (elution).
18. Adding another 2-3 ml elution buffer to the resin and separating by centrifugation enables one to obtain residual eluted protein.
19. The resulting 10-12 ml eluate is concentrated with the “ultrafree –15 centrifugal filter device” from Millipore by centrifugation at 4 °C (read manual). This step can take a long time (2-3 hrs) and needs to be monitored. Check every 20 min to be sure the concentration process didn't go too far. Also check the concentration. One can stop at around 120 μM.
20. For labeling the next day, a part of the concentrated protein is dialyzed overnight against 2 L of 50 mM NaH₂PO₄ (monobasic sodium phosphate) buffer, pH 7.5. A dialysis “bag” (Slide-A-Lyzer, from PIERCE) with a molecular weight cut-off of 3,500 kDa is used.
21. The concentration of CBD-EGFP is measured by taking a small aliquot (5-10 μL) and diluting into 50 mM Tris HCl (pH 7.5-8.0). We measure the absorbance at 280 and use 28260 (cm⁻¹/M⁻¹) as extinction coefficient for the protein. On average, 10-15 mg of CBD-EGFP is obtained per liter culture.

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

22. For storage, the rest of the protein is dialyzed over night against 2 L of storage buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 10% Glycerol) and is shock frozen on dry ice or liquid nitrogen.