

PBD (Reagent for Rac activation studies) Expression and Purification

Kraynov, V. S., C. E. Chamberlain, G. M. Bokoch, M. A. Schwartz, S. Slabaugh and **K.M. Hahn**. Localized Rac Activation Dynamics Visualized in Living Cells. *Science*, **290**:333-337, 2000.

Although they appear similar, there are important differences between this protocol and that for purification of CBD-EGFP.

PBD is expressed in the form of C-terminal 6His fusion from 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 PBD.

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).

Day 1

1. Competent BL21(DE3) cells are transformed with pET23-PBD according to standard protocols (Maniatis), and plated on LBamp plate.
2. The plate is incubated at 37°C overnight.

Day 2

3. The next morning, a single colony of cells is picked into 5 ml of LB media with 100 µg/ml carbenicillin (*carb*). 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.
4. The culture is grown in the shaker at 37°C for 6-8 hours (until dense), and 2 ml used to inoculate 50 ml of LB*carb*. The rest of the culture is diluted 1:1 with glycerol and frozen for permanent storage at –80°C.
5. The 50 ml culture is incubated in the shaker overnight at 37°C.

Day 3

6. Next morning 1-2 L of LB*carb* are inoculated with the overnight culture (15-20 mL culture/500mL media), and grown in the shaker (37°C) to OD₆₀₀ = 0.8-0.9 (about 2-3 hours).
7. The cultures are briefly chilled on ice to 30-32°C, then put back in the shaking incubator turned down to 30-32°C.
8. IPTG (1 M stock in water, kept at –20°C) is added to a final concentration of 0.4-0.5 mM, and the cultures are allowed to grow for another 4-5 hours at 30-32°C in the shaker.
9. The cells are collected by centrifugation (**8,000 rpm, 4 min**), and stored as a pellet at –20°C until use. Approximately 4-5 g of cells is usually obtained from each liter of culture.

Day 4

10. Purification of PBD-6His is performed essentially as described in the Clontech manual for the Talon affinity resin.
11. The cells (6 g) are thawed in 40-50 ml of the Lysis buffer [30 mM Tris HCl, pH 7.8 r.t., 250 mM NaCl, 10% glycerol, 5 mM MgCl₂, 2 mM β-ME, 1 mM PMSF], homogenized with a spatula and sonicated (4 pulses, 10-15 sec each).
12. T4 lysozyme and DNase are added in catalytic amount to help the lysis, and the suspension is incubated on ice with periodic mixing for 30 min.
13. The cells are then centrifuged at **12,500 rpm for 30 min**, and the supernatant containing PBD is carefully transferred into a 50 mL Falcon tube.

14. While the cells are spinning, 1.5-2 ml (dry volume) of Talon resin (Co²⁺ affinity, *Clontech*) is washed twice with 10 volumes of the lysis buffer in a 50 ml Falcon tube, centrifuging in the swinging bucket centrifuge in between to separate the resin (700 x g, 5 min).
15. The cell lysate is added to the 1.5-2ml of washed Talon resin in the 50 mL falcon tube, and inverted gently (orbit shaker) at r.t. for 20-30 min. The resin is then separated by centrifugation (700 x g, 5 min).
16. The supernatant is removed and saved (“unbound fraction”).
17. The resin is transferred into a new 50 mL Falcon tube and washed twice (10-15 min each, r.t., orbit shaker) with 25 mL of the lysis buffer, without PMSF and β-ME.
18. The third wash is performed with 20 ml lysis buffer + 10 mM imidazole (add 1 M stock in water, kept at –20°C) Invert gently on the orbit shaker at r.t. for 10 min.
19. After the final separation, the resin is resuspended in 2-3 mL of lysis buffer with 10 mM imidazole, and pipetted into a column 0.5 cm in diameter.
20. The resin is allowed to sediment by gravity flow until almost dry, and then another 3-5 mL of Lysis buffer with 10 mM imidazole are added to wash the column.
21. The elution is performed using Lysis buffer with 60 mM imidazole, and ca. 500 μL fractions are collected. PBD usually elutes in the fractions 5-13 (total volume about 3-4 mL). Fractions are analyzed by 12 or 15% SDS-PAGE. The fractions containing pure PBD are combined, and dialyzed twice against 1 L of 25 mM NaP buffer (pH 7.3) (NaH₂PO₄) using a Slide-A-Lyzer Dialysis Cassette (Pierce, Cat. No. 66330, molecular weight cut-off 3,500 kDa).
22. After dialysis, the bag is wiped with a KimWipe and buried in Aquacide powder for 15-45 min (depending on the volume of the sample in the bag) at 4°C. IMPORTANT: The concentration process should be monitored carefully, as complete drying may occur if the bag is left in the Aquacide for too long.
23. The powder is scraped gently from the bag every 10-15 min to facilitate water absorption. At one point the center of the cassette sticks together. This is ok as long as the entire contents are not dried.
24. When the sample reaches 0.5-1.5 mL in volume (3-10-fold concentration), the bag is cleaned of Aquacide and the sample is removed. Cut the bag at the top carefully, and

suck out the contents with a Pipetman, trying not to contaminate the sample with the residual Aquacide. Alternatively, one can carefully “inject” air into the bag to separate the membrane again and take the concentrated sample out of the bag with a syringe.

25. The sample is briefly centrifuged (*14,000 rpm, 2 min*) to separate precipitated material, and the supernatant is transferred into a new eppendorf tube.
26. The concentration of PBD is measured by taking a small aliquot (5-10 μL) and diluting into 25 mM NaP buffer (pH 7.5) or other appropriate buffer. The extinction coefficient of PBD at 280 nm is 8,250 (estimated from the primary sequence). On average, 1.5-2 mg of PBD is obtained per liter of cell culture.