

CMC Biosensors Initiative

Hahn Laboratory

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Generation of a Rac Biosensor for Activation Studies; - PBD Expression, Purification & Reactive Fluorophore Labeling

Introduction

This procedure describes the expression and purification of a fragment of p21-activated kinase (PBD), and its subsequent labeling with reactive fluorophore, in order to generate a biosensor for use in Rac activation studies (Kraynov et al., 2000). Fluorophore labeled PBD injected into cells expressing GFP-Rac binds selectively to GFP-Rac-GTP – the activated form of Rac. This binding enables the GFP and reactive fluorophore on the PBD to undergo fluorescence resonance energy transfer (FRET) and produce a unique fluorescence signal which signals the timing and location of Rac activation. For an explanation of how these probes work visit

http://www.cellmigration.org/resource/biosensors/biosen_approaches.shtml#dual and view this animation

http://stke.sciencemag.org/content/vol2003/issue165/images/data/pe3/DC1/figure_B.swf (Gaits & Hahn, 2003)

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). The highest levels of expression are observed when a plain T7 promoter (not T7/*lac*) 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. The dyes tested with this labeling procedure include TAMRA SE, Tetramethyl Rhodamine IAA, Alexa532 and Alexa546 (*Molecular Probes*).

The methodology as to how to use this biosensor to assay for Rac activity is described in Chamberlain et al., 2000 and Kraynov et al., 2000.

Materials

Reagents:

BL21(DE3) cells, pET23-PBD, LBamp plate, LB media, carbenicillin, IPTG, T4 lysozyme and DNase, imidazole, Aquacide powder, NaP buffer (pH 7.3 & 7.5), Reactive fluorophore of choice (i.e. TAMRA-SE, Tetramethylrhodamine-IAA, Alexa532 or Alexa546), β -ME, DMSO, 20-50mM Tris, methanol, foil, Aquacide powder.

Hahn laboratory plasmids are available at addgene, a non-profit constructs repository

<http://www.addgene.org/pgvec1?f=c&cmd=showcol&colid=177>

Lysis buffer

pH 7.8, room temp.

30 mM Tris HCl,

250 mM NaCl,

10% glycerol,

5 mM MgCl₂,

2 mM β -ME,

1 mM PMSF

Equipment:

Cell culture equipment, centrifuge, incubator, Spectrophotometer, vortex/stirrer, G25 gel filtration column

Time Taken

Four to five days

Procedure

PBD Expression & Purification

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, 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 room temperature 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, room temperature, 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) (NaH_2PO_4) 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, 1.5-2mg of PBD is obtained per liter of cell culture.

Labeling with Reactive Fluorophore

- 1) Prepare PBD at 2-3mg/ml or 250-300 μM in 50mM NaP buffer, pH 7.5 for the reaction with NH_2 -reactive forms of the dyes. For reactions with cysteine-reactive dyes use this buffer at 25mM or 20-50mM Tris.
- 2) Prepare fresh solutions of the dye in pure DMSO (or in dH_2O for the more water soluble Alexa dyes). Determine the exact concentration of the dye spectrophotometrically.
- 3) Make a 10,000-50,000 dilution of stock in methanol and use documented extinction coefficients, 78,000 at 528 nm for Alexa532.
- 4) Transfer a 200-300 μL aliquot of the fresh PBD preparation into an eppendorf tube wrapped in foil (to protect from light). Add the dye to the PBD solution in three-four aliquots (with gentle stirring or vortexing) to make the final molar dye:protein ratio in the reaction about 7:1 (usually, one needs to have about 2-4mM final dye concentration). 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.

- 5) Completely wrap the tube in foil and leave for 2-2.5 hours on a stirring wheel at room temperature. In the case of maleimide or iodoacetamide dye derivatives, after the reaction is complete add a small amount of the β -ME (to 5mM) and incubate the tube for 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.
- 6) Centrifuge the tube for 2 min at 14,000 rpm, to sediment insoluble material. Load the supernatant onto a small G25 gel filtration column (0.5 cm x 4 – 5cm) to separate the conjugate from the free dye.
- 7) Equilibrate the column and eluted with 50 mM NaP buffer, pH 7.5, or any other desired buffer. Collect approximately 300 μ L per fraction. The first colored band to elute contains PBD-dye conjugate.
- 8) Analyze fractions by 12 or 15% SDS-PAGE to confirm the presence of the modified PBD and the purity of the sample. Determine the concentration of the protein and dye:protein ratio 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:

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

$$[\text{PBD}] = (\text{OD}_{280} - 0.09 \cdot \text{OD}_{530}) / 8,250$$
- 9) Further concentrate the protein solution using Aquacide powder, as described in the protocol for PBD purification.
- 10) Aliquot into 20-30 μ L aliquots, flash freeze with dry ice or liquid nitrogen and store at -80°C until needed. Alternatively, PBD may be kept without freezing at 4°C for about a week.

Troubleshooting

1. The bacteria strain is critical. We use BL21(DE3). Do not use BL21(DE3)pLysS.
2. Use Talon resin (Co_2^+ affinity, Clontech) and not Ni-NTA resin! Don't use too much resin (2ml dry volume of talon resin for 6g of cell pellet).
3. Use the suggested buffers.
4. Use enough buffer during lysis, e.g. for 6g cell pellet use 50 ml total lysis buffer (divide between two 50 ml tubes).

Anticipated Results

On average, 1.5-2 mg of PBD is obtained per liter of cell culture.

References

Chamberlain CE, Kraynov VS, Hahn KM. Imaging spatiotemporal dynamics of Rac activation in vivo with FLAIR. *Methods Enzymol.* 2000;325:389-400. [PubMed](#)

Gaits F, Hahn K. Shedding light on Cell Signaling: Interpretation of FRET Biosensors. *Sci STKE* 2003;(165):PE3. [PubMed](#)