Coumarin-labeled Phosphate Binding Protein (MDCC-PBP): Purification and labeling

Plasmid

pET22b_PstS_1

PstS ORF encoding the mature *E. coli* (A197C)PstS protein (PBP) containing a stop codon at the end of the gene in pET22b (inserted between Nde1 and Xho1 sites in the MCS).

Expression produces MEA at the N-terminus of the mature PstS: So there is an extra M at the N-terminus over the natural, mature PstS. A stop codon was also added at the end of the sequence BEFORE the plasmid-derived His tag. This gives PLY at the C-terminus at the C-terminus of the expressed protein, as in the natural PstS. The amino-acid numbering has been left as based on positions in the natural, mature protein.

Cautionary words

This protocol is provided "as is", to be used together with any published procedures. It is at the discretion of the user whether to modify or implement as written.

Publications

(MDCC-PBP)
Brune M, Hunter JL, Corrie JET, Webb MR. Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. Biochemistry. 1994;33:8262-71.

Brune M, Hunter JL, Howell SA, Martin SR, Hazlett TL, Corrie JET, et al. Mechanism of inorganic phosphate interaction with phosphate binding protein from Escherichia coli. Biochemistry. 1998;37:10370-80.

(*this plasmid*)
Arnold LH, Kunzelmann S, Webb MR, Taylor IA. A continuous enzyme-coupled assay for triphosphohydrolase activity of HIV-1 restriction factor SAMHD1. Antimicrob Agents Chemother. 2015;59:186-92. doi: 10.1128/aac.03903-14.

(*Phosphate contamination*)
Nixon, A. E.; Hunter, J. L.; Bonifacio, G.; Eccleston, J. F.; Webb, M. R., Purine nucleoside phosphorylase: its use in a spectroscopic assay for inorganic phosphate and to remove inorganic phosphate with the aid of phosphodeoxyribomutase. Anal. Biochem. 1998, 265, 299-307.

Webb, M. R., A fluorescent sensor to assay inorganic phosphate. In Kinetic Analysis of Macromolecules: a Practical Approach, Johnson, K. A., Ed. Oxford University Press: Oxford, U.K., 2003; pp 131-152.

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PBP preparation

1) Transform competent BL21(DE3) cells with this plasmid.
2) Obtain single colonies from an L. agar plate, containing 100 µg mL-1 ampicillin.
3) Cell growth and expression is in L. broth, containing 100µg mL-1 ampicillin.
   a) Inoculate a 10-mL starter culture with a colony from a plate and incubate ~7 h with shaking.
   b) Add to 90mL L. broth and incubate overnight at 37 °C with vigorous shaking.
   c) Dilute to 4 l L. and incubate at 37 °C with vigorous shaking in 8 2-litre baffle flasks until the A_{600n}m is 1.
   d) Add IPTG to 400 µM and continue to incubate as before for 3 h.
4) Harvest the cells by centrifuging at 4000 rpm for 25 min at 4 °C.
5) Resuspend the pellets in 20 mM Tris.HCl pH 8.0, 1 mM EDTA, 5 mM DTT.
6) Freeze in dry ice and store at –80 °C in two equal amounts.
7) Thaw one portion of cells, disrupt by sonication,
8) Spin down cell debris at 40,000 rpm for 1 h at 4 °C.
9) Dilute to the correct, initial ionic strength for the subsequent column chromatography
10) Load onto a 120 mL Q Sepharose FF column, equilibrated in 10 mM Tris.HCl pH 7.6,
    a) Wash with two column volumes in the same buffer.
    b) Apply a 1 l gradient of 0-200 mM NaCl in this buffer.
11) Identify PBP by PAGE
12) Pool PBP and concentrate to 1-1.5 mM in a spin concentrator (YM10 membrane or similar).
13) Make suitable aliquots, quick freeze and store at –80 °C.

Labeling (A197C)PBP with MDCC

1) We recommend that the procedure given in Webb (2003) is used for labeling and characterization of MDCC-PBP, but a full experimental protocol is given below for ~100 mg scale labeling.
2) MDCC (N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide) can be obtained from several commercial sources.
3) Follow the extra precautions, as described separately above and in Webb (2003), particularly in relation to phosphate contamination.
4) Materials:
   a) (A197C)PBP
   b) 20 mM MEG (7-methylguanosine)

c) 1000 u mL^{-1} PNPase (purine nucleoside phosphorylase)
d) 20 mM MDCC in dimethylformamide
e) 1 M Tris.HCl pH 8.1 (Prepared Pi free)
f) 200 mM Pipes.NaOH pH 7.0 (Prepared Pi free)
g) 0.2µm Polyethersulfone membrane (Puradisc 25AS Whatman)
h) 50mL Q Sepharose FF column (G E Healthcare) at 4 °C, equilibrated in 10 mM Tris.HCl pH 8.0
i) Spin concentrator with YM10 membrane or similar

5) In a 50 ml tube with secure top, mix and incubate the following for 15 min at room temperature:
   a) 100µM A197C PBP in 20mM Tris.HCl pH 8.1
   b) 200µM MEG
   c) 0.2 u mL^{-1} PNPase
6) Add 150 µM MDCC in DMF (*Note that the solution may go cloudy initially*)
7) Incubate at 22 °C, protected from light, mixed end-over-end for 30 min
8) Pass the incubation mixture through a 0.2 µm membrane and chill on ice *(The mixture can be stored on ice overnight before the column)*
9) Pre-equilibrate the Q-Sepharose column in the loading buffer (10 mM Tris.HCl pH 8.0) at ~1 mL min^{-1}.
10) Dilute the MDCC-PBP to 10 mM Tris.HCl pH 8.0 by adding an equal volume of cold (4 °C) water.
11) Load the column, monitoring absorbance at 280 nm, while collect fractions in plastic tubes.
12) Wash to baseline with the loading buffer, then apply a 1.2 L gradient in 10 mM Tris.HCl pH from 0 to 50mM NaCl.
13) To obtain higher quality MDCC-PBP (at the expense of yield):
   a) Scan select fractions 220-520 nm
   b) Record the absorbance at 280 nm and at the wavelength of the maximum absorbance of the MDCC peak (~432 nm).
   c) Calculate Abs 280nm / Abs at MDCC λ_{max}
   d) This ratio is indicative of the degree of contamination with free MDCC, unlabeled PBP or doubly-labeled PBP.
      i) Pool and concentrate fractions with a ratio of 1.3 or above.
14) Concentrate the pools in the spin concentrator: rinse the concentrator with Pi-free water before use.
      i) There will be some free MDCC which passes through the membrane. Once concentrated, pass cold 10mM Tris.HCl pH 8.0 through the solution until the eluate is no longer yellow.
15) Make suitable aliquots, quick freeze and store at –80 °C.

Characterization: concentration

1) Measure absorbance spectrum
2) Calculate the concentration of both the coumarin and the protein:
   a) [diethylaminocoumarin] = Abs at λ_{max} x dilution / 46.8 (mM)
   b) Assuming an extinction coefficient (at λ_{max}) of 46800 M^{-1} cm^{-1}.
   c) [PBP] = Abs at 280 nm –(abs at max λ x 0.164) x dilution / 61.9 (mM)
      i) Where 0.164 is the correction factor for absorbance of MDCC at 280 nm
      ii) and assuming an extinction coefficient of 61880 M^{-1} cm^{-1} for the protein.

Characterization: fluorescence spectra

1) ~ 1mL of a 5 µM solution of MDCC-PBP in 10 mM Pipes.NaOH pH 7.0 is needed for the spectra and titration (below), if using a 3 mm x 3 mm cuvette. This solution is kept on ice.
   a) Run an absorbance scan of some of this to get an accurate concentration.
2) The cuvette is first treated with phosphate mop.
   a) To 1 ml 10 mM Pipes.NaOH pH 7.0 buffer add 5 µL 20 mM MEG and 1 µL 1000 u mL^{-1} PNPase
   b) Fill the fluorescence cuvette with the mop solution and leave at room temperature for 10-15 min.
   c) Discard the mop solution and wash the cuvette with Pi-free water, then dry with absolute ethanol and air.
3) With excitation at 430 nm and at 20 °C, scan the MDCC-PBP solution fluorescence from 440-550 nm.
4) Add and mix 1 µL 2 mM Pi (so 10 µM Pi final) Scan again.
5) Add and mix 2 µL 20 mM MEG and 0.5 µL 1000 u mL^{-1} PNPase. Scan after 2-3 min: the signal should go down, if the mop is working.*(This will not remove all Pi).*
6) Clean the cuvette by filling and emptying several times in Pi-free water and dry.
7) Scan a second aliquot of the same 5 µM MDCC-PBP.
8) Add and mix 2 µL 20 mM MEG and 0.5 µL 1000 u mL^{-1} PNPase. Leave 10 min before scanning. (This gives the true apo-protein fluorescence).