**Rhodamine-labeled Phosphate Binding Protein (rho-PBP): Purification and labeling**

**Plasmid**

pET22b\_PstS\_2

*PstS* ORF encoding the mature *E. coli* (A17C,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**

(rho-PBP)

Okoh MP, Hunter JL, Corrie JET, Webb MR. A biosensor for inorganic phosphate using a rhodamine-labeled phosphate binding protein. Biochemistry. 2006;45:14764-71.

(*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.
   1. Inoculate a 10-mL starter culture with a colony from a plate and incubate ~7 h with shaking.
   2. Add to 90mL L. broth and incubate overnight at 37 °C with vigorous shaking.
   3. Dilute to 4 l L. and incubate at 37 °C with vigorous shaking in 8 2-litre baffle flasks until the A600nm is 1.
   4. 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,
    1. 1 mM DTT is added to the column buffers just before using.
    2. Wash with two column volumes in the same buffer.
    3. 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 (A17C,A197C)PBP with 6-IATR

1. To 50 mg A17C A197C PBP in a maximum volume of 2 ml, add a 10-fold molar equivalent of DTT and leave at room temperature for 15 min, in a sealed tube with nitrogen over.
2. Apply to a PD10 column containing Sephadex G-25, equilibrated in 25 ml 10 mM Tris⋅HCl pH 8.0, which has been degassed prior to use. Collect 500 µl fractions, while eluting with the same buffer. Pool the fractions containing PBP. Determine the PBP concentration from the absorbance and dilute to 100 µM in the same buffer.
3. Add phosphate mop: 200 µM 7-methylguanosine, 0.2 unit mL-1 bacterial purine nucleoside phosphorylase (Sigma N8264). Seal and treat with nitrogen as before and incubate for 15 min at room temperature.
4. From a concentrated solution in DMF, add 6-IATR (Toronto Research Chemicals) to 400 µM (2-fold excess over cysteines). Treat with nitrogen as before, seal and protect from light. Incubate at 22 °C, mixing end-over-end, for 2 h.
5. Using 100 mM MESNA stock solution, make the incubation solution 800 µM. Continue to incubate for 20 min as before, but without nitrogen.
6. Pass the incubation mixture through a 0.2 µm Polysulfone membrane and load on a Q Sepharose FF column (20 ml), pre-equilibrated in 10 mM Tris⋅HCl pH 8.0 at 4 °C, running at 1 ml min−1. Wash the column with 2-4 column volumes of this buffer. Apply a linear gradient of 400 mL 0-200 mM NaCl in 10 mM Tris.HCl pH 8.0.
7. Scan absorbance spectra of fractions at the peak from 220-660nm, recording the absorbance at 280 nm, 526 nm (isosbestic point), 515 nm (mainly rhodamine dimer) and 555nm (mainly monomer). (Dilute fractions to scan if necessary.) Calculate 515/555 nm ratio. Pool the fractions containing 6IATR-PBP with a ratio of >2.5.
8. Concentrate in a Vivaspin concentrator (10mL) or in an Amicon pressure concentrator with a YM10 membrane to ~1 ml. Dilute 10x with 10mM Tris.HCl pH 8.0 buffer, re-concentrate to ~1 ml. Dilute 10x dilution and re-concentrate, by this stage the eluate should be clear (no pink). If still pink, dilute and concentrate.
9. Scan the absorbance spectrum of the final 6IATR-PBP, and store in aliquots at –80 °C.
10. Characterize the labeled protein by its fluorescence excitation and emission spectra and titration with phosphate, as below.

## Characterization of 6IATR-PBP: Absorbance spectra

1. Good check as the spectral change with Pi is very characteristic
2. ~900 µL of a 5µM solution of 6IATR-PBP in 10mM Pipes/NaOH pH 7.0 (buffer diluted fresh and Pi free) is required. This is kept on ice and the same solution can be used for all the measurements. A 50 µL fluorescence cuvette is used for the scans and a 3x3 mmcuvette for the titration, a 100µL absorbance cuvette for absorbance spectra.
3. To 994 µL 10 mM Pipes.NaOH pH7.0 buffer, add 5µL 20mM 7-methylguanosine (MEG) and 1 µL 1000 u mL-1 “bacterial” purine nucleoside phosphorylase (PNPase) (“Pi mop” – see final references for details). Mix, fill fluorescence cuvettes with the mop solution and leave at room temperature for 10-15 min. This ensures the cuvette is Pi free. The PNPase should be kept on ice and quick frozen on dry ice after use. Store at –80°C, in 50 µL aliquots to prevent excessive freeze-thawing damage.
4. Discard the mop solution and wash the cuvette with Pi-free water and dry with absolute ethanol and hot air.
5. Scan 120 µL of the 5 µM 6IATR-PBP against 10 mM Pipes.NaOH pH7.0 from 220 -620 nm. Add Pi mop ( 1.5µL 20mM MEG + 0.5µL 1000 unit mL-1 PNPase) leave 10 min at RT repeat scan, overlaying the spectrum.
6. Scan a second aliquot of the 5 µM 6IATR-PBP. Add 1 µL 1.2 mM Pi (so final 10 µM) and repeat scan. Add 1 µL 1.2 mM Pi (to 19.6 µM total) and repeat scan.
7. Note: Absorbance of MEG of the mop swamps the A280 peak. Typical spectra are shown below: “-Pi” has Pi mop present.
8. The concentration of labeled protein can be obtained from the extinction coefficient in this buffer at the isosbestic point (526 nm), 108 mM–1 cm–1 for the double label (2 rhodamines attached to PBP).
9. The concentration of protein can be determined from the absorbance at 280 nm and is used to calculate the percent labeling. The extinction coefficient of PBP is 60880 M–1cm–1. A correction is required for the absorbance of rhodamine at 280 nm (extinction coefficient for 2 rhodamines ~62000 M–1cm–1). However, the combination of the large correction and the possibilities of impurity absorbance at 280 nm makes this somewhat inaccurate.

## Characterization of 6IATR-PBP: Fluorescence spectra

1. Emission spectra are used: Excitation 555 nm, Emission 560-660 nm, Temp. 20 °C
2. Scan 60 µL ~5 µM 6IATR-PBP.
3. Add 1 µL 610 µM Pi (10µM Pi final). Mix by pipetting up and down three times without causing bubbles. Scan again. Repeat addition of 1µL 610µM Pi (19.68 µM final) and scan again. Print out.
4. Add 1 µL 20 mM MEG and 0.5 µL 1000 u mL-1 PNPase: mix as before. Scan after 2-3 min, the signal should have decreased close to the original level. If not, the mop is not working although the signal will not decrease completely to starting levels or below for 10-15 min.
5. Clean the cuvette by filling and emptying several times in Pi-free water and dry as before.
6. Scan a second aliquot of 200 µL 5 µM 6IATR-PBP.
7. Add 1 µL 20 mM MEG and 0.5 µL 1000 u mL-1 PNPase, mix as before, leave 10 min. Scan this spectrum for Pi-free PBP.