

RpMatB (His₆/C106A/R286C/Q457C/K488A/T303A): preparation and labeling

Plasmid

pTEV5_RpMatB_4

The same protocol is used for other variants:

pTEV5_RpMatB_1	RpMatB (His ₆ /C106A/R286C/Q457C/K488A)
pTEV5_RpMatB_2	RpMatB (His ₆ /C106A/R286C/Q457C/K488A/S200A)
pTEV5_RpMatB_3	RpMatB (His ₆ /C106A/R286C/Q457C/K488A/T167A)
pTEV5_RpMatB_4	RpMatB (His ₆ /C106A/R286C/Q457C/K488A/T303A)

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

RpMatB (His₆/C106A/R286C/Q457C/K488A):

Vancraenenbroeck, R.; Webb, M. R., A fluorescent, reagentless biosensor for ATP, based on malonyl-coenzyme A synthetase. ACS Chem. Biol. 2015, 10, 2650-2657.

Vancraenenbroeck R, Kunzelmann S, Webb MR. Development of a range of fluorescent reagentless biosensors for ATP, based on malonyl-coenzyme A synthetase. PLOS ONE. 2017;12(6):e0179547.

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Protein expression and purification

RpMatB variant was synthesized in *E. coli* OverExpress C41(DE3) cells (Lucigen). The cell pellet of a 5 ml overnight culture, grown at 37 °C and supplemented with 100 µg ml⁻¹ ampicillin, was resuspended in 0.5 l of lysogeny broth, supplemented with ampicillin, in 5 l shaker flasks and cultured via vigorous shaking at 30 °C to an optical density at 600 nm of 0.6 - 0.8 cm⁻¹. Then 0.5 mM isopropyl-β-D-thiogalactoside was added to start induction at 30 °C for 16 h.

After induction, the cultures were cooled to 4 °C and centrifuged at 3500 rpm for 30 min at 4 °C (rotor JS 4.2, Beckman). The cell pellet was washed with 30 ml of ice-cold buffer (10 mM Tris HCl pH 7.5, 300 mM NaCl), centrifuged at 3500 rpm for 30 min at 4 °C, the supernatant discarded and the pellet stored at -80 °C. Typically ~3 g wet weight of *E. coli* cells were harvested from 0.5 l culture.

The cell pellet was resuspended in 35 ml 30 mM Tris HCl, 300 mM NaCl, 10 mM imidazole, 3 mM tris(2-carboxyethyl)phosphine, 2 mM phenylmethanesulfonyl fluoride, pH 8.0 and sonicated on ice using an ultrasonicator (VC505, Sonics) at 200 W for 5 times 30 s with a 5 s on / 5 s off pulser. The soluble fraction was collected by centrifugation at 35000 rpm for 45 min at 4 °C (rotor 45 Ti, Beckman).

The His₆-tagged protein was purified at 4 °C by immobilized metal ion affinity chromatography (1 ml HisTrap HP column, GE Healthcare) using an Äkta system (GE Healthcare). The resin was equilibrated with Buffer A (30 mM Tris.HCl, 300 mM NaCl, 10 mM imidazole, 1 mM tris(2-carboxyethyl)phosphine, pH 8.0). The sample was filtered (0.45 µm Minisart NML filter, Sartorius) and loaded onto the column at 0.5 ml min⁻¹. The column was washed with 20 ml Buffer A and additionally with 20 ml of 95 % Buffer A and 5 % Buffer B (30 mM Tris.HCl, 300 mM NaCl, 250 mM imidazole, 1 mM tris(2-carboxyethyl)phosphine, pH 8.0) at a flow rate of 1 ml min⁻¹. The protein was eluted with 20 ml of Buffer B at a flow rate of 1 ml min⁻¹.

Protein fractions were pooled (~2-4 ml) and further purified via size exclusion chromatography at 4 °C using the HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) equilibrated with 30 mM Tris.HCl, 100 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid, 5 mM dithiothreitol, 1 mM NaN₃ at 1 ml min⁻¹.

Fractions containing the protein were pooled and concentrated (VivaSpin 20 MWCO 10 kDa cut off, GE Healthcare) to ~10 mg ml⁻¹.

The protein concentration was determined from the absorbance at 280 nm using the extinction coefficient at 280 nm of 46300 M⁻¹ cm⁻¹.

The protein was drop-frozen in liquid nitrogen and stored at -80 °C. Typically, 45 mg of protein was obtained from 3 g wet weight of cells.

Labeling and purification

Dithiothreitol was removed from ~40 mg of protein using a PD10 desalting column (GE Healthcare) pre-equilibrated with Buffer L (30 mM Tris HCl pH 7.5, 100 mM NaCl) at 20 °C.

50 µM protein was incubated at 20 °C with 225 µM 5-iodoacetamidotetramethylrhodamine (5-IATR, AnaSpec, CA) in Buffer L using an end-over-end mixer for 90 min. Then 2 mM sodium-2-mercaptoethanesulfonate was added and incubation continued for 15 min.

After centrifugation at 3500 rpm for 15 min at 4 °C (Heraeus Biofuge), the supernatant was filtered through a 0.2 µm syringe filter (Acrodisc Syringe Filter with HT Tuffryn Membrane, Pall Life Sciences) and loaded onto a PD10 desalting column, equilibrated with Buffer Q1 (30 mM Tris.HCl pH 8.0, 25 mM NaCl) at -20 °C to remove free label.

The labeled protein was further purified via ion exchange chromatography at 4 °C using a 1 ml HiTrap Q HP column (GE Healthcare), equilibrated in Buffer Q1 at 1 ml min⁻¹. After sample loading, the column was washed with 90 ml Buffer Q1. The protein was eluted using a gradient from 100 % Buffer Q1 to 50 % Buffer Q1 and 50 % Buffer Q2 (30 mM Tris.HCl pH 8.0, 1 M NaCl) over 25 ml followed by a gradient from 50 % Buffer Q1 and 50 % Buffer Q2 to 100 % Buffer Q2 over 10 ml. Fractions containing the protein were pooled and concentrated to ~5 mg ml⁻¹ using a concentrator (Amicon Ultra-4 10 kDa cut off, Millipore).

The labeled protein concentration was determined using the following extinction coefficients: RpMatB: ε₂₈₀ (46300 M⁻¹cm⁻¹) and tetramethylrhodamine:

ϵ_{280} (31000 M⁻¹ cm⁻¹) and ϵ_{528} (52000 M⁻¹ cm⁻¹). The protein was drop-frozen in liquid nitrogen and stored at -80 °C. Labeling yields were up to 35%.