

Entry Clone Source: MGC
Entry Clone Accession: IMAGE:5270012
SGC Construct ID: TTKA-c013
GenBank GI number: gi 23308722
Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]
Tags and additions: Tag sequence: mhhhhhssgvdlgtenlyfq*s (m) TEV-cleavable (*) N-terminal his6 tag.
Final protein sequence: <pre> mhhhhhssgvdlgtenlyfq*smSVKGR IYSILKQIGSGGSSKVFQVLNEKKQIYAI KYVNL EEADNQTLD SYRNEIAYLNKLQQH SDKIIRLYDYEITDQYIYMVMCEGNIDLN SWLKKKKSIDPWERKSYWKNM LEAVHTIH QHGIVHSDLKPANFLIVDGMLKLIDFGIA NQMQPD TTSVVKDSQVGTVNYMPPEAIKD MSSSRENGKSKSKISPKSDVWSLGCILYY MTYGKTPFQQIINQISKLHAIIDPNHEIE FPDIPEKDLQDVLKCCLKRDPKQRISIPE LLAHPYVQIQTHLVNQMAKG TTEE </pre> The construct contains a mutation P797L highlighted in red
Host: BL21 (DE3) phage resistant Rosetta strain
Growth medium, induction protocol: 1ml from a 10 ml overnight culture containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol was used to inoculate 1 liter of LB media containing the same concentration of antibiotics. Cultures were grown at 37°C until the OD₆₀₀ reached ~0.3. After that the temperature was adjusted to 20°C. Expression was induced for 4 hours using 1mM IPTG at an OD₆₀₀ of 0.6. The cells were collected by centrifugation and the pellet resuspended in binding buffer and frozen. Binding buffer: 50mM HEPES pH 7.5; 500 mM NaCl; 5% glycerol, 10 mM imidazole.
Extraction method: Cell pellets were lysed by C5 high pressure homogenizer (Avestin). The lysate was centrifuged at 21,000 rpm for 60 minutes and the supernatant collected for purification.
Column 1: Ni-affinity chromatography.
Buffers: Binding buffer: Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl , 5% Glycerol, 10mM Imidazole. Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl , 30 mM Imidazole , 5% glycerol. Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl , 50 to 250 mM imidazole, 5% Glycerol.
Procedure: 5 ml of 50% Ni-NTA slurry (Qiagen) was applied to a 1.5 x 10 cm gravity column. The column was equilibrated with 50 ml binding buffer. The lysate was applied to the column which was subsequently washed with 50 ml wash buffer 1 and 2. The protein was eluted by gravity flow by applying 5 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150mM, 250 mM); fractions were collected until essentially all protein was eluted. The eluted protein was analyzed by SDS-PAGE. DTT was added to the protein sample to a final concentration of 5mM.
Column 2: Size exclusion chromatography (Superdex S75, 60 x 1cm)
SEC-Buffers: 50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM DTT.

Procedure: The fractions eluted of the Ni-affinity chromatography were concentrated to about 4 ml using Centricon concentrators (10kDa cut off). The concentrated protein was applied to a Superdex S200 column equilibrated in SEC buffer at a flow rate of 0.8 ml/min. Eluted fractions were 95% pure as judged by SDS-PAGE.

Protein concentration: Centricon with a 30 kDa cut off in SEC-buffer.

Mass Spectrometry: The mass of the protein was calculated to be 36064 Da and experimentally determined mass was 36785 Da for the His tag containing protein. The identity of the protein was reconfirmed to be correct by DNA sequencing both DNA strands of this expression construct. It is therefore likely that the difference in Mass is due to posttranslational modifications of the protein in regions not visible in the electron density.

Crystallization: Crystals were obtained using the vapor diffusion method and a protein concentration of 11 mg/ml by mixing 150nl of the concentrated protein with 150 nl of a well solution containing: 55w/v PEG 300; 0.25M NaCl; 6.4 pH Na/K phosphate.
Crystals of the pyrrolo-pyridine inhibitor complex were grown in 40% PEG300, 0.25M NaCl, 0.1M Na/K phosphate pH 5.6.
Crystals of the complex with K00943a, 9-cyclopentyl- 2-(4- (4-hydroxypiperidin- 1-yl)- 2-methoxyphenylamino)- 5-methyl- 8,9-dihydro- 5H-pyrimido [5,4-b][1,4] diazepin- 6(7H)- one (III), was grown by mixing 75 nL of protein (11 mg/ml) and 1 mM of the inhibitor with 150 nL of reservoir solution containing 25 % PEG3350, 0.2 M MgCl₂ and 0.1 M Bis-Tris pH 5.5.
Crystals grew to diffracting quality within 2-3 weeks in all cases.

Data Collection: Crystals were cryo-protected using the well solution supplemented with an additional 25% ethylene glycol and flash frozen in liquid nitrogen. Crystals of the pyrrolo-pyridine complex were frozen using the crystallization condition supplemented with 40% PEG300. Diffraction data were collected at the SLS beam line SAX10. Resolution: 2.4, 2.8 and 2.6 Å, respectively.