Entry Clone Source: Site-directed mutagenesis

Entry Clone Accession: n/a

SGC Construct ID: PCTK1A-c031

GenBank GI number: gi|5453860

Vector: pNIC-CH. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

TTAAGAAGGAGATATACTATGGAGACCTAC
ATTAAGCTGGACAAACTGGGCGAGGGTACC
TATGCCACCGTCTACAAAGGCAAAAGCAAG
CTCACAGACAACCTTGTGGCACTCAAGGAG
ATCAGACTGGAACATGAAGAGGGGGCACCC
TGCACCGCCATCCGGGAAGTGTCCCTGCTC
AAGGACCTCAAACACGCCAACATCGTTACG
CTACATGACATTATCCACACGGAGAAGTCC
CTCACCCTTGTCTTTGAGTACCTGGACAAG
GACCTGAAGCAGTACCTGGATGACTGTGGG
AACATCATCAACATGCACAACGTGAAACTG
TTCCTGTTCCAGCTGCTCCGTGGCCTGGCC
TACTGCCACCGGCAGAAGGTGCTACACCGA
GACCTCAAGCCCCAGAACCTGCTCATCAAC
GAGAGGGGAGAGCTCAAGCTGGCTGACTTT
GGCCTGGCCCGAGCCAAGTCAATCCCAACA
AAGACATAC**GA**CAATGAGGTGGTGACACTG
TGGTACCGGCCCCCTGACATCCTGCTTGGG
TCCACGGACTACTCCACTCAGATTGACATG
TGGGGTGTGGGCTGCATCTTCTATGAGATG
GCCACAGGCCGTCCCCTCTTTCCGGGCTCC
ACGGTGGAGGAACAGCTACACTTCATCTTC
CGTATCTTAGGAACCCCAACTGAGGAGACG
TGGCCAGGCATCCTGTCCAACGAGGAGTTC
AAGACATACAACTACCCCAAGTACCGAGCC
GAGGCCCTTTTGAGCCACGCACCCCGACTT
GATAGCGACGGGGCCGACCTCCTCACCAAG
CTGTTGCAGTTTGAGGGTCGAAATCGGATC
TCCGCAGAGGATGCCATGAAACATCCATTC
TTCCTCAGTCTGGGGGAGCGGATCCACAAA
CTTCCTGACACTACTTCCATATTTGCACTA
AAGGAGATTCAGCTACAAAAGGAGGCCAGC
CTTCGGTCTGCGCACCATCATCACCACCAT
T

Expressed protein sequence:

METYIKLDKLGEGTYATVYKGKSKLTDNLV
ALKEIRLEHEEGAPCTAIREVSLLKDLKHA
NIVTLHDIIHTEKSLTLVFEYLDKDLKQYL
DDCGNIINMHNVKLFLFQLLRGLAYCHRQK
VLHRDLKPQNLLINERGELKLADFGLARAK
SIPTKTY**D**NEVVTLWYRPPDILLGSTDYST
QIDMWGVGCIFYEMATGRPLFPGSTVEEQL

HFIFRILGTPTEETWPGILSNEEFKTYNYP
KYRAEALLSHAPRLDSDGADLLTKLLQFEG
RNRISAEDAMKHPFFLSLGERIHKLPDTTS
IFALKEIQLQKEASLRSAHHHHHH
Engineered **S319D** phospho-mimetic mutation shown bold and underlined.

Host: BL21(DE3)-R3-pRARE2

Growth medium, induction protocol: A glycerol stock was used to inoculate a 10ml starter culture containing LB media with 50µg/ml Kanamycin and 34 µg/ml chloramphenicol. The starter culture was grown overnight at 37°C with shaking at 200 rpm. The following morning, four flasks containing 1 L LB/kanamycin/chloramphenicol were each inoculated with 5 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD600nm ≥ 0.7 was reached. The flasks were then cooled down to 18°C and 0.5mM IPTG added to induce protein expression overnight. Cells were harvested by centrifugation at 4500 rpm at 4°C for 15 min. Cell pellets from each flask were resuspended in 30ml binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole), transferred to 50 ml tubes, and stored at -20°C.

Extraction buffer, extraction method: The frozen cells were thawed and 0.5mM TCEP, 1mM PMSF added to the cell suspension. The cells were lysed by ultrasonication over 12 min with the sonicator pulsing ON for 5 sec and OFF for 15 sec. The cell lysate was spun down by centrifugation at 17000 rpm at 4°C for 1 h. The supernatant was recovered for purification.

Column 1: Ni-Affinity Chromatography**:** 5ml of 50 % Ni-sepharose slurry (Amersham) was applied onto a 1.5 x 10 cm column. The column was first washed with deionised distilled H_2O, and then equilibrated with binding buffer.

Column 1 Buffers:
Binding buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole
Wash buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole
Elution buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 50 to 250mM Imidazole

Column 1 Procedure: The supernatant was applied by gravity flow onto the Ni-sepharose column. The bound protein was eluted by applying a step gradient of imidazole (5 ml fractions of elution buffer supplemented with 50mM, 100mM, 150mM and 250mM imidazole). 10mM DTT was added to each fraction collected for overnight storage at 4°C.

Enzymatic treatment:: N/A

Column 2: Size Exclusion Chromatography - S200 HiLoad 16/60 Superdex run on ÄKTA-Express.

Column 2 Buffers: Gel Filtration buffer: 300mM NaCl, 50mM HEPES pH 7.5, 0.5mM TCEP

Column 2 Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. Eluted protein from the Ni-sepharose column was pooled and concentrated to 3ml using an Amicon Ultra-15 filter with a 10kDa cut-off. The concentrated protein was directly applied onto the equilibrated S200 16/60 column, and run at a flow-rate of 1 ml/min. Fractions containing the protein were pooled together, and 10mM DTT was added for overnight storage at 4°C.

Column 3: Anion Exchange Chromatography (monoQ)

Column 3 Buffers:
Buffer A: 50mM TRIS, pH 9.0

Buffer B: 50mM TRIS, pH 9.0; 1M NaCl

Column 3 Procedure: PCTK1 protein from gel filtration was buffer exchanged into 50mM Tris pH 9.0 and loaded onto a 1 ml monoQ anion exchange column equilibrated in the same buffer. A linear elution gradient was run from 0-1M NaCl. PCTK1 containing fractions were pooled and the buffer adjusted to 25 mM HEPES, pH 7.5, 250 mM NaCl, 5 % Glycerol, 10 mM DTT during concentration in a 5 kD MWCO Amicon Ultra concentrator.

Concentration: The protein was concentrated in a 5 kD MWCO Amicon Ultra concentrator to 15 mg/ml using an estimated extinction coefficient of 35870 and MW of 37252.

Mass spectrometry characterization: The purified native protein was homogeneous and had an experimental mass of 37252.74 (expected MW = 37252). Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser.

Crystallisation: Protein was buffered in 25 mM HEPES, pH 7.5, 250 mM NaCl, 5 % Glycerol, 10 mM DTT. Protein was concentrated to 15 mg/ml in the presence of indirubin E804 (final inhibitor concentration of 1 mM). Crystals were grown by micro-seeding at 20°C in 130 nl sitting drops mixing 96 nl protein solution with 10 nl micro-seed solution (crystals prepared from the same precipitant) and 24 nl of a reservoir solution containing 2.1M Na-formate pH 7.0, 0.1 M Bis-Tris pH 7.0. On mounting crystals were cryo-protected with reservoir solution mixed with 25% glycerol.

Data Collection: Resolution: Resolution: 2.4 Å, X-ray source: Diamond I03. The native crystal diffracted to a resolution of 2.4 Å.