Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: PKMYT1A-c004

GenBank GI number: gi|33383241

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Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

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CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGCACCAGTTGCAACCTCGT
CGTGTTTCTTTTCGCGGCGAGGCTTCAGAA
ACCTTGCAGAGCCCAGGTTATGATCCGTCC
CGCCCAGAAAGTTTCTTTCAACAGTCGTTC
CAACGCCTCAGCCGTCTGGGACATGGTTCT
TACGGCGAAGTATTCAAAGTACGCTCCAAA
GAGGACGGCCGTTTGTATGCGGTGAAACGC
TCAATGAGTCCGTTTCGTGGCCCCAAAGAT
CGCGCCCGTAAGCTGGCGGAAGTTGGCTCT
CACGAGAAAGTCGGCCAGCATCCGTGTTGC
GTACGCCTGGAGCAAGCCTGGGAAGAAGGC
GGTATTCTGTACCTGCAGACAGAGCTGTGC
GGCCCTTCACTGCAACAGCACTGTGAGGCG
TGGGGTGCAAGTTTGCCAGAAGCTCAAGTG
TGGGGTTATCTGCGTGACACCTTGCTGGCC
TTGGCACATCTGCATTCGCAGGGTTTGGTT
CACCTGGATGTCAAGCCCGCCAACATCTTC
CTGGGCCCTCGTGGTCGTTGTAAACTGGGC
GATTTTGGCCTGTTGGTGGAGTTGGGCACT
GCGGGTGCTGGTGAAGTACAAGAAGGCGAT
CCGCGTTACATGGCACCAGAATTGCTGCAG
GGCTCTTATGGTACAGCCGCGGATGTTTTC
AGCCTGGGCTTGACTATTCTGGAGGTAGCT
TGCAATATGGAGCTGCCCCACGGCGGCGAA
GGTTGGCAACAGTTGCGCCAAGGTTACCTG
CCCCCTGAGTTTACCGCCGGTTTGTCATCC
GAGTTGCGCAGTGTACTGGTCATGATGCTG
GAGCCCGATCCGAAACTGCGCGCAACAGCT
GAAGCATTGCTGGCTTTGCCCGTTTTGCGC
CAACCATGACAGTAAAGGTGGATACGGATC
CGAA
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Final protein sequence (tag sequence in lowercase):

mhhhhhhssgvdlgtenlyfq^smHQLQPRRVSFR
GEASETLQSPGYDPSRPESFFQQSFQRL
SRLGHGSYGEVFKVRSKEDGRLYAVK
RSMSPFRGPKDRARKLAEVGSHEKVG
QHPCCVRLEQAWEEGGILYLQTELCGP
SLQQHCEAWGASLPEAQVWGYLRDTL

LALAHLHSQGLVHLDVKPANIFLGPRG
RCKLGDFGLLVELGTAGAGEVQEGDPR
YMAPELLQGSYGTAADVFSLGLTILEVA
CNMELPHGGEGWQQLRQGYLPPEFTA
GLSSELRSVLVMMLEPDPKLRATAEAL
LALPVLRQP

^ TEV cleave site

Tags and additions: Cleavable N-terminal His6 tag.

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain)

Growth medium, induction protocol: 5ml from a 50ml overnight culture containing 50µg/ml kanamycin & 34µg/ml chloramphenicol were used to inoculate each of two 1 litre cultures of LB containing 50µg/ml kanamycin & 34µg/ml chloramphenicol. Cultures were grown at 37°C until the OD_{600} reached ~0.5 then the temperature was adjusted to 18°C. Expression was induced overnight using 0.5 mM IPTG at an OD_{600} of 0.9. The cells were collected by centrifugation and the pellet re-suspended in binding buffer and frozen.
Binding buffer: 50 mM HEPES pH 7.5; 500 mM NaCl; 5 mM imidazole, 5 % glycerol.
Extraction buffer, extraction method: Frozen pellets were thawed and fresh 0.5 mM TCEP added to the lysate. Cells were lysed using C5 high pressure homogenizer (Avestin). The lysate was centrifuged at 16,500 rpm for 60 minutes and the supernatant collected for purification.

Column 1: Ni-affinity. Ni-Sepharose (Amersham), 5 ml of 50 % slurry in 1.5 x 10 cm column, washed with binding buffer.

Column 1 Buffers:
Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% Glycerol.
Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 25 mM Imidazole, 5% glycerol.
Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 50 to 250 mM Imidazole, 5% Glycerol (step elution).

Column 1 Procedure: The lysate supernatant was loaded by gravity flow on the Ni-sepharose column. The column was then washed with 30 ml wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5-ml portions of elution buffer with increasing concentration of imidazole (50mM, 100mM, 150mM and 250mM); fractions were collected until essentially all protein was eluted.

Column 2: Size Exclusion Chromatography. Superdex S200 16/60 HiLoad

Column 2 Buffer: 25 mM HEPES, pH 7.5; 500 mM NaCl, 0.5 mM TCEP

Column 2 Procedure: The protein was concentrated and applied to an S200 16/60 HiLoad gel filtration column equilibrated in 25 mM HEPES, pH 7.5; 500 mM NaCl, 0.5 mM TCEP using an ÄKTA express system.

Mass spectrometry characterization : LC- ESI -MS TOF revealed that the recombinant protein had a mass of 34535 correlating well with the estimated mass of 34453.2 plus 1 phosphorylation site.

Protein concentration: Protein was concentrated to 12 mg/ml using an Amicon 10 kDa cut-off concentrator.

Crystallisation: Crystals in complex with K00032 ("Calbiochem (EMD) #266788") were grown at 4°C in 300 nl sitting drops from a 1:2 ratio of reservoir solution ("0.20M Na2SO4; 0.1M BTProp pH 6.5; 20.0% PEG 3350; 10.0% EtGly") and protein.

Data Collection: Crystals were cryo-protected using the well solution supplemented with 22 % Ethylene glycol and flash frozen in liquid nitrogen.
X-ray source: Diffraction data were collected from a single crystal for each dataset on Diamond beamline IO4 at a single wavelength.