Entry Clone Source: n/a

Entry Clone Accession: n/a

SGC Construct ID: MMACHCA-c001

GenBank GI number: gi|153070822

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

Amplified construct sequence:

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TACTTCCAATCCATGGAGCCGAAAGT
CGCAGAGCTGAAGCAGAAGATCGAGG
ACACGCTATGTCCTTTTGGCTTCGAG
GTTTACCCCTTCCAGGTGGCATGGTA
CAATGAACTCTTGCCTCCAGCCTTCC
ACCTACCGCTGCCAGGACCTACCCTG
GCCTTCCTGGTACTCAGCACGCCTGC
CATGTTTGACCGGGCCCTCAAGCCCT
TCTTGCAGAGCTGCCACCTCCGAATG
CTGACTGACCCAGTGGACCAGTGTGT
GGCCTACCATCTGGGCCGTGTTAGAG
AGAGCCTCCCAGAGCTGCAGATAGAA
ATCATTGCTGACTACGAGGTACACCC
CAACCGACGCCCCAAGATCCTGGCCC
AGACAGCAGCCCATGTAGCTGGGGCT
GCTTACTACTACCAACGACAAGATGT
GGAGGCTGACCCATGGGGGAACCAGC
GCATATCAGGTGTGTGCATACACCCC
CGATTTGGGGGCTGGTTTGCCATCCG
AGGGGTAGTGCTGCTGCCAGGGATAG
AGGTGCCAGATCTGCCACCCAGAAAA
CCTCATGACTGTGTACCTACAAGAGC
TGACCGTATCGCCCTACTCGAAGGCT
TCAATTTCCACTGGCGTGATTGGACT
TACCGGGATGCTGTGACACCCCAGGA
GCGCTACTCAGAAGAGCAGAAGGCCT
ACTTCTCCACTCCACCTGCCCAACGA
TTGGCCCTATTGGGCTTGGCTCAGCC
CTCAGAGAAGCCTAGTTCTCCCTCCC
CGGACCTTCCCTTTACCACACCCGCC
CCCAAGAAGCCTGGGAATCCCAGCAG
AGCCCGGAGCTGGCTCAGCCCCAGGG
TCTCACCACCTGCATCCCCTGGCCCT
TGACAGTAAAGGTGGATA
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smEP
KVAELKQKIEDTLCPFGFEVYPFQVA
WYNELLPPAFHLPLPGPTLAFLVLST
PAMFDRALKPFLQSCHLRMLTDPVDQ
CVAYHLGRVRESLPELQIEIIADYEV
HPNRRPKILAQTAAHVAGAAYYYQRQ
DVEADPWGNQRISGVCIHPRFGGWFA
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IRGVVLLPGIEVPDLPPRKPHDCVPT
RADRIALLEGFNFHWRDWTYRDAVTP
QERYSEEQKAYFSTPPAQRLALLGLA
QPSEKPSSPSPDLPFTTPAPKKPGNP
SRARSWLSPRVSPPASPGP

^ TEV cleavage site

Tags and additions: Cleavable N-terminal His_6 tag.

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

Growth medium, induction protocol: The construct DNA was transformed into competent cells of the expression strain by a standard heat shock procedure. One colony from the transformation was used to inoculate 1ml of TB media containing 50µg/ml kanamycin and 34µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture. A glycerol stock was used to inoculate 50ml of TB media containing 50µg/ml kanamycin and 34µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day cells were pelleted, resuspended in minimal media (MM) and subsequently inoculated into 12L of MM (10ml starter culture per 1L). When OD_{600} reached ~0.5, cells were supplemented with 100mg/l of Lys, Thr and Phe, 50mg/l of Leu, Ile and Val and 25mg/l Se-Met. Cells were grown until OD_{600} reached ~0.8. Temperature was then reduced to 18°C and after a further 30 minutes cells were induced by adding 0.1 mM IPTG, and were further supplemented with 50mg/l Se-Met. The expression was continued overnight. Cells were harvested by centrifugation at 6000g after which the supernatant was poured out and the cell pellet either placed in a -20°C freezer or used directly for purification.
Lysis buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 10 mM Imidazole; 5% Glycerol; 0.5 mM TCEP; 1 tablet per 50ml protease inhibitor cocktail EDTA-free (Roche).
Extraction buffer, extraction method: Cell pellets were dissolved in approximately 100ml lysis buffer and broken by passing through a high pressure homogenizer at 15,000psi for 4 cycles. The cell debris was pelleted at 35,000g and the supernatant used for further purification.

Column 1: Ni-NTA (2.5ml volume in a gravity-flow column).

Column 1 Buffers:
Binding buffer : 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 10 mM Imidazole; 0.5 mM TCEP.
Wash buffer : 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 40 mM Imidazole; 0.5 mM TCEP.
Elution buffer : 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 250 mM Imidazole; 0.5 mM TCEP.

Column 1 Procedure: The clarified cell extract was incubated with 2.5ml of Ni-NTA pre-equilibrated with lysis buffer for 1 hour at 4°C with rotation after which it was passed through a glass column. The column was then washed with 100ml of Binding Buffer and 50ml of Wash Buffer. The protein was eluted with 15ml of Elution Buffer in 5ml fractions.

Column 2: Superdex 200 16/60 Gel Filtration.

GF buffer: 10 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 0.5 mM TCEP.

Column 2 Procedure: The elution buffer fractions from column 1 were pooled and concentrated to 4ml with a 10kDa mwco spin concentrator and injected onto an S200 16/60

column (pre-equilibrated in GF Buffer) at 1.2ml/min. 1.8ml fractions were collected.

Enzymatic treatment: Eluted protein fractions were pooled and incubated with 1:20 mol:mol TEV protease overnight at 4°C. Then protein plus TEV was passed through a column containing 0.5ml Ni-NTA pre-equilibrated with GF Buffer. Column was washed 1ml of GF Buffer. Flow-through and wash were pooled.

Column 3: 1ml Resource Q Cation Exchange.

Column 3 Buffers:
Buffer A : 50 mM MES, pH 6.0; 50 mM NaCl; 5% glycerol; 1 mM TCEP.
Buffer B : 50 mM MES, pH 6.0; 2 M NaCl; 5% glycerol; 1 mM TCEP.

Column 3 Procedure: Protein from flow-through and wash were concentrated to 700µl using a 10kDa mwco spin concentrator, diluted to 10ml using Buffer A and injected into a 1ml Resource S Column. Protein was eluted using a linear gradient of 0-100% Buffer B over 35 column volumes at 1ml/min. 1.0ml fractions were collected.

Protein concentration: Fractions containing protein were pooled and concentrated to 15mg/ml using a 10kDa MWCO concentrator.

Mass spectrometry characterization: after TEV protease digestion:
Measured mass: 31957.0Da
Expected mass: 31956.2Da

Crystallisation: Crystals were grown by vapour diffusion at 20°C in sitting drops containing 50nl protein (17mg/ml; pre-incubated with 1 mM adenosylcobalamin) and 100nl mother liquor containing 22-24% (v/v) PEG3350, 0.25 mM ammonium citrate pH 5.0. Crystals were cryo-protected in mother liquor containing 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data collection:
Resolution: 2.40Å
X-ray source: Diamond Light Source beamline I24.