Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: ACACAA-c104

GenBank GI number: gi|38679960

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

Amplified construct sequence:

ATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACC
TGTACTTCCAATCCATGTCCGGCTTG
CACCTGGTTAAGCAGGGTCGCGACCG
TAAGAAAATCGATTCTCAACGCGACT
TTACTGTCGCGTCACCAGCAGAGTTC
GTAACACGTTTTGGCGGTAATAAGGT
GATCGAGAAAGTTTTGATTGCCAACA
ATGGCATCGCTGCGGTCAAGTGTATG
CGCAGTATTCGTCGCTGGTCGTACGA
AATGTTCCGTAACGAGCGCGCCATCC
GTTTCGTGGTAATGGTGACCCCTGAG
GACCTCAAGGCAAACGCTGAGTATAT
TAAGATGGCCGACCATTACGTTCCGG
TCCCCGGCGGCCCAAATAACAATAAC
TATGCGAACGTCGAGCTGATCTTGGA
TATTGCAAAGCGCATCCCTGTGCAGG
CTGTTTGGGCCGGTTGGGGCCACGCG
AGCGAGAATCCGAAACTGCCCGAACT
CCTCCTCAAGAACGGTATCGCTTTTA
TGGGCCCACCTTCCCAGGCCATGTGG
GCACTGGGTGACAAAATTGCATCTTC
AATCGTCGCTCAAACTGCCGGCATTC
CGACACTGCCCTGGAGTGGTAGCGGC
TTGCGTGTGGATTGGCAGGAAAATGA
CTTCTCCAAACGCATCCTGAACGTAC
CCCAAGAGTTGTACGAAAAGGGTTAT
GTTAAAGATGTCGACGATGGCCTGCA
GGCAGCCGAGGAAGTGGGTTATCCCG
TAATGATTAAGGCGTCGGAGGGCGGC
GGCGGTAAAGGCATCCGTAAAGTAAA
CAATGCTGACGATTTTCCGAACCTGT
TCCGCCAAGTTCAGGCCGAAGTCCCA
GGTTCTCCCATCTTTGTGATGCGTTT
GGCAAAGCAGTCACGCCATCTGGAGG
TACAAATCTTGGCGGACCAGTATGGC
AATGCTATCAGTCTGTTCGGTCGTGA
TTGCAGCGTTCAACGCCGTCACCAGA
AAATTATCGAAGAGGCCCCTGCAACC
ATTGCGACTCCGGCTGTATTCGAACA
CATGGAGCAGTGTGCCGTGAAGTTGG
CAAAGATGGTGGGCTACGTATCCGCG
GGTACCGTTGAATATCTGTACTCGCA

AGACGGCTCGTTTTACTTCCTGGAGT
TGAACCCACGCCTGCAGGTCGAACAC
CCCTGCACAGAAATGGTAGCTGATGT
CAATTTGCCTGCCGCACAACTGCAGA
TCGCGATGGGTATTCCGCTGTATCGT
ATCAAAGACATTCGCATGATGTACGG
TGTTTCACCCTGGGGCGATAGCCCCA
TCGACTTTGAGGATAGTGCTCATGTC
CCTTGTCCGCGTGGTCACGTGATCGC
CGCTCGCATTACCTCCGAAAACCCAG
ACGAGGGCTTCAAGCCCTCGTCTGGT
ACTGTACAAGAATTGAACTTTCGTTC
AAATAAGAACGTTTGGGGCTATTTCA
GCGTCGCCGCAGCGGGCGGCCTGCAT
GAGTTCGCTGATTCCCAGTTTGGTCA
CTGCTTCAGTTGGGGCGAAAATCGCG
AGGAAGCCATCTCTAACATGGTAGTG
GCCTTGAAGGAGCTGAGCATTCGTGG
TGACTTTCGCACAACCGTAGAATACT
TGATCAAACTGCTGGAGACTGAATCG
TTCCAGATGAACCGTATTGATACCGG
CTGGTTGGACCGCCTGATCTGA

Final protein sequence (Tag sequence in lowercase):

mhhhhhhssgvdlgtenlyfq^smSG
LHLVKQGRDRKKIDSQRDFTVASPAE
FVTRFGGNKVIEKVLIANNGIAAVKC
MRSIRRWSYEMFRNERAIRFVVMVTP
EDLKANAEYIKMADHYVPVPGGPNNN
NYANVELILDIAKRIPVQAVWAGWGH
ASENPKLPELLLKNGIAFMGPPSQAM
WALGDKIASSIVAQTAGIPTLPWSGS
GLRVDWQENDFSKRILNVPQELYEKG
YVKDVDDGLQAAEEVGYPVMIKASEG
GGGKGIRKVNNADDFPNLFRQVQAEV
PGSPIFVMRLAKQSRHLEVQILADQY
GNAISLFGRDCSVQRRHQKIIEEAPA
TIATPAVFEHMEQCAVKLAKMVGYVS
AGTVEYLYSQDGSFYFLELNPRLQVE
HPCTEMVADVNLPAAQLQIAMGIPLY
RIKDIRMMYGVSPWGDSPIDFEDSAH
VPCPRGHVIAARITSENPDEGFKPSS
GTVQELNFRSNKNVWGYFSVAAAGGL
HEFADSQFGHCFSWGENREEAISNMV
VALKELSIRGDFRTTVEYLIKLLETE
SFQMNRIDTGWLDRLI

^ 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 this starter culture was used to inoculate 6L of TB media (7.5 ml starter culture used per 1L) containing 50µg/ml kanamycin. When the OD_{600} reached approximately 1.0 the temperature was reduced to 18°C and after a further 30 minutes the cells were induced by the addition of 0.1 mM IPTG. 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 7.4; 500 mM NaCl; 5% glycerol; 10 mM Imidazole; 0.5 mM TCEP; 1 tablet per 50ml protease inhibitor cocktail EDTA-free (Roche).
Extraction buffer, extraction method: Cell pellets were dissolved in approximately 200ml lysis buffer and broken by sonication at 35% power for 15 min. The cell debris was pelleted at 35,000g and the supernatant used for further purification.

Column 1: Ni-NTA (3.0ml volume in a gravity-flow column).

Column 1 Buffers:
Binding buffer: 50 mM HEPES, pH 7.4; 500 mM NaCl; 5% glycerol; 10 mM Imidazole; 0.5 mM TCEP.
Wash buffer: 50 mM HEPES, pH 7.4; 500 mM NaCl; 5% glycerol; 40 mM Imidazole; 0.5 mM TCEP.
Elution buffer: 50 mM HEPES, pH 7.4; 500 mM NaCl; 5% glycerol; 250 mM Imidazole; 0.5 mM TCEP.

Column 1 Procedure: The clarified cell extract was incubated with 3.0ml 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 60ml of Binding Buffer and 50ml of Wash Buffer. The protein was eluted with 30ml of Elution Buffer in 5x6ml fractions.

Column 2: Superdex 200 16/60 Gel Filtration.

Column 2 Buffer: 10 mM HEPES, pH 7.4; 500 mM NaCl; 5% glycerol; 0.5 mM TCEP.

Column 2 Procedure: The wash buffer fractions and elution buffer fractions from column 1 were pooled separately and concentrated to 5ml with a 30kDa mwco spin concentrator and injected onto an S200 16/60 column (pre-equilibrated in GF Buffer) at 1.0ml/min. 1.0ml fractions were collected.

Enzymatic treatment: Protein from fractions eluted at 80-90ml from S200 gel filtration 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 was pooled.

Column 3: 1ml Resource Q Cation Exchange.

Column 3 Buffers:
Buffer A: 50 mM HEPES, pH 7.5; 50 mM NaCl.
Buffer B: 50 mM HEPES, pH 7.5; 2 M NaCl.

Column 3 Procedure: Protein from flow-through, approximately 10ml, was diluted to 100ml 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: Two fractions of protein eluted at 4-6% Buffer B were pooled and concentrated to 14mg/ml using a 30kDa mwco concentrator.

Mass spectrometry characterization: After TEV protease digestion:
Measured mass: 62536.2Da
Expected mass: 62638.0Da

Crystallisation: Crystals were grown by vapour diffusion in sitting drop at 20°C. A sitting drop consisting of 75nl protein and 75nl well solution was equilibrated against well solution containing 30% (v/v) LMW PEG smear and 0.1 M Tris pH 8.5. Crystals were mounted in the presence of 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data collection:
Resolution: 2.10Å.
X-ray source: Diamond Light Source beamline I02.