Entry Clone Source: MGC

Entry Clone Accession: gi|4821265

SGC Construct ID: KIAA1576A-c212

GenBank GI number: gi|4821265

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

Amplified construct sequence:

CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGGAGATG
CGCGCGGTGGTGCTGGCTGGCTTCGG
GGGGCTCAACAAGCTGCGGCTCTTCA
GGAAGGCCATGCCCGAGCCTCAGGAC
GGCGAGCTCAAGATCCGCGTCAAAGC
CTGTGGATTAAACTTCATTGACTTGA
TGGTGCGACAAGGGAATATTGACAAC
CCTCCCAAGACTCCCCTGGTGCCAGG
ATTTGAGTGTTCTGGGATTGTTGAAG
CTCTGGGGGACAGCGTGAAAGGATAT
GAGATTGGAGACCGTGTCATGGCATT
TGTCAATTACAATGCCTGGGCAGAGG
TGGTCTGCACACCAGTGGAGTTTGTC
TACAAGATCCCGGATGACATGAGCTT
CTCGGAGGCTGCTGCATTCCCCATGA
ACTTCGTCACAGCCTATGTGATGCTG
TTTGAAGTTGCCAACCTCCGGGAAGG
GATGTCTGTGCTCGTGCACTCAGCTG
GTGGGGGCGTGGGTCAAGCTGTGGCT
CAGCTGTGTTCCACTGTCCCCAACGT
GACTGTCTTTGGAACAGCCTCTACTT
TCAAGCATGAAGCAATCAAAGACTCT
GTGACCCACCTCTTTGACAGAAATGC
AGACTACGTGCAAGAAGTTAAAAGAA
TCTCTGCTGAAGGTGTGGACATCGTT
TTGGATTGCCTCTGTGGGGACAACAC
TGGAAAAGGTCTCAGTCTTCTCAAAC
CCCTGGGAACCTACATTTTATATGGC
TCATCCAACATGGTAACTGGAGAGAC
CAAGAGCTTCTTCAGCTTTGCAAAAT
CATGGTGGCAGGTGGAGAAGGTGAAC
CCCATCAAGCTGTATGAGGAGAACAA
AGTCATCGCGGGGTTTTCCCTTTTAA
ATCTGCTCTTCAAACAAGGCCGGGCG
GGCCTCATTCGGGGAGTGGTGGAAAA
ACTCATAGGGCTCTACAACCAGAAGA
AGATCAAGCCTGTGGTGGACTCCTTG
TGGGCTCTGGAGGAGGTGAAGGAGGC
CATGCAGCGGATTCACGACCGAGGGA
ACATTGGCAAGTTAATTCTGGATGTA

GAAAAGACCCCAACTCCACTGTGACA
GTAAAGGTGGATACGGATCCGAA

Final protein sequence (Tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq^smEM
RAVVLAGFGGLNKLRLFRKAMPEPQD
GELKIRVKACGLNFIDLMVRQGNIDN
PPKTPLVPGFECSGIVEALGDSVKGY
EIGDRVMAFVNYNAWAEVVCTPVEFV
YKIPDDMSFSEAAAFPMNFVTAYVML
FEVANLREGMSVLVHSAGGGVGQAVA
QLCSTVPNVTVFGTASTFKHEAIKDS
VTHLFDRNADYVQEVKRISAEGVDIV
LDCLCGDNTGKGLSLLKPLGTYILYG
SSNMVTGETKSFFSFAKSWWQVEKVN
PIKLYEENKVIAGFSLLNLLFKQGRA
GLIRGVVEKLIGLYNQKKIKPVVDSL
WALEEVKEAMQRIHDRGNIGKLILDV
EKTPTPL

^ TEV cleavage site

Tags and additions: N-terminal, TEV protease cleavable hexahistidine tag

Host: BL21(DE3)pLysS

Transformation: The construct DNA was transformed into competent cells of the expression strain by a standard heat shock procedure.

Glycerol stock preparation: One colony from the transformation was used to inoculate 1 ml of TB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture.

Expression: A glycerol stock was used to inoculate 50 ml of TB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day this starter culture was used to inoculate 12L of TB media (3.5 ml starter culture used per 1L) containing 50 µg/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

Cell harvest: Cells were harvested by centrifugation at 6000 x g after which the supernatant was poured out and the cell pellet either placed in a -20°C freezer or used directly for purification.

Cell Lysis: Cell pellets were dissolved in approximately 150ml lysis buffer and broken by homogenization by 5 passes at 12,000 psi. The cell debris was pelleted at 35,000 x g and the supernatant used for further purification
Lysis buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 0.5 mM TCEP, 1 tablet per 50 ml protease inhibitor cocktail EDTA-free (Roche)
Extraction buffer, extraction method: Frozen pellets were thawed and supplemented with TCEP, Benzonase and protease inhibitors. Cells were lysed by sonication. Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine), then centrifuged for further 30 minutes at 17,000rpm.

Column 1: Ni-NTA (2.5 ml 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 pH 7.4, 0.5 mM TCEP
Wash buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.4, 0.5 mM TCEP
Elution buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4, 0.5 mM TCEP

Column 1 Procedure: The clarified cell extract was incubated with 2.5 ml 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 Binding Buffer (60 ml) and Wash Buffer (50 ml). The protein was eluted with 25 ml of Elution Buffer in 5 x 5 ml fractions.

Column 2: Superdex 200 16/60 Gel Filtration.

Column 2 GF Buffer: 10 mM Hepes pH 7.4, 500 mM NaCl, 0.5 mM TCEP, 5% Glycerol

Column 2 Procedure: The wash buffer fractions and elution buffer fractions from column 1 were pooled separately and concentrated to 5 ml with a 10 kDa mwco spin concentrator and injected onto an s200 16/60 column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.0 ml fractions were collected.
The protein eluted at between 80 ml and 90 ml volume.

Column 3: TEV cleavage/ Ni-NTA rebind.

Column 3 Procedure: Protein from fractions eluted at 80-90 ml 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 0.5 ml Ni-NTA pre-equilibrated with GF Buffer. Column was washed 2ml of GF Buffer and 2ml Elution Buffer.Cut protein from the flow-through and GF Buffer fractions were pooled.

Column 4: 1ml Resource Q Anion Exchange

Column 4 Buffer A: 50 mM Tris pH 8.5, 50 mM NaCl

Column 4 Buffer B: 50 mM Tris pH 8.5, 2 M NaCl

Column 4 Procedure: Protein from Ni-rebind elution, approximately 12 ml, was concentrated to 5ml with a 10kDa mwco spin concentrator and diluted to 50 ml using Buffer A and injected into a 1 ml Resource Q column. Protein was eluted using a linear gradient of 0-25% Buffer B over 35 column volumes at 1ml/min. 1.0 ml fractions were collected.

The protein was found in the flow-through and eluted at 1-5% Buffer B

Concentration: The fraction that was found in the flow-through was concentrated to 14 mg/ml using a 10 kDa mwco concentrator.

Mass spectrometry characterization:
Observed mass: 38353.8 Da
Expected mass: 38353.5 Da

Crystallisation: Crystals were grown by vapour diffusion in sitting drop at 20°C. A sitting drop consisting of 100 nl protein pre-mixed with 5 mM NADPH and 50 nl well solution was equilibrated against well solution containing 20% (v/v) PEG3350, 10% (v/v) ethylene glycol,

0.2 M sodium bromide and 0.1 M Bis-Tris propane pH 6.5. Crystals were mounted in the presence of 20% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

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