Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: ASPHA-c008

GenBank GI number: gi|14589866

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

Amplified construct sequence:

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CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGCGCTCA
CTCTACAATGTGAATGGACTGAAAGC
ACAGCCTTGGTGGACCCCAAAAGAAA
CGGGCTACACAGAGTTAGTAAAGTCT
TTAGAAAGAAACTGGAAGTTAATCCG
AGATGAAGGCCTTGCAGTGATGGATA
AAGCCAAAGGTCTCTTCCTGCCTGAG
GATGAAAACCTGAGGGAAAAAGGGGA
CTGGAGCCAGTTCACGCTGTGGCAGC
AAGGAAGAAGAAATGAAAATGCCTGC
AAAGGAGCTCCTAAAACCTGTACCTT
ACTAGAAAAGTTCCCCGAGACAACAG
GATGCAGAAGAGGACAGATCAAATAT
TCCATCATGCACCCCGGGACTCACGT
GTGGCCGCACACAGGGCCCACAAACT
GCAGGCTCCGAATGCACCTGGGCTTG
GTGATTCCCAAGGAAGGCTGCAAGAT
TCGATGTGCCAACGAGACCAAGACCT
GGGAGGAAGGCAAGGTGCTCATCTTT
GATGACTCCTTTGAGCACGAGGTATG
GCAGGATGCCTCATCTTTCCGGCTGA
TATTCATCGTGGATGTGTGGCATCCG
GAACTGACACCACAGCAGAGACGCAG
CCTTCCAGCAATTTGACAGTAAAGGT
GGATACGGATCCGAA
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smRS
LYNVNGLKAQPWWTPKETGYTELVKS
LERNWKLIRDEGLAVMDKAKGLFLPE
DENLREKGDWSQFTLWQQGRRNENAC
KGAPKTCTLLEKFPETTGCRRGQIKY
SIMHPGTHVWPHTGPTNCRLRMHLGL
VIPKEGCKIRCANETKTWEEGKVLIF
DDSFEHEVWQDASSFRLIFIVDVWHP
ELTPQQRRSLPAI
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^ TEV cleavage site

Tags and additions: Cleavable N-terminal His6 tag.

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

Growth medium, induction protocol: 10µl of BL21(DE3)-R3 glycerol stock were inoculated into 5ml of Terrific broth medium supplemented with 50µg/ml kanamycin and 34µg/ml chloramphenicol and grown overnight at 37°C, 200rpm. In the morning 1L of TB supplemented with the same antibiotics was inoculated with 10ml of the overnight culture and incubated at 37°Cwith intensive shaking (160rpm). After the OD_{600} reached 1.5, the temperature was changed to 18°C and IPTG was added to the final concentration of ~0.1mM. The culture was incubated at 18°C with shaking (160rpm) for additional 18h. The following morning the 3Ll culture was harvested and centrifuged for 10min at 4000rpm. Supernatant was discarded and the cell pellets were resuspended in 75ml of a lysis buffer and frozen at -80°C.
Lysis buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5 mM Imidazole; 5% Glycerol; EDTA-free Complete (1 tablet/50ml).
Extraction buffer, extraction method: The thawed cells were broken by 5 passes at 16,000psi through a high pressure homogeniser, followed by centrifugation for 45mins at 20,000rpm at 4°C.

Column 1: Ni-affinity, HisTrap CrudeFF 5ml (GE Healthcare).

Column 1 Buffers:
Start buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 20 mM Imidazole; 1 mM PMSF; 0.5 mM TCEP.
Wash buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 40 mM Imidazole; 1 mM PMSF; 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 cell extract was loaded on the column at 4ml/min on an ÄKTA Express system (GE Healthcare). The column was washed with 10 volumes of lysis buffer, and then eluted with elution buffer at 4ml/min. The eluted peak of A_{280} was automatically collected.

Column 2: Superdex S200 Column, HiPrep 16/60 (Amersham).

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

Column 2 Procedure: The eluted fractions from the Ni-affinity HisTrap column was loaded on the gel filtration column in buffer at 1.2ml/min. Eluted proteins were collected in 2ml fractions and analyzed on SDS-PAGE.

Column 3: Ion exchange, 5ml SP Sepharose.

IEX Buffers:
Buffer A: 25 mM Tris, pH 7.0; 25 mM NaCl.
Buffer B: 25 mM Tris, pH 7.0; 1 M NaCl.

Column 3 Procedure: ASPH containing fractions were pooled and diluted with IEX buffer A to a concentration of NaCl of 25 mM. The protein was loaded onto a HiTrap SP Sepharose column, and eluted with a NaCl gradient (0-25% buffer B). Fractions containing protein were analysed by SDS-PAGE.

Mass spectrometry characterization: The calculated mass of the construct was 25601Da, and the observed mass (ESI-MS) was 23320Da, suggesting loss of several amino acids at the N-Terminus.

Protein concentration: Using Amicon Ultra-15 concentrators with 3kDa cutoff, the sample was concentrated to 9.5mg/ml. Concentrations were determined from the absorbance at 280nm using a NanoDrop spectrophotometer.

Crystallisation: Crystals were grown by vapor diffusion at 20°C in 150nl sitting drops. NOG(S00653) was added to a final concentration of 1 mM prior to crystallisation. The drops were prepared by mixing 100nl of protein solution and 50nl of precipitant consisting 50 mM Na Malate; 20% PEG3350; 30% dextran sulfate (sodium salt) (2µl/20µl of well conditions. 1µl of 200 mM $NiSO_4$/20µl of well conditions). Crystals were flash-cooled in liquid nitrogen with 25% glycerol as cryoprotectant.

Data collection: Resolution: 2.0Å.
X-ray source: Swiss Light source (SLS), beamline X-10.