Entry Clone Source: MGC

Entry Clone Accession: IMAGE:5122305

SGC Construct ID: LTB4DHA-c007

GenBank GI number: gi|28570172

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

Amplified construct sequence:

CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGACTAAG
ACATGGACCCTGAAGAAGCACTTTGT
TGGCTATCCTACTAATAGTGACTTTG
AGTTGAAGACATCTGAGCTCCCACCC
TTAAAAAATGGAGAGGTCCTGCTTGA
AGCTTTGTTCCTCACCGTGGATCCCT
ACATGAGAGTGGCAGCCAAAAGATTG
AAGGAAGGTGATACAATGATGGGGCA
GCAAGTGGCCAAAGTTGTGGAAAGTA
AAAATGTAGCCCTACCAAAAGGAACT
ATTGTACTGGCTTCTCCAGGCTGGAC
AACGCACTCCATTTCTGATGGGAAAG
ATCTGGAAAAGCTGCTGACAGAGTGG
CCAGACACAATACCACTGTCTTTGGC
TCTGGGGACAGTTGGCATGCCAGGCC
TGACTGCCTACTTTGGCCTACTTGAA
ATCTGTGGTGTGAAGGGTGGAGAAAC
AGTGATGGTTAATGCAGCAGCTGGAG
CTGTGGGCTCAGTCGTGGGGCAGATT
GCAAAGCTCAAGGGCTGCAAAGTTGT
TGGAGCAGTAGGGTCTGATGAAAAGG
TTGCCTACCTTCAAAAGCTTGGATTT
GATGTCGTCTTTAACTACAAGACGGT
AGAGTCTTTGGAAGAAACCTTGAAGA
AAGCGTCTCCTGATGGTTATGATTGT
TATTTTGATAATGTAGGTGGAGAGTT
TTCAAACACTGTTATCGGCCAGATGA
AGAAATTTGGAAGGATTGCCATATGT
GGAGCCATCTCTACATATAACAGAAC
CGGCCCACTTCCCCCAGGCCCACCCC
CAGAGATTGTTATCTATCAGGAGCTT
CGCATGGAAGCTTTTGTCGTCTACCG
CTGGCAAGGAGATGCCCGCCAAAAAG
CTCTGAAGGACTTGCTGAAATGGGTC
TTAGAGGGTAAAATCCAGTACAAGGA
ATATATCATTGAAGGATTTGAAAACA
TGCCAGCCGCATTTATGGGAATGCTG
AAAGGAGATAATTTGGGGAAGACAAT
AGTGAAAGCATAACAGTAAAGGTGGA
TACGGATCCGAA

Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smTK
TWTLKKHFVGYPTNSDFELKTSELPP
LKNGEVLLEALFLTVDPYMRVAAKRL
KEGDTMMGQQVAKVVESKNVALPKGT
IVLASPGWTTHSISDGKDLEKLLTEW
PDTIPLSLALGTVGMPGLTAYFGLLE
ICGVKGGETVMVNAAAGAVGSVVGQI
AKLKGCKVVGAVGSDEKVAYLQKLGF
DVVFNYKTVESLEETLKKASPDYDCY
FDNVGGEFSNTVIGQMKKFGRIAICG
AISTYNRTGPLPPGPPPEIVIYQELR
MEAFVVYRWQGDARQKALKDLLKWVL
EGKIQYKEYIIEGFENMPAAFMGMLK
GDNLGKTIVKA
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^ TEV cleavage site

Tags and additions: TEV Cleavable N-terminal His6 tag.

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

Growth medium, induction protocol: 10µl of a glycerol stock was inoculated into 3ml of TB medium (supplemented with 50µg/µl kanamycin) in a 15ml culture tube and cultured at 37°C o/n in a shaking incubator (275rpm). Next day 1ml of o/n culture was used to inoculate 1 litre of TB medium and grown at 37°C with vigorous shaking (180rpm) until the culture reaches an OD_{600} of 1.5. Temperature was reduced to 18°C, and cells were induced with IPTG at concentration of 0.5 mM and cultivated for 16 hours. Cells were harvested, centrifuged at 6500rpm for 10 minutes, and the pellet was stored at -20°C until further use.
Binding buffer: 50 mM HEPES pH 7.5; 500 mM NaCl; 5 mM Imidazole, 5% Glycerol.
Extraction buffer, extraction method: Thawed cell pellets were dissolved in 30-40ml of binding buffer. Cells were lysed by sonication (3x2 minutes) in a 50ml conical tube. After lysis the cell lysate was centrifuged at 4°C for 45 minutes at 21,000rpm.

Column 1: Ni-sepharose resin.

Column 1 Buffer:
Wash Buffer: 50 mM Tris-HCl pH 7.5; 500 mM NaCl; 5% glycerol; 30 mM Imidazole.
Elution Buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 250 mM Imiidazole.

Column 1 Procedure: The clear supernatant after centrifugation was passed through a Ni-sepharose (2.5ml resin) column twice. The column was washed with 50ml of wash buffer, and protein was eluted with 15ml of elution buffer.

Column 2: HiLoad 16/60 Superdex 200 prep grade 120ml, GE Healthcare.

Column 2 Buffers: 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 columns were loaded on the gel filtration column in GF buffer at 1.0ml/min. Eluted proteins were collected in 1ml fractions.

Protein concentration: 11.2mg/ml using Vivaspin 10K concentrators

Mass spectrometry characterization: The protein had an experimental mass of 38183 Da corresponds to theoretical mass, 38183.2 Da, as determined by ESI-TOF MS.

Crystallisation: Crystals were grown by vapour diffusion in sitting drops at 4°C. Before crystallisation setup protein was incubated with 5 mM of NADP and 2.0 mM of Raloxifene. A sitting drop consisting of 50µl protein and 100µl well solution was equilibrated against well solution containing 10% PEG 10K, 8% EtGly, 0.1 M HEPES pH 7.5. Crystals were mounted in the presence of 25% ethylene glycol and flash-cooled in liquid nitrogen.

Data Collection:
Resolution: 2.3 Å
X-ray source: SLS-X10, single wavelength.