Entry Clone Source: IMAGE

Entry Clone Accession: IMAGE:4822768

SGC Construct ID: KLHL11A-c003

GenBank GI number: gi|8922528

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

Amplified construct sequence:

```
CATATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACCTGT
ACTTCCAATCCATGGAAGCCGAGGATTTC
GAGTGCAGCTCTCACTGCTCAGAGCTGTC
CTGGCGGCAGAACGAGCAGCGGCGCCAGG
GCCTCTTCTGCGACATTACCCTGTGCTTC
GGCGGGGCTGGAGGCCGCGAGTTCCGGGC
CCACCGCTCGGTACTGGCTGCCGCCACCG
AGTACTTCACGCCCCTGCTCTCGGGCCAG
TTTTCCGAGTCCCGCTCGGGACGGGTGGA
GATGCGCAAGTGGAGCTCCGAGCCGGGGC
CCGAACCCGACACAGTGGAAGCCGTAATC
GAGTACATGTACACCGGGCGCATCCGCGT
CAGCACGGGCAGCGTGCACGAGGTGCTGG
AGTTGGCCGACAGGTTCCTACTCATTCGT
TTAAAAGAATTTTGTGGAGAATTTCTCAA
GAAAAAACTTCATCTCTCAAATTGTGTGG
CAATTCATAGCTTAGCACACATGTACACC
CTGAGCCAACTTGCTCTGAAGGCTGCTGA
TATGATACGGAGAAATTTCCACAAAGTGA
TTCAGGATGAAGAATTTTATACGTTACCT
TTCCATCTCATTAGAGACTGGCTTTCAGA
TTTGGAAATTACAGTTGATTCTGAAGAGG
TTCTCTTTGAAACCGTTTTGAAATGGGTT
CAGAGAAATGCTGAAGAGAGAGAGAGATA
CTTTGAAGAACTTTTTAAATTGCTCAGGT
TGTCCCAGATGAAACCTACCTACCTTACT
CGACATGTCAAACCAGAGAGGCTGGTAGC
CAATAATGAAGTTTGTGTCAAGTTGGTCG
CTGACGCAGTGGAGAGACATGCTCTGAGA
GCTGAGAATATACAATCTGGCACATGACA
GTAAAGGTGGATACGGATCCGAA
```

Tags and additions: Tag sequence: `mhhhhhhssgvdlgtenlyfq*s(m)` TEV-cleavable (*) N-terminal his6 tag.

Expressed sequence (tag sequence in lowercase):

```
mhhhhhhssgvdlgtenlyfq*sMEAEDF
ECSSHCSELSWRQNEQRRQGLFCDITLCF
GGAGGREFRAHRSVLAAATEYFTPLLSGQ
FSESRSGRVEMRKWSSEPGPEPDTVEAVI
EYMYTGRIRVSTGSVHEVLELADRFLLIR
LKEFCGEFLKKKLHLSNCVAIHSLAHMYT
LSQLALKAADMIRRNFHKVIQDEEFYTLP
FHLIRDWLSDLEITVDSEEVLFETVLKWV
QRNAEERERYFEELFKLLRLSQMKPTYLT
RHVKPERLVANNEVCVKLVADAVERHALR
AENIQSGT
```

Host: BL21(DE3)-R3-pRARE2

Growth medium, induction protocol: The expression plasmid was transformed into the host strain and plated on LB-agar containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol. Several colonies were combined to inoculate a 1-ml culture in TB (+ 50 µg/ml kanamycin, 35 µg/ml chloramphenicol). The culture was grown overnight, glycerol was added to 15% v/v (from a 60% stock), and the resulting glycerol stock was frozen at -80°C in 100 µl aliquots. A loopful of cells from the glycerol stock was inoculated into 6x 10-ml of LB medium containing 100 µg/ml kanamycin and 35 µg/ml chloramphenicol and grown overnight at 37°C. Cultures were harvested by centrifugation and washed twice with M9 minimal medium and resuspended in 10 ml M9 minimal medium. 4x 1L M9 minimal medium (containing 0.4% glucose, 2mM $MgSO_4$, 0.1mM $CaCl_2$, 50 µg/ml kanamycin) were each inoculated with 10 ml resuspended cells and grown in 2.5L UltraYield baffled flasks until OD_{600} of 0.80. Selenomethionine was added to 25mg/L along with leucine, isoleucine and valine to 50mg/L and lysine, threonine, and phenylalanine to 100mg/L (all amino acids dissolved in 0.2M HEPES pH 7.5). Cultures were grown for a further 1.5 hours until OD_{600} of 1.2 and then cooled to 18°C for 1 hour. Additional selenomethionine was added (final total concentration of 75mg/L) and IPTG was added to 0.1 mM, and growth continued at 18°C overnight. The cells were collected by centrifugation then the pellets were scraped out and transferred to 50-ml Falcon tubes and frozen at -80°C.

Cell extraction : **Lysis buffer:** 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP, 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution), and 15 units/ml Benzonase. **2x Lysis buffer** contains the same components at double concentration. Frozen cell pellets (17.2g) were thawed briefly in a bath of warm water (20 - 37°C) then transferred to ice. One volume (i.e. 1 ml for every gram of cells) of 2x lysis buffer was added, followed by 1x lysis buffer to a total volume of 50-ml. The cells were resuspended by agitating and disrupted by high pressure homogenization (20 kpsi). Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine) from a 5% (w/v, pH 7.5) stock, stirring for 15 minutes, then centrifugation for 20 minutes at 25,000 x *g* . The supernatant was then further clarified by filtration (Acrodisc filters, 0.2 µm).

Column 1 : Ni-affinity, HisTrap Crude FF, 5 ml (GE Healthcare)

Solutions: Affinity buffer: 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% Glycerol, 0.5 mM TCEP; **Wash buffer:** 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 30 mM imidazole, 5% Glycerol, 0.5 mM TCEP; **Elution buffer:** 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 300 mM imidazole, 5% Glycerol, 0.5 mM TCEP.

Procedure: The cell extract was loaded on the column at 4 ml/minute on an AKTA-express system (GE Healthcare). The column was washed with 10 volumes of lysis buffer, 10 volumes of wash buffer, and then eluted with elution buffer at 4 ml/min. The eluted peak of A280 was automatically collected.

Column 2 : Gel filtration, Hiload 16/60 Superdex S75 prep grade, 120 ml (GE Healthcare)

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

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

Concentration method: The purified protein was concentrated in a VivaSpin4 (5 K MWCO) to 6 mg/ml and stored at 4°C. The protein concentration was determined spectrophotometrically using ε280 = 33920.

Mass spec characterization : Observed mass with histidine tag, 34802 Da (calculated mass with selenomethionines with histidine tag, 34803).

Crystallization: Crystals were obtained using the vapor diffusion method. The selenomethionine-labelled protein was concentrated in gel fitration buffer to a protein concentration of 6 mg/ml. Sitting drops comprising 100 nl of the concentrated protein mixed with 50nl of a well solution (0.25M K thiocyanate, 25% w/v PEG3350, 5% v/v ethylene glycol, 0.1M bis-tris propane pH7.5) were equilibrated against well solution at 4°C. Crystals were cryo-protected in well solution containing 25% v/v ethylene glycol.

Data Collection: X-ray source: Swiss Light Source, station X10SA, using monochromatic radiation at wavelength 0.99586; **Resolution:** 2.6.