

Entry Clone Source: IMAGE

Entry Clone Accession: IMAGE:3504538

SGC Construct ID: GYG1A-c003

GenBank GI number: gi|20127457

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

Amplified construct sequence:

```
CATATGCACCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGACAGAT
CAGGCCTTTGTGACACTAACCACAAA
CGATGCCTACGCCAAAGGTGCCCTGG
TCCTGGGATCATCTCTGAAACAGCAC
AGGACCACCAGGAGGCTGGTCGTGCT
CGCCACCCCTCAGGTCTCAGACTCCA
TGAGAAAAGTTTTAGAGACAGTCTTT
GATGAAGTCATCATGGTAGATGTCTT
GGACAGTGGCGATTCTGCTCATCTAA
CCTTAATGAAGAGGCCAGAGTTGGGT
GTCACGCTGACAAAGCTCCACTGCTG
GTCGCTTACACAGTATTCAAATGTG
TATTCATGGATGCAGATACTCTGGTC
CTAGCAAATATTGATGATCTTTTTGA
CAGAGAAGAATTGTCAGCAGCACCAG
ACCCAGGGTGGCCTGACTGCTTCAAT
TCCGGAGTCTTCGTTTATCAGCCTTC
AGTTGAAACATACAATCAGCTGTTGC
ATCTTGCTTCTGAGCAAGGTAGTTTT
GATGGTGGGGACCAAGGCATACTGAA
CACATTTTTTAGCAGCTGGGCAACAA
CAGATATCAGAAAACACCTGCCGTTT
ATTTATAACCTAAGCAGCATCTCTAT
ATACTCCTACCTCCCGGCATTTAAAG
TGTTTGGTGCAAGTGCCAAAGTTGTG
CATTCCTGGGACGAGTCAAACCATG
GAATTATACTTATGATCCCAAAACAA
AAAGTGTCAAAGTGAGGCCCATGAT
CCCAACATGACTCATCCAGAGTTTCT
CATCCTGTGGTGGAACATCTTTACCA
CCAACGTTTTACCTCTGCTTCAATGA
CAGTAAAGGTGGATACGGATCCGAA
```

Final protein sequence (Tag sequence in lowercase):

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mhhhhhhsqgvdltgtenlyfq^smTD
QAFVTLTTNDAYAKGALVLGSSLKQH
RTTRRLVVLATPQVSDSMRKVLETVF
DEVIMVDVLDSGDSAHLTLMKRPELG
VTLTKLHCWSLTQYSKCVFMDADTLV
LANIDDLFDREELSAAPDPGWPDCFN
SGVFVYQPSVETYNQLLHLASEQGSF
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DGGDQGILNTFFSSWATTDIRKHLPF
IYNLSSISISIYSYLPFAFKVFGASAKVV
HFLGRVKPWNYYTDPKTKSVKSEAHD
PNMTHPEFLILWWNIFTTNVLP LLQ

^ TEV cleavage site

Tags and additions: Cleavable N-terminal His₆ tag.

Host: E. coli M226 (CGSC #4997) lysogenized with λDE3

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 1 ml 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 3L of TB media (7.5ml starter culture used per 1L) containing 50μg/ml kanamycin. When the OD₆₀₀ reached approximately 0.8 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 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.

Lysis buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 10 mM Imidazole; 5% Glycerol; 0.5 mM TCEP; 1 tablet per 50 ml protease inhibitor cocktail EDTA-free (Roche).

Extraction buffer, extraction method: Cell pellets were dissolved in approximately 150ml lysis buffer and broken by passing through a high pressure homogenizer at 15,000 psi for 4 cycles. The cell debris was pelleted at 35,000 x g and the supernatant used for further purification.

Column 1: Ni-NTA (1.5ml 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 1.5ml 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 (50ml) and Wash Buffer (50ml). The protein was eluted with 25ml of Elution Buffer in 5ml fractions.

Column 2: Superdex 75 16/60 Gel Filtration.

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

Column 2 Procedure: The elution buffer fractions from column 1 were pooled and concentrated to 2ml with a 10kDa mwco spin concentrator and injected onto an S75 16/60 column (pre-equilibrated in GF Buffer) at 1.0ml/min. 1ml fractions were collected.

Enzymatic treatment: Protein from fractions eluted at 50-55ml from S75 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 and wash fractions were pooled.

Protein concentration: 10.5mg/ml using Millipore 10kDa mwco concentrators.

Mass spectrometry characterization: After TEV protease digestion:

Measured mass: 29583.8Da

Expected mass: 29582.8Da

Crystallisation: Prior to crystallization, protein was pre-incubated with 1 mM MnCl_2 and 1 mM uridine diphosphate UDP. Crystals were grown by vapour diffusion in sitting drop at 20°C. A sitting drop consisting of 100nl protein and 50nl well solution was equilibrated against well solution containing 2 M ammonium sulfate and 0.1 M Bis-Tris pH 6.5. Crystals were mounted in the presence of 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data collection:

Resolution: 2.00Å.

X-ray source: FRE superbright, single wavelength.