Entry Clone Source: MGC

Entry Clone Accession: gi|3504538

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

Amplified DNA sequence:

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CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGACAGAT
CAGGCCTTTGTGACACTAACCACAAA
CGATGCCTACGCCAAAGGTGCCCTGG
TCCTGGGATCATCTCTGAAACAGCAC
AGGACCACCAGGAGGCTGGTCGTGCT
CGCCACCCCTCAGGTCTCAGACTCCA
TGAGAAAAGTTTTAGAGACAGTCTTT
GATGAAGTCATCATGGTAGATGTCTT
GGACAGTGGCGATTCTGCTCATCTAA
CCTTAATGAAGAGGCCAGAGTTGGGT
GTCACGCTGACAAAGCTCCACTGCTG
GTCGCTTACACAGTATTCAAAATGTG
TATTCATGGATGCAGATACTCTGGTC
CTAGCAAATATTGATGATCTTTTTGA
CAGAGAAGAATTGTCAGCAGCACCAG
ACCCAGGGTGGCCTGACTGCTTCAAT
TCCGGAGTCTTCGTTTATCAGCCTTC
AGTTGAAACATACAATCAGCTGTTGC
ATCTTGCTTCTGAGCAAGGTAGTTTT
GATGGTGGGGACCAAGGCATACTGAA
CACATTTTTTAGCAGCTGGGCAACAA
CAGATATCAGAAAACACCTGCCGTTT
ATTTATAACCTAAGCAGCATCTCTAT
ATACTCCTACCTCCCGGCATTTAAAG
TGTTTGGTGCAAGTGCCAAAGTTGTG
CATTTCCTGGGACGAGTCAAACCATG
GAATTATACTTATGATCCCAAAACAA
AAAGTGTCAAAAGTGAGGCCCATGAT
CCCAACATGACTCATCCAGAGTTTCT
CATCCTGTGGTGGAACATCTTTACCA
CCAACGTTTTACCTCTGCTTCAATGA
CAGTAAAGGTGGATACGGATCCGAA
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smTD
QAFVTLTTNDAYAKGALVLGSSLKQH
RTTRRLVVLATPQVSDSMRKVLETVF
DEVIMVDVLDSGDSAHLTLMKRPELG
VTLTKLHCWSLTQYSKCVFMDADTLV
LANIDDLFDREELSAAPDPGWPDCFN
SGVFVYQPSVETYNQLLHLASEQGSF
DGGDQGILNTFFSSWATTDIRKHLPF
IYNLSSISIYSYLPAFKVFGASAKVV
HFLGRVKPWNYTYDPKTKSVKSEAHD
PNMTHPEFLILWWNIFTTNVLPLLQ
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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: 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 6L of TB media (7.5 ml starter culture used per 1L) containing 50 µg/ml kanamycin. When the OD_{600} 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.
Binding 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: 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 (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: 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 (100 ml) and Wash Buffer (50 ml). The protein was eluted with 25 ml of Elution Buffer in 5 ml fractions

Column 2: Superdex 75 16/60 Gel Filtration

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

Column 2 Procedure: The elution buffer fractions from column 1 were pooled and concentrated to 2 ml with a 10 kDa mwco spin concentrator and injected onto an s75 16/60 column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1 ml fractions were collected. The protein eluted at between 50 ml and 55 ml volume.

Column 3: Ni-NTA rebind

Column 3 Procedure: Protein fractions eluted at 50-55 ml 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 0.5 ml Ni-NTA pre-equilibrated with GF Buffer. Column was washed 1ml of GF Buffer. Flow-through and wash were pooled

Protein concentration: 9.95 mg/ml using Millipore 10k mwco concentrators.

Mass spectrometry characterization:
Measured mass: 29584.9 Da
Expected mass: 29582.8 Da

Crystallisation: Prior to crystallization, protein was pre-incubated with 1mM $MnCl_2$ and 1mM uridine diphosphate (UDP). Crystals were grown by vapour diffusion in sitting drop at 20°C. A sitting drop consisting of 75 nl protein and 75 nl well solution was equilibrated against well solution containing 0.2M potassium sodium tartate, 0.1M sodium citrate tribasic pH 5.6 and

2.0M ammonium sulfate. Crystals were mounted in the presence of 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen

Data collection:
X-ray source: FRE superbright, single wavelength
Resolution: 1.9Å