Entry Clone Source: MGC

Entry Clone Accession: BC003565

SGC Construct ID: HGSA-c001

GenBank GI number: gi|4758528

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

Amplified construct sequence:

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TACTTCCAATCCATGGGGCGAGGCAG
CGGCACCTTCGAGCGTCTCCTAGACA
AGGCGACCAGCCAGCTCCTGTTGGAG
ACAGATTGGGAGTCCATTTTGCAGAT
CTGCGACCTGATCCGCCAAGGGGACA
CACAAGCAAAATATGCTGTGAATTCC
ATCAAGAAGAAAGTCAACGACAAGAA
CCCACACGTCGCCTTGTATGCCCTGG
AGGTCATGGAATCTGTGGTAAAGAAC
TGTGGCCAGACAGTTCATGATGAGGT
GGCCAACAAGCAGACCATGGAGGAGC
TGAAGGACCTGCTGAAGAGACAAGTG
GAGGTAAACGTCCGTAACAAGATCCT
GTACCTGATCCAGGCCTGGGCGCATG
CCTTCCGGAACGAGCCCAAGTACAAG
GTGGTCCAGGACACCTACCAGATCAT
GAAGGTGGAGGGGCACGTCTTTCCAG
AATTCAAAGAGAGCGATGCCATGTTT
GCTGCCGAGAGAGCCCCAGACTGGGT
GGACGCTGAGGAATGCCACCGCTGCA
GGGTGCAGTTCGGGGTGATGACCCGT
AAGCACCACTGCCGGGCGTGTGGGCA
GATATTCTGTGGAAAGTGTTCTTCCA
AGTACTCCACCATCCCCAAGTTTGGC
ATCGAGAAGGAGGTGCGCGTGTGTGA
GCCCTGCTACGAGCAGCTGAACAGGA
AAGCGGAGGGATGACAGTAAAGGTGG
ATA
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smGR
GSGTFERLLDKATSQLLLETDWESIL
QICDLIRQGDTQAKYAVNSIKKKVND
KNPHVALYALEVMESVVKNCGQTVHD
EVANKQTMEELKDLLKRQVEVNVRNK
ILYLIQAWAHAFRNEPKYKVVQDTYQ
IMKVEGHVFPEFKESDAMFAAERAPD
WVDAEECHRCRVQFGVMTRKHHCRAC
GQIFCGKCSSKYSTIPKFGIEKEVRV
CEPCYEQLNRKAEG
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^ TEV cleavage site

Tags and additions: Cleavable N-terminal His_6 tag.

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

Growth medium, induction protocol: A glycerol stock was used to inoculate a 10ml starter culture containing LB media with 50µg/ml Kanamycin and 34µg/ml Chloramphenicol. The starter culture was grown overnight at 37°C with shaking at 200 rpm. The following morning, three flasks containing 1L LB/Kanamycin were each inoculated with 1 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD_{600}nm = 0.4 was reached. The flasks were then cooled down to 18°C to an OD_{600}nm = 0.7 and added 0.4mM IPTG to induce protein expression overnight. Cells were harvested by centrifugation at 4500 rpm at 4°C for 15 min. Cell pellets from each flask were resuspended in 25ml Binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole).
Extraction buffer, extraction method: The cells were lysed by ultrasonication over 7 min with the sonicator pulsing ON for 5 sec and OFF for 10 sec. The cell lysate was spun down by centrifugation at 18000 rpm at 4°C for 1 h. The supernatant was recovered for purification.

Column 1: Ni-Affinity Chromatography twinned with DE-52 column

Column 1 Buffers:
Binding buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 5 mM Imidazole.
Wash buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 30 mM Imidazole.
Elution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol, 50 to 250 mM Imidazole (step elution).

Column 1 Procedure: 2.5g of DE-52 resin dissolved in 25ml 2.5M NaCl was applied onto a drip column and equilibrated with Binding buffer. 5ml of 50 % Ni-IDA slurry () was applied onto a 1.5 x 10 cm column. The column was first washed with deionised distilled H_2O, and then equilibrated with Binding buffer. DE-52 column was mounted on top of the Ni column. The supernatant was applied by gravity flow onto the DE-52 column and then passed onto the Ni column. The Ni column was washed with Wash buffer and the bound protein was eluted by applying a step gradient of Imidazole (5 ml fractions of Elution buffer supplemented with 50mM, 100mM, 150mM and 2x10ml fractions with 250mM Imidazole). 10mM Arginine/Glutamic Acid was added to each fraction collected for overnight storage at 4°C.

Enzymatic treatment:TEV protease cleavage. Fractions containing HGS were treated with TEV protease overnight at 4°C.

Column 2: Ni-Affinity Chromatography

Column 2 Buffers:
Binding buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 5 mM Imidazole.
Wash buffer: 50 mM HEPES, 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 Imidazole (step elution).

Column 2 Procedure: 500µl of 50 % Ni-IDA slurry was applied onto a BioRad Poly-Prep disposable drip column. The column was first washed with deionised distilled H_2O, and then equilibrated with Binding buffer. The HGS pool treated with TEV was applied by gravity flow onto the Ni-IDA column. The flow through was collected, washed the column with 10ml Wash buffer and the bound impurities were eluted with 500µl of elution buffer with 250mM Imidazole.

Column 3: Size Exclusion Chromatography. Superdex S200 16/60 HiLoad (GE Healthcare).

Column 3 Buffer: 50 mM HEPES, pH 7.5; 300 mM NaCl; 0.5 mM TCEP.

Column 3 Procedure: The Superdex S200 column was first equilibrated with Gel Filtration buffer. Concentrated the protein fraction from above step to <5ml using Vivaspin filter with a 3kDa cut-off, syringe injected onto the column and eluted with Gel Filtration buffer. Clean fractions containing the protein were pooled together.

Column 4: Ni-Affinity Chromatography

Column 4 Buffers:
Binding buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 5 mM Imidazole.
Wash buffer: 50 mM HEPES, 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 Imidazole.

Column 4 Procedure: 500µl of 50 % Ni-IDA slurry was applied onto a BioRad Poly-Prep disposable drip column. The column was first washed with deionised distilled H_2O, and then equilibrated with Binding buffer. Protein from Gel Filtration step was applied by gravity flow onto the Ni-IDA column. The flow through was collected, washed the column with 10ml Wash buffer and the bound impurities were eluted with 500µl of elution buffer with 250mM Imidazole. Added 10mM DTT and 10mM Arginine/Glutamic Acid to the flow through.

Column 5: Cation Exchange Chromatography - HiTrap SP HP column

Column 5 Buffers:
IEX Buffer I: 50mM HEPES pH 7.5
IEX Buffer II: 1M NaCl, 50mM HEPES pH 7.5

Column 5 Procedure: The HiTrap SP HP column was first washed with IEX buffer II and then equilibrated with IEX buffer I. Concentrated the protein fraction from above step to 5ml using an Amicon Ultra-15 filter with a 10kDa cut-off. The 5ml fraction was made up to 50ml with IEX buffer I and applied onto the column using a super loop. Bound protein was eluted in 0%-100% gradient with IEX buffer II. Clean fractions containing the protein were pooled together.

Protein concentration: The protein was concentrated in an Amicon Ultra-4 filter with a 10 kDa cut-off.

Mass spectrometry characterization: Pending analysis

Crystallisation: Protein was buffered in 40mM NaCl, 50mM HEPES pH 7.5, 10mM DTT and 10mM Arginine/Glutamic acid. Protein was concentrated to 11.7 mg/ml (calculated using extinction co-efficient of 26930). Native crystals were grown at 20°C in 150nl sitting drops mixing 50nl protein solution with 100nl of a reservoir solution containing 25% PEG 3350, 0.1M NH_4SO_4, 0.1M Bis-Tris pH 7.0. On mounting crystals were cryo-protected with an additional 25% Ethylene glycol.

Data collection:
X-ray source: Diamond I04 (native)
Resolution: 1.5Å