Entry Clone Source: Origene

Entry Clone Accession: NM_000020 variant

SGC Construct ID: ACVRL1A-c079

GenBank GI number: gi|4557243

Vector: pFB-LIC-Bse. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

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TACTTCCAATCCATGCAGAGGACAGTGGCA
CGGCAGGTTGCCTTGGTGGAGTGTGTGGGA
AAAGGCCGCTATGGCGAAGTGTGGCGGGGC
TTGTGGCACGGTGAGAGTGTGGCCGTCAAG
ATCTTCTCCTCGAGGGATGAACAGTCCTGG
TTCCGGGAGACTGAGATCTATAACACAGTG
TTGCTCAGACACGACAACATCCTAGGCTTC
ATCGCCTCAGACATGACCTCCCGCAACTCG
AGCACGCAGCTGTGGCTCATCACGCACTAC
CACGAGCACGGCTCCCTCTACGACTTTCTG
CAGAGACAGACGCTGGAGCCCCATCTGGCT
CTGAGGCTAGCTGTGTCCGCGGCATGCGGC
CTGGCGCACCTGCACGTGGAGATCTTCGGT
ACACAGGGCAAACCAGCCATTGCCCACCGC
GACTTCAAGAGCCGCAATGTGCTGGTCAAG
AGCAACCTGCAGTGTTGCATCGCCGACCTG
GGCCTGGCTGTGATGCACTCACAGGGCAGC
GATTACCTGGACATCGGCAACAACCCGAGA
GTGGGCACCAAGCGGTACATGGCACCCGAG
GTGCTGGACGAGCAGATCCGCACGGACTGC
TTTGAGTCCTACAAGTGGACTGACATCTGG
GCCTTTGGCCTGGTGCTGTGGGAGATTGCC
CGCCGGACCATCGTGAATGGCATCGTGGAG
GACTATAGACCACCCTTCTATGATGTGGTG
CCCAATGACCCCAGCTTTGAGGACATGAAG
AAGGTGGTGTGTGTGGATCAGCAGACCCCC
ACCATCCCTAACCGGCTGGCTGCAGACCCG
GTCCTCTCAGGCCTAGCTCAGATGATGCGG
GAGTGCTGGTACCCAAACCCCTCTGCCCGA
CTCACCGCGCTGCGGATCAAGAAGACACTA
CAAAAAATTAGCAACAGTCCAGAGTGACAG
TAAAGGTGGATA
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Final protein sequence (tag sequence in lowercase)

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mghhhhhhssgvdlgtenlyfq*SMQRTVA
RQVALVECVGKGRYGEVWRGLWHGESVAVK
IFSSRDEQSWFRETEIYNTVLLRHDNILGF
IASDMTSRNSSTQLWLITHYHEHGSLYDFL
QRQTLEPHLALRLAVSAACGLAHLHVEIFG
TQGKPAIAHRDFKSRNVLVKSNLQCCIADL
GLAVMHSQGSDYLDIGNNPRVGTKRYMAPE
VLDEQIRTDCFESYKWTDIWAFGLVLWEIA
RRTIVNGIVEDYRPPFYDVVPNDPSFEDMK
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KVVCVDQQTPTIPNRLAADPVLSGLAQMMR
ECWYPNPSARLTALRIKKTLQKISNSPE

Tags and additions: mghhhhhhssgvdlgtenlyfq. N-terminal hexahistidine tag cleavable by TEV protease.

Host: SF9 Spodoptera frugiperda Insect cells

Growth medium, induction protocol: Cells at the density of 2millions/ml were infected. Cells were harvested by centrifugation at 4500 rpm at 4°C for 15 min. Cell pellets from each flask (1l volume) were resuspended in 20 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole), transferred to 50 ml tubes, and stored at -20°C.

Extraction buffer, extraction method: The frozen cells were thawed and protease inhibitor SET V (Calbiochem) added to the cell suspension at 1:100 dilution. The cells were lysed by homogenization. The cell lysate was spun down by centrifugation at 21K rpm at 4°C for 1 h. The supernatant was recovered for purification.

Column 1: Anion-exchange for Nucleic acid removal with DEAE cellulose (DE52, Whatmann)10 g of resin was suspended in 50 ml 0.3 M NaCl, and then applied onto a 2.5 x 20 cm column. The resin was then equilibrated with 50 ml binding buffer prior to loading the sample.

Column 1 Buffers:
Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole 0.5mM TCEP
Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole 0.5mM TCEP

Column 1 Procedure: The supernatant was first applied onto the column by gravity flow, which was followed by a wash with 100 ml wash buffer. The column flow-through and wash was directly applied onto a Ni-sepharose column.

Column 2: Ni-Affinity Chromatography. 5 ml of 50 % Ni-sepharose slurry was applied onto a 1.5 x 10 cm column. The column was equilibrated with binding buffer (25ml).

Column 2 Buffers:
Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole 0.5mM TCEP
Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole 0.1mM TCEP
Elution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 50 to 250 mM imidazole 0.5mM TCEP

Column 2 Procedure: The flow-through from column 1 (DE52) was applied by gravity flow onto the Ni-sepharose column. The bound protein was eluted by applying a step gradient of imidazole - using 12 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 250 mM). 10 mM DTT was added during overnight storage at 4°C.

Enzymatic treatment: 0.1mg of TEV protease was added to the eluted protein to remove the His-tag.

Column 3: Size Exclusion Chromatography - S200 HiLoad 16/60 Superdex run on ÄKTA-Express

Column 3 Buffers: Gel Filtration buffer: 300 mM NaCl, 50 mM Tris HCl, 0.5 mM TCEP, pH 7.5

Column 3 Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. The protein was concentrated to 3 ml using an Amicon Ultra-15 filter with a 30 kDa cut-off. The concentrated protein was directly applied onto the equilibrated S200 16/60 column, and run at a flow-rate of 1 ml/min. The protein was eluted at 80 - 95 ml. Fractions containing the protein were pooled together.

Mass spectrometry characterization: The purified protein was homogeneous and had an experimental mass of 34.766 kDa, as expected from primary sequences. Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% methanol in water with 0.1% formic acid.

Protein concentration: Protein was concentrated to 10 mg/ml using an Amicon 30 kDa cut-off concentrator.

Crystallisation: Protein was concentrated to 10 mg/ml in gel filtration buffer supplemented with 2% glycerol. LDN-193189 was added to the final sample at a concentration of 1mM. Crystals were grown at 20°C in 300 nl sitting drops mixing 200 nl protein solution with 100 nl of a reservoir solution containing 16% PEG3350, 0.2M Na/KPO_4, 5% ethylene glycol, 2% glycerol. On mounting crystals were cryo-protected with an additional 25% ethylene glycol.

Data collection: Resolution: 2.65 Å resolution; **X-ray source:** Diamond Light Source, station I24, using monochromatic radiation at wavelength 0.9779 Å