

Molecular Biology

Entry Clone Accession: IMAGE:4574938

Entry Clone Source: MGC

SGC Construct ID: GBE1A-c106

Protein Region: L38-L700

Vector: pFB-LIC-Bse

Tag: N-6HIS; TEV cleavage site

Host: DH10Bac

Sequence (with tag(s)):

MGHHHHHHSSGVDLG TENLYFQSMLKPYAVDFQRRYKQFSQILKNIGENEGGIDKFSRG
YESFGVHRCADGGLYCKEWAPGAEGVFLTGFDFNGWNPFSYPYKKLDYGWELYPKQ
NKSVLVPHGSKLKVVITSKSGEILYRISPWAKYVVREGDNVNYDWHWDPEHSYEFKHS
RPPKPRSLRIYESHVGISSHEGKVASYKHFTCNVLPRIKGLGYNCIQLMAIMEHAYYASFG
YQITSFFAASSRYGSPEELQELVDTAHSMGIIVLLDVVHSHASKNSADGLNMF DGT DSCY
FHSGPRGTHDLWDSRLFAYSSWEVLRFLLSNIRWWLEEYRFDGFRFDGVTSMLYHHHGV
GQGFSGDYSEYFGLQVDEDALTYLMLANHLVHTLCPDSITIAEDVSGMPALCSPISQGGG
GFDYRLAMAIPDKWIQLLKEFKDEDWNMGDIVYTLTNRRYLEKCIAYAESH DQALV GDK
SLAFWLMDAEMYTNMSVLTPFTPVIDRGIQLHKMIRLITHGLGGEGYLNFMGNEFGHPE
WLD FPRKGNNESYHYARRQFH LTDDDLLRYKFLNNFDRDMNRLEERYGWLAAPQAYVS
EKHEGNKIIAFERAGLLFIFNFHPSKSYTDYRVGTALPGKFKIVLDS DAAEYGGHQRLDHS
TDDFFSEAFEHNGRPYSLLVYIPSRVALILQNVDL

Sequence after tag cleavage:

SMLKPYAVDFQRRYKQFSQILKNIGENEGGIDKFSRGYESFGVHRCADGGLYCKEWAPG
AEGVFLTGFDFNGWNPFSYPYKKLDYGWELYPKQ NKSVLVPHGSKLKVVITSKSGEIL
YRISPWAKYVVREGDNVNYDWHWDPEHSYEFKHSRPPKPRSLRIYESHVGISSHEGKV
ASYKHFTCNVLPRIKGLGYNCIQLMAIMEHAYYASFGYQITSFFAASSRYGSPEELQELVD
TAHSMGIIVLLDVVHSHASKNSADGLNMF DGT DSCYFHSGPRGTHDLWDSRLFAYSSWE
VLRFLLSNIRWWLEEYRFDGFRFDGVTSMLYHHHGVGQGFSGDYSEYFGLQVDEDALT
YLMLANHLVHTLCPDSITIAEDVSGMPALCSPISQGGGGFDYRLAMAIPDKWIQLLKEFK
DEDWNMGDIVYTLTNRRYLEKCIAYAESH DQALV GDKSLAFWLMDAEMYTNMSVLTPF
TPVIDRGIQLHKMIRLITHGLGGEGYLNFMGNEFGHPEWLD FPRKGNNESYHYARRQFH
LTDDDLLRYKFLNNFDRDMNRLEERYGWLAAPQAYVSEKHEGNKIIAFERAGLLFIFNFH
PSKSYTDYRVGTALPGKFKIVLDS DAAEYGGHQRLDHSTDDFFSEAFEHNGRPYSLLVYIP
SRVALILQNVDL

DNA Sequence:

CCATGGGCCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTG
TACTTCCAATCCATGTTGAAGCCCTACGCCGTGGACTTCCAGCGCAGGTATAAGCAGT
TTAGCCAAATTTTGAAGAACATTGGAGAAAATGAAGGTGGTATTGATAAGTTTTCCAG
AGGCTATGAATCATTTGGCGTCCACAGATGTGCTGATGGTGGTTTATACTGCAAAGAAT
GGGCCCCGGGAGCAGAAGGAGTTTTTCTTACTGGAGATTTTAATGGTTGGAATCCATT
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CAGAATAAATCTGTACTCGTGCCTCATGGATCCAAATTAAAGGTAGTTATTACTAGTAA
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TTCCAGACCAAAGAAGCCACGGAGTCTAAGAATTTATGAATCTCATGTGGGAATTTCT
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CAAAGGCCTTGGATACA ACTGCATTCAGTTGATGGCAATCATGGAGCATGCTTACTATG
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GAAGAGCTACAAGAACTGGTAGACACAGCTCATTCCATGGGTATCATAGTCCTCTTAG
ATGTGGTACACAGCCATGCTTCAAAAAATT CAGCAGATGGATTGAATATGTTTGATGG
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GATTGTTTGCCTACTCCAGCTGGGAAGTTTTAAGATTCCTTCTGTCAAACATAAGATGG
TGGTTGGAAGAATATCGCTTTGATGGATTTCGTTTTGATGGTGTACGTCCATGCTTTAT
CATCACCATGGAGTGGGTCAAGGTTTCTCAGGTGATTACAGTGAATATTTTCGGACTAC
AAGTAGATGAAGATGCCTTGACTTACCTCATGTTGGCAAATCATTGGTTACACGCT
GTGTCCCGATTCTATAACAATAGCTGAGGATGTATCAGGAATGCCAGCTCTGTGCTCTC
CAATTTCCCAGGGAGGGGGTGGTTTTGACTATCGACTAGCCATGGCAATTCCAGATAA
GTGGATTACGCTACTTAAAGAGTTTAAAGATGAAGACTGGAACATGGGCGATATAGTA
TACACGCTCACAAACAGGCGCTACCTTGAAAAGTGCATTGCTTATGCAGAGAGCCATG
ATCAGGCATTGGTTGGGGATAAGTCGCTGGCATTTTGGTTGATGGATGCCGAAATGTAT
ACAAACATGAGTGTCTGACTCCTTTTACTCCAGTTATTGATCGTGGAATACAGCTTCA
TAAAATGATTCGACTCATTACGCATGGGCTTGGTGGAGAAGGCTATCTCAATTTTCATGG
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TTACCATTATGCCAGGCGGCAGTTTCATTTAAGTACGACGACCTTCTTCGCTACAAGT
TCCTAAATAATTTTGACAGGGATATGAATAGATTGGAAGAAAGATATGGTTGGCTTGCA
GCTCCACAGGCCTACGTGAGTGAAAAACATGAAGGCAATAAGATCATTGCTTTTGAA
AGAGCAGGTCTTCTTTTCATTTTCAACTTCCATCCAAGCAAGAGCTACACTGACTACC
GAGTTGGAACAGCATTGCCAGGGAAATTCAAAATTGTGCTAGATTTCAGATGCAGCGG
AATATGGAGGGCATCAGAGACTGGACCACAGCACTGACTTTTTTTCTGAGGCTTTTGA
ACATAATGGGCGTCCCTATTCTCTTTTGGTGTACATTCCAAGCAGAGTGGCCCTCATCC
TTCAGAATGTGGATCTGTGACAGTAAAGGTGGATACGGATCCGAATTCGAGCTCCGTC
GACAAGCTT

Protein Expression

Medium: Sf9 medium

Procedure: High five cells were grown in Sf9 medium at 27°C. Cells were infected at a density of 2x10⁶/ml with recombinant baculovirus (virus stock P2; 1ml of virus stock/1l of cell culture) Culture was supplemented with FCS to final concentration 1%. 120 hours post infection the cultures were collected and centrifuged for 20min at 1500 xg. Cellular pellets were discarded and supernatants were used as a source of a protein.

Protein Purification

Cell Lysis

Cell pellets were dissolved in approximately 50ml lysis buffer and broken by sonication for 10min @ 35% amplitude followed by homogenization by 2 passes at 12,000 psi. The cell debris was pelleted at 40,000 x g and the supernatant used for further purification.

Lysis 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)

Column 1

Ni-NTA (1.5 ml volume in a gravity-flow column).

The clarified cell extract was added to 1.5 ml of Ni-NTA pre-equilibrated with lysis buffer and passed through a glass column. The column was then washed with Binding Buffer (2 x 15 ml) and Wash Buffer (2 x 15 ml). The protein was eluted with 25 ml of Elution Buffer in 5 x 2.5 ml fractions.

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: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4, 0.5 mM TCEP

Column 2

Superdex 200 16/60 Gel Filtration.

The first three elution fractions from column 1 were pooled and concentrated to 5 ml with a 30 kDa mwco spin concentrator and injected into an s200 16/60 column (preequilibrated in GF Buffer) at 1.0 ml/min. 1.0 ml fractions were collected.

The protein eluted at between 80 ml and 90 ml volume.

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

Concentration

Pooled protein fractions were concentrated to 16.5 mg/ml using a 30 kDa mwco concentrator.

Mass-spec Verification: Yes

Measured mass: 78939.2 Da

Expected mass: 79023.3 Da

Structure Determination

Crystallization: Crystals were grown by vapour diffusion in sitting drop at 4°C. A sitting drop consisting of 50 nl protein (pre-incubated with 5 mM acarbose) and 100 nl well solution was equilibrated against well solution containing 0.05 M sodium-succinate and 22% PEG 3350 supplemented with 4% formamide (Hampton additive screen). Crystals were mounted in the presence of 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data collection: Resolution: 2.70 Å; X-ray source: Diamond I04