Entry Clone Source: MGC

Entry Clone Accession: BC009970

SGC Construct ID: TKTA-c604

GenBank GI number: gi|4507521

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

Amplified construct sequence:

CCATGGGCCACCATCATCATCATCATTCTT
CTGGTGTAGATCTGGGTACCGAGAACCTGT
ACTTCCAATCCATGCAGAAGCTGCAGGCCT
TGAAGGACACGGCCAACCGCCTACGTATCA
GCTCCATCCAGGCCACCACTGCGGCGGGCT
CTGGCCACCCCACGTCATGCTGCAGCGCCG
CAGAGATCATGGCTGTCCTCTTTTTCCACA
CCATGCGCTACAAGTCCCAGGACCCCCGGA
ATCCGCACAATGACCGCTTTGTGCTCTCCA
AGGGCCATGCAGCTCCCATCCTCTACGCGG
TCTGGGCTGAAGCTGGTTTCCTGGCCGAGG
CGGAGCTGCTGAACCTGAGGAAGATCAGCT
CCGACTTGGACGGGCACCCGGTCCCGAAAC
AAGCTTTCACCGACGTGGCCACTGGCTCCC
TGGGCCAGGGCCTCGGGGCCGCTTGTGGGA
TGGCCTACACCGGCAAATACTTCGACAAGG
CCAGCTACCGAGTCTATTGCTTGCTGGGAG
ATGGGGAGCTGTCAGAGGGCTCTGTATGGG
AGGCCATGGCCTTCGCCAGCATCTATAAGC
TGGACAACCTTGTGGCCATTCTAGACATCA
ATCGCCTGGGCCAGAGTGACCCGGCCCCGC
TGCAGCACCAGATGGACATCTACCAGAAGC
GGTGCGAGGCCTTCGGTTGGCATGCCATCA
TCGTGGATGGACACAGCGTGGAGGAGCTGT
GCAAGGCCTTTGGCCAGGCCAAGCACCAGC
CAACAGCCATCATTGCCAAGACCTTCAAGG
GCCGAGGGATCACGGGGGTAGAAGATAAGG
AGTCTTGGCATGGGAAGCCCCTCCCCAAAA
ACATGGCTGAGCAGATCATCCAGGAGATCT
ACAGCCAGATCCAGAGCAAAAAGAAGATCC
TGGCAACCCCTCCACAGGAGGACGCACCCT
CAGTGGACATTGCCAACATCCGCATGCCCA
GCCTGCCCAGCTACAAAGTTGGGGACAAGA
TAGCCACCCGCAAGGCCTACGGGCAGGCAC
TGGCCAAGCTGGGCCATGCCAGTGACCGCA
TCATCGCCCTGGATGGGGACACCAAAAATT
CCACCTTCTCGGAGATCTTCAAAAAGGAGC
ACCCGGACCGCTTCATCGAGTGCTACATTG
CCGAGCAGAACATGGTGAGCATCGCGGTGG
GCTGTGCCACCCGCAACAGGACGGTGCCCT
TCTGCAGCACTTTTGCAGCCTTCTTCACGC
GGGCCTTTGACCAGATTCGCATGGCCGCCA
TCTCCGAGAGCAACATCAACCTCTGCGGCT

CCCACTGCGGCGTTTCCATCGGGGAAGACG
GGCCCTCCCAGATGGCCCTAGAAGATCTGG
CTATGTTTCGGTCAGTCCCCACATCAACTG
TCTTTTACCCAAGTGATGGCGTTGCTACAG
AGAAGGCAGTGGAACTAGCCGCCAATACAA
AGGGTATCTGCTTCATCCGGACCAGCCGCC
CAGAAAATGCCATCATCTATAACAACAATG
AGGACTTCCAGGTCGGACAAGCCAAGGTGG
TCCTGAAGAGCAAGGATGACCAGGTGACCG
TTATCGGGGCTGGGGTGACCCTGCACGAGG
CCTTGGCCGCTGCCGAACTGCTGAAGAAAG
AAAAGATCAACATCCGCGTGCTGGACCCCT
TCACCATCAAGCCCCTGGACAGAAAACTCA
TTCTCGACAGCGCTCGTGCCACCAAGGGCA
GGATCCTCACCGTGGAGGACCATTATTATG
AAGGTGGCATTGGTGAGGCTGTGTCCAGTG
CAGTAGTGGGCGAGCCTGGCATCACTGTCA
CCCACCTGGCAGTTAACCGGGTACCAAGAA
GTGGGAAGCCAGCTGAGCTGCTGAAGATGT
TTGGTATCGACAGGGATGCCATTGCACAAG
CTGTGAGGGGCCTCATCTGACAGTAAAGGT
GGATACGGATCCGAATTCGAGCTCCGTCGA
CAAGCTT

Final protein sequence:
smQKLQALKDTANRLRISSIQATTAAGSGH
PTSCCSAAEIMAVLFFHTMRYKSQDPRNPH
NDRFVLSKGHAAPILYAVWAEAGFLAEAEL
LNLRKISSDLDGHPVPKQAFTDVATGSLGQ
GLGAACGMAYTGKYFDKASYRVYCLLGDGE
LSEGSVWEAMAFASIYKLDNLVAILDINRL
GQSDPAPLQHQMDIYQKRCEAFGWHAIIVD
GHSVEELCKAFGQAKHQPTAIIAKTFKGRG
ITGVEDKESWHGKPLPKNMAEQIIQEIYSQ
IQSKKKILATPPQEDAPSVDIANIRMPSLP
SYKVGDKIATRKAYGQALAKLGHASDRIIA
LDGDTKNSTFSEIFKKEHPDRFIECYIAEQ
NMVSIAVGCATRNRTVPFCSTFAAFFTRAF
DQIRMAAISESNINLCGSHCGVSIGEDGPS
QMALEDLAMFRSVPTSTVFYPSDGVATEKA
VELAANTKGICFIRTSRPENAIIYNNNEDF
QVGQAKVVLKSKDDQVTVIGAGVTLHEALA
AAELLKKEKINIRVLDPFTIKPLDRKLILD
SARATKGRILTVEDHYYEGGIGEAVSSAVV
GEPGITVTHLAVNRVPRSGKPAELLKMFGI
DRDAIAQAVRGLI

Tags and additions: N-terminal His6-tag with a TEV protease cleavage site (*):
MHHHHHHSSGVDLGTENLYFQ(*)SM

Host: *Trichoplusia Ni* (High five)

Expression: High five cells were grown in Insect Express Medium. Cells were infected at a density of 2x106/ml with recombinant baculovirus (virus stock P2; 1ml of virus stock/100 ml

of cell culture). Cells were shaken at 120 rpm at 27°C in the innova shaker. After 48 hours post-infection the cultures were collected and centrifuged for 30min at 2000rpm. The cell pellet was resuspended in cold PBS and centrifugation was repeated.

Extraction: The pellet was resuspended in lysis buffer (20ml per 1L of cell pellet) Cells were broken using Dounce homogenizer followed by centrifugation for 1 hour at 15000rpm at 4°C.

Lysis Buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol containing 0.2% of Triton X-100 and benzonase (Novagen) (25u/10ml of lysate).

Binding Buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP

Column 1: Ni-affinity. Ni-sepharose (Amersham), 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.

Column 1 Buffers:
Wash Buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM Imidazole, 5% glycerol, 1 mM PMSF, 0.5 mM TCEP.
Elution Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole, 0.5 mM TCEP**.**

Column 1 Procedure: The supernatant was diluted with binding buffer and centrifuged for 30min at 2000rpm. The clarified supernatant was incubated with Ni-Sepharose for 1 hour at 4°C with gentle rotation. Lysate containing resin was loaded on the gravity column and after removing the unbound protein extract resin was washed with 50 ml of wash buffer. The protein was eluted with 3x5 ml of elution buffer.

Column 2: Gel filtration. Hiload S200 16/60

Column 2 Buffer: 10 mM Hepes pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP

Column 2 Procedure: The eluted fractions from Ni-sepharose were filtered (Acrodisc filters, 0.2 mm) and then loaded on the gel filtration column pre-equilibrated in GF buffer at 1.2ml/min. Eluted proteins were collected in 1.8ml fractions and analysed by SDS-PAGE.

Column 3: HiTrap Q Sepharose column (anion exchange).

Column 3 Buffers:
Buffer A: 50 mM Tris, pH 9.0; 10 mM NaCl.
Buffer B: 50 mM Tris, pH 9.0; 1M NaCl.

Procedure: Protein was diluted with 50mM Tris buffer pH 9.0 to the final NaCl concentration of 16 mM. The protein was loaded onto the HiTrap Q Sepharose column, and eluted with a linear NaCl gradient. Fractions containing protein were analysed by SDS-PAGE

Mass spec. characterisation:
Measured: 69 118 Da (ESI-MS)
Expected: 69 202 Da

Protein concentration: Protein was concentrated to 11mg/ml and frozen at -80°C. Concentrations were determined from the absorbance at 280 nm using NanoDrop.

Crystallisation: Crystals were grown at 20°C by vapour diffusion in sitting drops mixing protein (11 mg/ml) and well solution containing 0.2M ammonium acetate, 18% PEG 10000, 0.1M Bis-Tris pH 5.5 at a protein to precipitant ratio of 2:1. Prior to crystallization, protein was pre-incubated with 2mM Xylulose 5-phosphate, 2mM Thiamine pyrophosphate and 2mM

MgCl2. Crystals were cryo-protected using 20% (v/v) ethylene glycol supplemented to the well solution and flash cooled in liquid nitrogen.

Data Collection:
Resolution: 2.05 Å
X-ray source: Diamond Light Source beamline I 24