Entry Clone Source: MGC

Entry Clone Accession: BC039585

SGC Construct ID: KLHL7B-c009

GenBank GI number: gi|170784846

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

Coding DNA sequence:

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ATGGCAGCCTCTGGGGTGGAGAAGAGCAGC
AAGAAGAAGACCGAGAAGAAACTTGCTGCT
CGGGAAGAAGCTAAATTGTTGGCGGGTTTC
ATGGGCGTCATGAATAACATGCGGAAACAG
AAAACGTTGTGTGACGTGATCCTCATGGTC
CAGGAAAGAAAGATACCTGCTCATCGTGTT
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TTAATGTTCACAACTAACATGCTTGAATCA
AAGTCCTTTGAACTAGAACTCAAAGATGCT
GAACCTGATATTATTGAACAACTGGTGGAA
TTTGCTTATACTGCTAGAATTTCCGTGAAT
AGCAACAATGTTCAGTCTTTGTTGGATGCA
GCAAACCAATATCAGATTGAACCTGTGAAG
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GCAGTCAGGTGGTTGAAATACGATGAACCT
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GCTAAAGTCAGGTTTCCTCTTATATCAAAG
AATTTCTTAAGTAAAACGGTACAAGCTGAA
CCACTTATTCAAGACAATCCTGAATGCCTT
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CACACAAAGCCCAGCATGCTGACCCAGCGC
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AGGAAGAATCATGGGCTGGTATTTGTAAAA
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GACAAGATATTTGCTGTGGGTGGTCAGAAT
GGTTTAGGTGGTCTGGACAATGTGGAATAT
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ATTTGTGTTGTCGATACTTGTGGAGCAAAT
GAAGAGACCCTTGAAACATGAAAAATGAGT
GGACTTCAGACTCATCAGAGACTCTAA

Tags and additions: MHHHHHHSSGVDLGTENLYFQ*S(M), TEV-cleavable (*) N-terminal hexahistidine tag.

Expressed sequence (tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfqsmGTRPRRK
KHDYRIALFGGSQPQSCRYFNPKDYSWTDI
RCPFEKRRDAACVFWDNVVYILGGSQLFPI
KRMDCYNVVKDSWYSKLGPPTPRDSLAACA
AEGKIYTSGGSEVGNSALYLFECYDTRTES
WHTKPSMLTQRCSHGMVEANGLIYVCGGSL
GNNVSGRVLNSCEVYDPATETWTELCPMIE
ARKNHGLVFVKDKIFAVGGQNGLGGLDNVE
YYDIKLNEWKMVSPMPWKGVTVKCAAVGSI
VYVLAGFQGVGRLGHILEYNTETDKWVANS
KVRAFPVTSCLICVVDTCGANEETLET

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

Growth medium, induction protocol: A glycerol stock was used to inoculate a 50 ml starter culture containing LB media and 34 µg/ml chloramphenicol and 50 µg/ml kanamycin. The starter culture was grown overnight at 37°C with shaking at 250 rpm. The following morning three flasks containing 1 L LB media with 34 µg/ml chloramphenicol and 50 µg/ml kanamycin were each inoculated with 10 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD_{600} ~0.5 was reached.

The flasks were then cooled down to 18°C and 0.5 mM IPTG added 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 30 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 0.5 mM TCEP, and 1 mM PMSF were added to the cell suspension. The cells were lysed by ultrasonication over 10 min with the sonicator pulsing ON for 5 sec and OFF for 15 sec. The cell lysate was spun down by centrifugation at 17000 rpm and 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 100 ml 2.5 M NaCl, and then applied onto a 2.5 x 20 cm column. The resin was then equilibrated with 100 ml binding buffer prior to loading the sample.

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

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-IDA column.

Column 2: Ni-Affinity Chromatography
5 ml of 50 % Ni-IDA slurry (Genscript) was applied onto a 1.5 x 10 cm column. The column was first washed with deionised destilled H2O, and then equilibrated with binding buffer.

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
Procedure: The flow-through from column 1 (DE52) was applied by gravity flow onto the Ni-IDA column. The bound protein was eluted by applying a step gradient of imidazole using 7 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 250 mM). 10 mM DTT was added to each fraction collected for overnight storage at 4°C.
Enzymatic treatment: TEV protease cleavage. Fractions containing KLHL7B were treated with TEV protease overnight at 4°C.
Column 3: Size Exclusion Chromatography S200 HiLoad 16/60 Superdex run on AKTA-Express
Buffer: Gel Filtration buffer: 300 mM NaCl, 50 mM Hepes pH 7.5, 0.5 mM TCEP
Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. Eluted protein from the Ni-IDA column was pooled and 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 90 - 110 ml. Fractions containing the protein were pooled together, and 10 mM DTT was added for overnight storage at 4°C.
Column 4: Cation-exchange - MonoS 5/50 GL column on AKTA-Purifier
Buffers: Buffer A (low salt): 50 mM Hepes pH 7.5; **Buffer B (high salt):** 50 mM Hepes, pH 7.5; 1 M NaCl
Procedure: Prior to applying the protein, the MonoS column was washed with buffer B before equilibration with buffer A. The eluted protein from gel filtration was diluted with buffer A to 50 ml then applied to a MonoS column, and run at a flow-rate of 1 ml/min with a linear gradient. The protein was eluted at 9.8 % buffer B. Fractions containing the protein were pooled and 10 mM DTT was added. Protein was stored at 4°C.
Concentration: The protein was concentrated in an Amicon Ultra-4 filter with a 10 kDa cut-off.
Mass spectrometry characterization : The purified protein was homogeneous and had an experimental mass of 34.039 kDa, as expected from its primary structure. 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 % acetonitrile in water with 0.1 % formic acid.
Crystallization: Protein was buffered in 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM DTT. Protein was concentrated down to 10.10 mg/ml (calculated using an extinction coefficient of 66350). Crystals were grown at 20°C in 150 nl sitting drops mixing 50 nl protein solution with 100 nl of a reservoir solution containing 0.1M MES, pH 6.5, 12% PEG 20K. On mounting crystals were cryo-protected with an additional 30% ethylene glycol.
Data Collection: Resolution: 1.6Å, **X-ray source:** Rigaku FR-E Superbright