Entry Clone Source: TKC

Entry Clone Accession: n/a

SGC Construct ID: LIMK1A-c056

GenBank GI number: gi|4505001

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

Amplified construct sequence:

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TACTTCCAATCCATGCCACACCGCAT
CTTCCGGCCGTCGGACCTCATCCACG
GGGAGGTGCTGGGCAAGGGCTGCTTC
GGCCAGGCTATCAAGGTGACACACCG
TGAGACAGGTGAGGTGATGGTGATGA
AGGAGCTGATCCGGTTCGACGAGGAG
ACCCAGAGGACGTTCCTCAAGGAGGT
GAAGGTCATGCGATGCCTGGAACACC
CCAACGTGCTCAAGTTCATCGGGGTG
CTCTACAAGGACAAGAGGCTCAACTT
CATCACTGAGTACATCAAGGGCGGCA
CGCTCCGGGGCATCATCAAGAGCATG
GACAGCCAGTACCCATGGAGCCAGAG
AGTGAGCTTTGCCAAGGACATCGCAT
CAGGGATGGCCTACCTCCACTCCATG
AACATCATCCACCGAGACCTCAACTC
CCACAACTGCCTGGTCCGCGAGAACA
AGAATGTGGTGGTGGCTGACTTCGGG
CTGGCGCGTCTCATGGTGGACGAGAA
GACTCAGCCTGAGGGCCTGCGGAGCC
TCAAGAAGCCAGACCGCAAGAAGCGC
TACACCGTGGTGGGCAACCCCTACTG
GATGGCACCTGAGATGATCAACGGCC
GCAGCTATGATGAGAAGGTGGATGTG
TTCTCCTTTGGGATCGTCCTGTGCGA
GATCATCGGGCGGGTGAACGCAGACC
CTGACTACCTGCCCCGCACCATGGAC
TTTGGCCTCAACGTGCGAGGATTCCT
GGACCGCTACTGCCCCCCAAACTGCC
CCCCGAGCTTCTTCCCCATCACCGTG
CGCTGTTGCGATCTGGACCCCGAGAA
GAGGCCATCCTTTGTGAAGCTGGAAC
ACTGGCTGGAGACCCTCCGCATGCAC
CTGGCCGGCCACCTGCCACTGGGCCC
ACAGCTGGAGCAGCTGGACAGAGGTT
TCTGGGAGACCTACCGGCGCGGCGAG
AGCTGACAGTAAAGGTGGATA
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Final protein sequence (Tag sequence in lowercase):

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mghhhhhhssgvdlgtenlyfq^smP
HRIFRPSDLIHGEVLGKGCFGQAIKV
THRETGEVMVMKELIRFDEETQRTFL
KEVKVMRCLEHPNVLKFIGVLYKDKR
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LNFITEYIKGGTLRGIIKSMDSQYPW
SQRVSFAKDIASGMAYLHSMNIIHRD
LNSHNCLVRENKNVVVADFGLARLMV
DEKTQPEGLRSLKKPDRKKRYTVVGN
PYWMAPEMINGRSYDEKVDVFSFGIV
LCEIIGRVNADPDYLPRTMDFGLNVR
GFLDRYCPPNCPPSFFPITVRCCDLD
PEKRPSFVKLEHWLETLRMHLAGHLP
LGPQLEQLDRGFWETYRRGES

^ TEV cleavage site

Tags and additions: Cleavable N-terminal His6 tag.

Host: SF9 *Spodoptera frugiperda* Insect cells

Growth medium, induction protocol: Cells at a density of $2x10^6$/ml were infected with recombinant baculovirus (virus stock P3; 2ml of virus stock/100ml of cell culture). Cells were harvested after 72 hours by centrifugation at 800g for 20 minutes at 4°C.
Lysis buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5 mM Imidazole; 5% Glycerol; 0.5 mM TCEP; 1:1000 protease inhibitor SET V (Calbiochem)).
Extraction buffer, extraction method: Cell pellets from each flask (1L volume) were resuspended in 20ml of Lysis Buffer. The cells were lysed by 2 minute sonication. The cell lysate was spun down by centrifugation at 21K rpm at 4°C for 1h. The supernatant was recovered for purification and passed through a 1.2 µM filter.

Column 1: Ni-Affinity chromatography. 7ml of 50% Ni-sepharose slurry applied onto a 1.5x10cm 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; 50-250 mM Imidazole.

Column 1 Procedure: 7ml of 50% Ni-sepharose slurry was equilibrated with binding buffer and mixed with the LIMK1 supernatant in batch. The mix was left on a rotating platform for 1 hour. Subsequently, the beads was pelleted at 800g for 10 mins and washed with 50ml binding buffer. The wash was repeated with 30ml binding buffer and this slurry applied onto a 1.5x10cm column. The column was then washed again with 50ml wash buffer. The bound protein was eluted by applying a step gradient of imidazole - using 10 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 250 mM). SDS PAGE revealed some protein elution in both the 30 mM imidazole wash as well as the subsequent elution fractions. Both fractions were kept and concentrated to 5ml. A further 10ml binding buffer was then added to dilute the imidazole together with 10mM DTT and 0.1mg TEV protease for overnight tag cleavage.

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

Column 2: Size Exclusion Chromatography - S200 HiLoad 16/60 Superdex run on ÄKTA-Express.

Gel Filtration Buffers: 50 mM HEPES, pH 7.5; 300 mM NaCl; 5% glycerol; 5 mM L-arginine; 5 mM L-glutamate; 0.5 mM TCEP, pH 7.5; 1:1000 protease inhibitor SET V (Calbiochem).

Column 2 Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. The protein was concentrated to 2ml using an Amicon Ultra-15 filter with a 10kDa cut-off. The concentrated protein was directly applied onto the equilibrated S200 16/60 column, and run at a flow-rate of 1ml/min. Elution fractions containing the protein were pooled together.

Column 3: Ni-Affinity Chromatography. 0.5ml of 50% Ni-sepharose slurry applied onto a disposable Bio-Rad 9cm Poly-prep column.

Column 3 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.

Column 3 Procedure: A Ni-rebinding step was performed to remove minor contaminants. LIMK1 protein from column 2 was applied onto the column under gravity flow. In the absence of the cleaved Histag, the LIMK1 protein was collected by washing out the beads with a further 3ml of binding buffer and 8ml of wash buffer. Contaminants were mostly separated as judged by SDS PAGE analysis of a subsequent elution fraction containing 250 mM imidazole.

Column 4: Ion Exchange Chromatography - HiTrap SP HP 5ml column (Pharmacia).

Column 4 Buffer:
Buffer A: 50 mM HEPES, pH 7.5; 50 mM NaCl; 5% glycerol; 5 mM L-arginine; 5 mM L-glutamate; 0.5 mM TCEP, pH 7.5; 1:1000 protease inhibitor SET V (Calbiochem)..
Buffer B: 50 mM HEPES, pH 7.5; 2 M NaCl; 5% glycerol; 5 mM L-arginine; 5 mM L-glutamate; 0.5 mM TCEP, pH 7.5; 1:1000 protease inhibitor SET V (Calbiochem).

Column 4 Procedure: A further ion exchange step was performed (primarily to remove some contaminants arising from the 30 mM imidazole elution fraction from column 1). Prior to applying the protein, the HiTrap SP HP column was washed and equilibrated with buffer A (low salt). The protein from column 4 (Ni-rebinding) was concentrated to 4ml and combined with 45ml buffer A to reduce ionic strength. The diluted protein was loaded onto the HiTrap SP HP column and eluted with a gradient from buffer A to buffer B. Elution fractions containing the LIMK1 protein were pooled, concentrated to >2mg/ml and stabilized with 25% glycerol before flash freezing for storage at -80°C.

Column 5: PD10 Desalting column.

PD10 Buffer: 50 mM HEPES, pH 7.5; 300 mM NaCl; 5% glycerol; 5 mM L-arginine; 5 mM L-glutamate; 0.5 mM TCEP, pH 7.5; 1 mM EDTA; 1:1000 protease inhibitor SET V (Calbiochem).

Column 5 Procedure: Protein from -80°C storage was thawed and applied onto a PD10 desalting column. The protein was eluted in PD10 buffer and concentrated to 8mg/ml using an Amicon Ultra-15 filter with a 30kDa cut-off.

Protein concentration: Protein was concentrated to 8mg/ml using an Amicon 30kDa cut-off concentrator.

Mass spectrometry characterization: The purified protein was homogeneous and had an experimental mass of 35940Da, as expected from the primary sequence. 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.

Crystallisation: Staurosporine was added to the concentrated LIMK1 protein to a final concentration of 1.5 mM. Crystals were grown at 20°C in 200nl sitting drops mixing 150nl protein solution with 50nl of a reservoir solution containing 24% MPD, 90 mM Tris pH 7.2, 10 mM Phenol. On mounting crystals were cryo-protected with mother liquor raised to 30% MPD and supplemented with 10% glycerol.

Data collection: 1.65Å resolution.
X-ray source: Diamond Light Source beamline I03, using monochromatic radiation at wavelength 0.97625Å.