NEK-1 (4B9D) Materials & Methods

Entry Clone Source: SGC Oxford

SGC Construct ID: NEK1A-c011

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

DNA sequence:

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ATGCACCATCATCATCATCATTCTTCTGG
TGTAGATCTGGGTACCGAGAACCTGTACT
TCCAATCCATGGAGAAGTATGTTAGACTA
CAGAAGATTGGAGAAGGTTCATTTGGAAA
AGCCATTCTTGTTAAATCTACAGAAGATG
GCAGACAGTATGTTATCAAGGAAATTAAC
ATCTCAAGAATGTCCAGTAAAGAAAGAGA
AGAATCAAGGAGAGAAGTTGCAGTATTGG
CAAACATGAAGCATCCAAATATTGTCCAG
TATAGAGAATCATTTGAAGAAAATGGCTC
TCTCTACATAGTAATGGATTACTGTGAGG
GAGGGGATCTGTTTAAGCGAATAAATGCT
CAGAAAGGCGTTTTGTTTCAAGAGGATCA
GATTTTGGACTGGTTTGTACAGATATGTT
TGGCCCTGAAACATGTACATGATAGAAAA
ATTCTTCATCGAGACATTAAATCTCAGAA
CATATTTTTAACTAAAGATGGAACAGTAC
AACTTGGAGATTTTGGAATTGCTAGAGTT
CTTAATAGTACTGTAGAGCTGGCTCGAGC
TTGCATAGGGACCCCATACTACTTGTCAC
CTGAAATCTGTGAAAACAAACCTTACAAT
AATAAAAGTGACATTTGGGCTCTGGGGTG
TGTCCTTTATGAGCTGTGTACACTTAAAC
ATGCTTTTGAAGCTGGCAGTATGAAAAAC
CTGGTACTGAAGATAATATCTGGATCTTT
TCCACCTGTGTCTTTGCATTATTCCTATG
ATCTCCGCAGTTTGGTGTCTCAGTTATTT
AAAAGAAATCCTAGGGATAGACCATCAGT
CAACTCCATATTGGAGAAAGGTTTTATAG
CCAAACGCATTGAAAAGTTTCTCTCTCCT
CAGCTTATTGCAGAAGAATTTTGTCTAAA
AACATTTTCGAAGTTTGGATCACAGCCTA
TACCAGCTAAAAGACCAGCTTCAGGACAA
AACTCGATTTCTGTTATGCCTGCTCAGAA
AATTACAAAGCCTGCCGCTAAATATGGAA
TACCTTTAGCATATAAGAAATATGGAGAT
AAAAAATGA
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^sMEK
YVRLQKIGEGSFGKAILVKSTEDGRQ
YVIKEINISRMSSKEREESRREVAVL
ANMKHPNIVQYRESFEENGSLYIVMD
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YCEGGDLFKRINAQKGVLFQEDQILD
WFVQICLALKHVHDRKILHRDIKSQN
IFLTKDGTVQLGDFGIARVLNSTVEL
ARACIGTPYYLSPEICENKPYNNKSD
IWALGCVLYELCTLKHAFEAGSMKNL
VLKIISGSFPPVSLHYSYDLRSLVSQ
LFKRNPRDRPSVNSILEKGFIAKRIE
KFLSPQLIAEEFCLKTFSKFGSQPIP
AKRPASGQNSISVMPAQKITKPAAKY
GIPLAYKKYGDKK

^ TEV protease recognition site (Met1 to Lys328, T162A mutant)
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Tags and additions: Cleavable N-terminal His6 tag

Host: BL-21(DE3)-R3-lambda-ppase (A homemade phage resistant version of BL21(DE3) that carries a plasmid for co-expression of lambda phosphatase).

Transformation: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure.

Expression: Colonies were used to inoculate 50 ml of LB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol in a 250 ml baffled shaker flask, which was placed in a 37°C shaker overnight. The next day 3x 10 ml of this starter culture was used to inoculate 3x 1L of LB media containing 35 µg/ml kanamycin in 2L baffled shaker flasks. When the OD_{600} was approximately 0.45, the temperature was reduced to 20°C and when the OD_{600} was approximately 0.6 the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight.

Cell harvest: Cells were spun at 4000rpm for 10 mins and the pellets resuspended in Lysis Buffer and then frozen at -20°C.

Cell Lysis: The resuspended cell pellet was thawed and lysed by sonication. PEI (polyethyleneimine) was added to a final concentration of 0.15 %. The cell debris and precipitated DNA were spun down.
Lysis buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 20 mM Imidazole, 0.5 mM TCEP, 0.2 mM PMSF.

Purification

Column 1: 5 ml of Ni-Sepharose in a 2 cm diameter gravity flow column.

Column 1 Buffers:
Binding buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 20 mM Imidazole, 0.5 mM TCEP.
Wash buffer 1: As Binding Buffer except 1 M NaCl and 40 mM imidazole.
Wash buffer 2: As Binding Buffer except 60 mM imidazole.
Elution buffer: As Binding Buffer except 250 mM imidazole.

Column 1 Procedure: The clarified supernatant was passed through the column. The column was washed with 50 ml of Binding Buffer and 50 ml each of Wash Buffer 1 and 2. 25 ml of Elute Buffer was passed through to elute the protein.

Column 2: S200 16/60 Gel Filtration (GE Healthcare)

Column 2 Buffers:
Gel filtration buffer: 50 mM Hepes pH 7.4, 300 mM NaCl, 0.5 mM TCEP

Column 2 Procedure: The eluted protein was concentrated to 5 ml volume and injected onto the column

Concentration: The NEK1 was concentrated to 19.8 mg/ml (measured by 280 nm absorbance).

Mass spec characterization:
Expected: 39850.1
Observed: 39851.4

Crystallization: The compound was added to the concentrated protein sample to a concentration of 1 mM. Crystals grew from a 2:1 ratio of protein and precipitant solution (0.2 M Na/KPO_4, 20% PEG3350, 10% Ethylene Glycol), using the vapour diffusion method.

Data Collection:
Resolution: Crystals were cryo-protected by equilibration into precipitant solution containing 25% ethylene glycol, and then flash frozen in liquid nitrogen. Data was collected at Diamond, beamline IO4-1.