

Molecular Biology

Entry Clone Accession:

Entry Clone Source: Tony Pawson

SGC Construct ID: WNK3B-c016

Protein Region: A133-E414

Vector: pNIC28-Bsa4

Tag: N-6HIS;N-TEV

Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):

MHHHHHHSSGVDLGTENLYFQSM AEMKAVATSPSGRFLKFDIELGRGAFKTVYKGLDTE
TWVEVAWCELQDRKLTAEQQRFKEEA EMLKGLQHPNIVRFYDSWESILKGKKCIVLVT
ELMTSGTLKTYLKRFKVMPKVLRSWCRQILKGLQFLHTRTPPIIHRDLKCDNIFITGPTG
SVKIGDLGLATLMRTSFAKSVIGTPEFMAPEMYEEHYDESVDVYAFGMCMLEMATSEYP
YSECQNAAQIYRKVTSGIKPASFNKVTDPEVKEIIEGCIRQNKSERLSIRDLLNHAFFAEDT
GLRVE

Sequence after tag cleavage:

SMAEMKAVATSPSGRFLKFDIELGRGAFKTVYKGLDTETWVEVAWCELQDRKLTAEQQ
RFKEEA EMLKGLQHPNIVRFYDSWESILKGKKCIVLVTELMTSGTLKTYLKRFKVMPKV
VLRSWCRQILKGLQFLHTRTPPIIHRDLKCDNIFITGPTGSVKIGDLGLATLMRTSFAKSVI
GTPEFMAPEMYEEHYDESVDVYAFGMCMLEMATSEYPYSECQNAAQIYRKVTSGIKPAS
FNKVTDPEVKEIIEGCIRQNKSERLSIRDLLNHAFFAEDTGLRVE

DNA Sequence:

ATGGCCACTGATTCAGGGGATCCAGCCAGCACAGAAGATTCTGAGAAACCTGATGGA
ATTTCATTTGAACAGAGTTCCCCAGGTCGCTGCAACTTTGACAGTAGAAGCTAGAC
TAAAGGAGAAAAACAGTACCTTCTCTGCTTCTGGGGAAACTGTAGAAAGGAAGAGAT
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GCCACAATCCCTCCTGGTCCTAAATGA

Protein Expression

Medium: LB

Antibiotics: Kanamycin/Chloramphenicol

Procedure: A glycerol stock was used to inoculate a starter culture which was supplemented with Kanamycin (50 µg/ml)/ Chloramphenicol (34 µg/ml). These were then grown overnight at 37 °C with shaking at 240 RPM. The starter culture was used to inoculate LB flasks supplemented with Kanamycin/Chloramphenicol. The cultures were then grown at 37 °C with shaking at 160 RPM to an OD₆₀₀ of between 0.6-0.8. The cultures were then cooled to 18 °C, induced with 0.25 mM IPTG and the protein was expressed overnight at 18 °C /160 RPM. Cultures were harvested, pellets made up to 40 ml with Nickel affinity binding buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5% Glycerol) and 40 µl of SET III protease inhibitors (Merck) added before storage at -20 °C.

Protein Purification

Procedure: Cells were lysed by sonication, 0.125% polyethyleneimine added, and lysates were clarified by centrifugation at 50,000G. The supernatant was then applied to a Nickel-NTA gravity column and washed and eluted with 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, and 10-300 mM imidazole. Protein containing fractions were then treated with TEV protease overnight at 4 °C prior to gel filtration on an S200 gel filtration column using low salt 50 mM HEPES pH 7.5, 50 mM NaCl, 1 mM TCEP as the running buffer. Fractions containing the protein were pooled, concentrated and loaded onto a 5ml HiTrap S IEX column, and eluted with 50 mM HEPES pH 6.5, 1mM TCEP, 0-1M NaCl. The protein was then buffer exchanged into 50 mM HEPES pH 7.5, 50 mM NaCl, 1 mM TCEP, and concentrated to 12.9 mg/ml using a spin concentrator.

Columns: Column 1: Ni-NTA Gravity Column; Column 2: S200 Gel Filtration; Column 3: Hi-trap S, 5 ml

Concentration: 12 mg/ml

Mass-spec Verification: Intact mass was correct and verified that the protein was diphosphorylated

Structure Determination

Crystallization: Crystals were grown using the sitting drop vapor diffusion method at 20 °C. The inhibitor PP-121 (K06398a) was added to the protein to a concentration of 1mM and 2.3% DMSO final. Crystals were grown in 150 nl drops consisting of 1:1 mother liquor (0.2M magnesium chloride -- 25% PEG3350 -- 0.1M bis-tris pH 5.5) to protein (9.0 mg/ml). 25% Ethylene glycol was added and the crystal was then mounted and flash frozen in liquid nitrogen.

Data Collection: Data was collected at beamline I04 at 100K.

Data Processing: Data was processed to a resolution of approximately 2.0 Å using XDS and either xia2 or autoPROC, or DIALS.