

Vector: pNIC-Zb

Amplified construct sequence:

ATGCACCATCATCATCATCATCATCTTC
TGGTGTGGATAACAAGTTCAACAAGG
AGCGTCGAAGAGCTGCCGTGAAATT
CGCCATCTGCCGAACCTGAACCGCGA
ACAGCGTCGCGCATTATTTCGCAGCC
TGCAGATGCCGAGCCAGAGCGCG
AACCTGCTGGCGGAAGCGAAGAAGCT
GAACGATGCGCAGCCGAAGGGTACCG
AGAACCTGTACTTCCAATCCATGGGG
CCGGAGGATGAGCTGCCGACTGGGC
CGCCGCCAAAGAGTTTACCAAGAAGT
ACGACCCTAAGGACGTACCGGCAGA
GGAGTGAGCTCTGTTCCGCCGTTG
TGTTCATCGAGCTACTGCCACGAGT
TTGCGGTGAAGATTATGGAAGTGACA
GCTGAGCGGCTGAGTCCTGAGCAGCT
GGAGGAGGTGCGGGAAAGCCACACGGC
GAGAGACACACATCCTCGCCAGGT
GCCGCCACCCCCACATCATCACCC
CATCGATTCTACGAGTCTCTAGCT
TCATGTTCTGGTGTGTTGACCTGATG
CGGAAGGGAGAGCTGTTGACTATCT
CACAGAGAAGGTGGCCCTCTGAAA
AGGAAACCAGGTCCATCATGCGGT
CTGCTGGAAGCAGTGAGCTTCTCCA
TGCCAACAAACATTGTGCATCGAGATC
TGAAGCCCGAGAATATTCTCTAGAT
GACAATATGCAGATCCGACTTCAGA
TTTCGGGTTCTCCTGCCACTGGAAC
CTGGCGAGAAGCTTCGAGAGTTGT
GGGACCCCAGGGTATCTAGGCCAGA
GATCCTAAATGCTCCATGGATGAAA
CCCACCCAGGCTATGGCAAGGAGGT
GACCTCTGGCCTGTGGGTGATCTT
GTTCACACTCCTGGCTGGCTGCCAC
CCTTCTGGCACCGGGCAGATCCTG
ATGTTACGCATGATCATGGAGGGCA
GTACCAGTTCAAGTCCCCGAGTGGG
ATGACCGTCCAGCACTGTCAAAGAC
CTGATCTCAGGCTGCTGCAGGTGGA
TCCTGAGGCACGCCTGACAGCTGAGC
AGGCCCTACAGCACCCCTTTGAG
CGTTGA

Final protein sequence (Tag sequence in lowercase):

smgPEDELPDWAAAKEFYQKYDPKDV
IGRGVSSVVRRCVHRATGHEFAVKIM
EVTAERLSPEQLEEVREATRRETHIL
RQVAGHPHIITLIDSYESSSFMFLVF
DLMRKGELFDYLTEKVALSEKETRSI
MRSILLEAVSFLHANNIVHRDLKPENI

LLDDNMQIRLSDFGFSCHLEPGEKLR
ELCGTPGYLAPEILKCSMDETHPGYG
KEVDLWACGVILFTLLAGSPPFWHRR
QILMLRMIMEGQYQFSSPEWDRSST
VKDLISRLLQVDPEARLTAEQALQHP
FFER

(Gly6 to Arg293)

The N-terminal residues, sm, derive from the vector following TEV protease digestion to remove the expression tag.

Tags and additions: N-terminal, TEV cleavable hexahistidine tag and Zbasic tag

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

Growth medium, induction protocol: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure. A number of colonies from the transformation were used to inoculate 1ml of LB media containing 50 μ g/ml kanamycin and 34 μ g/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture. A glycerol stock was used to inoculate 40ml of LB media containing 50 μ g/ml kanamycin and 34 μ g/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day 2x10ml of this starter culture was used to inoculate 2x1L of LB media containing 50 μ g/ml kanamycin in 2x2L baffled shaker flasks. When the OD₆₀₀ was approximately 0.5, the temperature was reduced to 20°C and the cells were induced by the addition of 0.5mM IPTG. The expression was continued overnight. Cells were spun at 5500rpm for 10 mins and the pellets resuspended in Lysis Buffer and then frozen at -20°C.

Lysis buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM Imidazole; 0.5 mM TCEP; 0.5 mM PMSF.

Extraction buffer, extraction method: The resuspended cell pellet was thawed and lysed by sonication. The sample was diluted to 90ml volume and PEI (polyethyleneimine) was added to a final concentration of 0.15%. The cell debris and precipitated DNA were spun down (17000rpm, JA17 rotor, 45 min).

Column 1: 3ml of Ni-NTA in a 1cm diameter gravity flow column.

Column 1 Buffers:

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

Wash Buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 25 mM Imidazole; 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 clarified supernatant was passed through the column. The column was washed with 60ml of binding Buffer and 50ml of Wash Buffer. 15ml of Elution Buffer was passed through to elute the protein.

Column 2: 5ml HiTrap S

Column 2 Buffers:

Buffer A: 25 mM HEPES, pH 7.0; 200 mM NaCl; 0.5 mM TCEP.

Buffer B: 25 mM HEPES, pH 7.0; 2 mM NaCl; 0.5 mM TCEP.

Column 2 Procedure: The eluted protein was diluted with 25ml HEPES pH 7.0 to a NaCl concentration of approximately 200 mM. The protein was loaded onto a 5ml HiTrap S column at 4ml/min (pre-equilibrated in Buffer A), and eluted with a gradient to Buffer B.

Enzymatic treatment: The fractions containing PHKG2A were pooled and TEV protease was added. The sample was left at 4°C overnight.

Column 3: S200 16/60 Gel Filtration

Column 2 Buffer: 25 mM HEPES, pH 7.4; 500 mM NaCl; 0.5 mM TCEP.

Column 2 Procedure: The protein was concentrated to 5ml volume and injected onto an S200 16/60 GF column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.75ml fractions were collected.

Protein concentration: The pooled fractions from column 3 were concentrated to 4.5mg/ml (measured by 280nm absorbance).

Mass spectrometry characterization:

Measured: 33394.8

Expected: 33394.3

Crystallisation: Crystals grew from a 2:1 ratio of protein and precipitant solution (25% PEG3350, 0.1M BisTris pH 5.5), using the vapour diffusion method.

Data collection: 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 I03.