Entry Clone Source: MGC

Entry Clone Accession: IMAGE:5269036

SGC Construct ID: CBLBA-c024

GenBank GI number: gi|54112420

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

Amplified construct sequence:

TTAAGAAGGAGATATACTATGCAAGC
TGCCGCAGATCGCAGGACCGTGGAGA
AGACTTGGAAGCTCATGGACAAAGTG
GTAAGACTGTGCCAAAATCCCAAACT
TCAGTTGAAAAATAGCCCACCATATA
TACTTGATATTTTGCCTGATACATAT
CAGCATTTACGACTTATATTGAGTAA
ATATGATGACAACCAGAAACTTGCCC
AACTCAGTGAGAATGAGTACTTTAAA
ATCTACATTGATAGCCTTATGAAAAA
GTCAAAACGGGCAATAAGACTCTTTA
AAGAAGGCAAGGAGAGAATGTATGAA
GAACAGTCACAGGACAGACGAAATCT
CACAAAACTGTCCCTTATCTTCAGTC
ACATGCTGGCAGAAATCAAAGCAATC
TTTCCCAATGGTCAATTCCAGGGAGA
TAACTTTCGTATCACAAAAGCAGATG
CTGCTGAATTCTGGAGAAAGTTTTTT
GGAGACAAAACTATCGTACCATGGAA
AGTATTCAGACAGTGCCTTCATGAGG
TCCACCAGATTAGCTCTGGCCTGGAA
GCAATGGCTCTAAAATCAACAATTGA
TTTAACTTGCAATGATTACATTTCAG
TTTTTGAATTTGATATTTTTACCAGG
CTGTTTCAGCCTTGGGGCTCTATTTT
GCGGAATTGGAATTTCTTAGCTGTGA
CACATCCAGGTTACATGGCATTTCTC
ACATATGATGAAGTTAAAGCACGACT
ACAGAAATATAGCACCAAACCCGGAA
GCTATATTTTCCGGTTAAGTTGCACT
CGATTGGGACAGTGGGCCATTGGCTA
TGTGACTGGGGATGGGAATATCTTAC
AGACCATACCTCATAACAAGCCCTTA
TTTCAAGCCCTGATTGATGGCAGCAG
GGAAGGATTTTATCTTTATCCTGATG
GGAGGAGTTATAATCCTGATTTAACT
GGATTAGCAGAGAACCTCTACTTCCA
ATC

Final protein sequence (Tag sequence in lowercase):

MQAAADRRTVEKTWKLMDKVVRLCQN
PKLQLKNSPPYILDILPDTYQHLRLI
LSKYDDNQKLAQLSENEYFKIYIDSL

MKKSKRAIRLFKEGKERMYEEQSQDR
RNLTKLSLIFSHMLAEIKAIFPNGQF
QGDNFRITKADAAEFWRKFFGDKTIV
PWKVFRQCLHEVHQISSGLEAMALKS
TIDLTCNDYISVFEFDIFTRLFQPWG
SILRNWNFLAVTHPGYMAFLTYDEVK
ARLQKYSTKPGSYIFRLSCTRLGQWA
IGYVTGDGNILQTIPHNKPLFQALID
GSREGFYLYPDGRSYNPDLTGLAENL
YFq^shhhhhhdykddddk

^ TEV cleave site

Tags and additions: TEV-cleavable C-terminal His tag.

Host: BL21 (DE3)R3-pRARE2.

Growth medium, induction protocol: A glycerol stock was used to inoculate a 10ml starter culture containing LB media with 50µg/ml Kanamycin and 34 µg/ml chloramphenicol. The starter culture was grown overnight and subsequently used to inoculate flasks containing 1L LB with 50µg/ml of kanamycin and 34µg/ml of chloramphenicol (total 6L). The cells were cultured at 37°C until the OD reached 0.620 at which point the temperature was decreased to 18°C. IPTG was added at 0.2mM (final concentration) and the culture kept at 18°C for overnight protein expression.
Extraction buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 5 mM Imidazole. Complete Protease Inhibitor Cocktail Tablets (Roche) were added (one tablet/50ml buffer).
Extraction method: The cells were harvested by centrifugation at 4,000 g for 10 min. The pellet from 1 L culture was resuspended in 25 ml of extraction buffer. The sample was lysed using an ultrasonic processor and then centrifuged at 37505 g for 30 minutes. The supernatant was kept for further purification.

Column 1: Ni-sepharose (Amersham), 6mil of 50% slurry in 1.5 x 10cm column, washed with binding buffer.

Column 1 Buffer:
Binding buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 5 mM Imidazole.
Elution Buffer I: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 30 mM Imidazole.
Elution Buffer II: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 60 mM Imidazole.
Elution Buffer III: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 250 mM Imidazole.

Column 1 Procedure: The sample was divided into two and applied by gravity flow onto two Ni-sepharose columns. The column was washed with 20 ml of binding buffer. The protein was then eluted with 5 ml of elution buffer I, II & 6 ml of elution buffer III respectively.

Enzymatic treatment: 600µl of TEV protease (6mg/ml) were added into the sample from Ni-sepharose purification (elutions I, II and III). The sample was incubalted at 4°C overnight.

Column 2: Superdex 200 Hiload 16 60

Column 2 Buffers: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 0.5 mM TCEP.

Column 2 Procedure: The TEV-cleaved sample was concentrated to 3ml and injected onto an S200 gel filtration column ran at 4°C on an ÄKTA Purifier. Elution fractions were analyzed by SDS-PAGE. The purest fractions were pooled.

Column 3: Ni-sepharose

Column 3 Buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 0.5 mM TCEP.

Column 3 Procedure: The sample was loaded onto the column (packed from 1ml of Ni-NTA slurry). The flow through was collected and the column was then washed with 5ml of binding buffer (also collected).

Protein concentration: Sample from Column 3 was concentrated to 9mg/ml using a Centricon (30 kDa cut off). The final buffer contained 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM DTT.

Mass spectrometry characterization: The purified native protein was homogeneous and had an experimental mass of 36884.7 Da (expected MW = 36882.5 Da). 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 of a C3 column with a gradient of 5-95% isopropanol in water with 0.1% formic acid.

Crystallisation: Crystals were obtained at 20°C in 150nl sitting drops containing 100nl protein (9.0mg/ml CBLB protein with 1mM of EGFR p Y1069 piptide) and 50nl of reservoir solution containing 0.30 M ammonium sulphate, 0.1 M Bis-Tris pH 6.5 and 30% (v/v) PEG3350. Crystals were vitrified in well solution supplemented with 22.5% ethylene glycol

Data Collection:
Resolution: The native crystal diffracted to a resolution of 2.27 Å
X-ray source: Diamond Light Source beamline I03