Entry Clone Source: Synthetic

Entry Clone Accession: GI:38788274

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

Amplified DNA sequence:
CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGCAGTGT
CAGAGCACCGAAGATGCCATGACCGT
GCTGACCCCGCTGACCGAAAAAGATT
ATGAAGGTCTGAAACGTGTTCTGCGC
AGCCTGCAGGCGCATAAAATGGCGTG
GCCGTTTCTGGAACCGGTTGATCCGA
ATGATGCGCCGGATTATTATGGCGTG
ATTAAAGAACCGATGGATCTGGCGAC
CATGGAAGAACGTGTTCAGCGTCGCT
ATTATGAAAAACTGACCGAATTTGTG
GCGGATATGACCAAAATTTTTGATAA
CTGCCGTTATTATAATCCGAGCGATA
GCCCGTTTTATCAGTGCGCGGAAGTT
CTGGAAAGCTTTTTTGTGCAGAAACT
GAAAGGCTTTAAAGCCAGCCGCAGCC
ATTGACAGTAAAGGTGGATACGGATC
CGAA

Final protein sequence (Tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq^smQC
QSTEDAMTVLTPLTEKDYEGLKRVLR
SLQAHKMAWPFLEPVDPNDAPDYYGV
IKEPMDLATMEERVQRRYYEKLTEFV
ADMTKIFDNCRYYNPSDSPFYQCAEV
LESFFVQKLKGFKASRSH

^ TEV cleavage site

Tags and additions: Cleavable N-terminal His6 tag.

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

Growth medium, induction protocol: 10 ml from a 50 ml overnight culture containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol were used to inoculate each of two 1 liter cultures of TB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. Cultures were grown at 37°C until the OD_{600} reached ~2.5 then the temperature was adjusted to 18°C. Expression was induced overnight using 0.1 mM IPTG at an OD_{600} of 3.0. The cells were collected by centrifugation and the pellet re-suspended in binding buffer and frozen.
Binding buffer: PBS, 1% Triton-X100, 100 mM + protease inhibitor cocktail
Extraction buffer, extraction method: Frozen pellets were thawed and fresh 0.5 mM TCEP, 1 mM PMSF added to the lysate. Cells were lysed using sonication. DNA was precipitated with 0.15% PEI. The lysate was centrifuged at 17,000 rpm for 60 minutes and the supernatant collected for purification.

Column 1: Ni-affinity. Ni-sepharose (Amersham), 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer

Column 1 Buffers:
Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% glycerol
Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM Imidazole, 5% glycerol
Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 50 to 250 mM Imidazole (step elution).

Column 1 Procedure: The supernatant was loaded by gravity flow on the Ni-sepharose column. The column was washed first with 30 ml of binding buffer then with 30 ml of wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 200 and 250 mM); fractions were collected until essentially all protein was eluted

Enzymatic treatment: The N-terminal His tag was cleaved by treatment with TEV protease, overnight.

Column 2: Size Exclusion Chromatography; Superdex S75 16/60 HiLoad

Column 2 Buffers:
Buffer: 10 mM HEPES, pH 7.5; 500 mM NaCl, 5% glycerol

Column 2 Procedure: The protein was concentrated and applied to an S75 16/60 HiLoad gel filtration column equilibrated in 10 mM HEPES, pH 7.5; 500mM NaCl, 5% glycerol using an ÄKTAexpress system.

Protein concentration: The protein was concentrated to 4.1 mg/ml using an Amicon 3 kDa cut-off concentrator.

Mass spectrometry characterization:
Measured mass:: 14796.9 Da
LC- ESI -MS TOF gave a measured mass of 14796.9 Da for the TEV cleaved protein as predicted from the sequence of this protein.

Crystallisation: Protein buffer was exchanged to 10 mM HEPES pH7.5 and 300 mM NaCl. The protein was concentrated to 8.4 mg/ml using an Amicon 3 kDa cut-off concentrator. Crystals were grown at 4°C in 150 nl sitting drops from a 2:1 ratio of protein to reservoir solution containing 0.20M Na/KPO4, 20.0% PEG 3350, 10.0% EtGly

Data collection:
Resolution: 1.54Å

Crystals were cryo-protected using the well solution supplemented by 25% ethylene glycol and flash frozen in liquid nitrogen. Data were collected in house on a Rigaku FRE-Superbright with an RAXIS-IV detector at a wavelength of 1.542Å

Phasing: The structure was solved by molecular replacement using an ensemble of known bromodomain structures as a starting model