Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: ASH1LA-c031

GenBank GI number: gi|8922081

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

Amplified construct sequence:
CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGGAAGTGGCGCGTGCGGCG
CGTCTGGCCCAGATTTTTAAAGAAATTTGC
GATGGCATTATTAGCTATAAAGATAGCAGC
CGTCAGGCGCTGGCCGCCCCGCTGCTGAAC
CTGCCGCCGAAAAAAAAAAATGCCGATTAT
TATGAAAAAATTAGCGATCCGCTGGATCTG
ATTACCATCGAAAAACAGATTCTGACCGGT
TATTATAAAACCGTGGAAGCGTTTGATGCC
GATATGCTGAAAGTGTTTCGTAATGCCGAA
AAATATTATGGTCGCAAAAGCCCGGTTGGC
CGCGATGTTTGTCGCCTGCGTAAAGCGTAT
TATAACGCCCGTCATGAAGCGAGCGCCCAG
ATTGATGAAATTGTGGGCGAAACCGCCAGC
GAATGACAGTAAAGGTGGATACGGATCCGA
A

Final protein sequence:
MHHHHHHSSGVDLGTENLYFQ^SMEVARAA
RLAQIFKEICDGIISYKDSSRQALAAPLLN
LPPKKKNADYYEKISDPLDLITIEKQILTG
YYKTVEAFDADMLKVFRNAEKYYGRKSPVG
RDVCRLRKAYYNARHEASAQIDEIVGETAS
E
^ TEV cleave 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 OD600 of 3.0. The cells were collected by centrifugation and the pellet re-suspended in binding buffer and frozen.
Binding buffer: 50 mM HEPES pH 7.5; 500 mM NaCl; 10 mM imidazole, 5% glycerol.

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. 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 then washed with 30 ml wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5-ml 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: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.

Mass spectrometry characterization: LC- ESI -MS TOF gave a measured mass of 14641 for this construct as predicted from the sequence of this protein.

Protein concentration: Protein was concentrated to 14.6 mg/ml using an Amicon 3 kDa cut-off concentrator.

Crystallisation: Crystals were grown at 4 °C in 300 nl sitting drops from a 2:1 ratio of protein (at 5.0 mg/ml) to reservoir solution containing 0.15 M $NaNO_3$, 20 % PEG3350 and 10 % EtGly.

Data collection: Crystals were cryo-protected using the well solution supplemented by 25 % EtGly and flash frozen in liquid nitrogen.
X-ray source: Diffraction data were collected from a single crystal at Diamond beamline I04 at a single wavelength of 0.9762 Å and the structure was refined to 2.54 Å.
Phasing: The structure was solved by molecular replacement using an ensemble of known bromodomain structures as a starting model.