Entry Clone Source: in house ingeniering

Entry Clone Accession: n/a

SGC Construct ID: BRD2A-c013

GenBank GI number: gi|4826806

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

Amplified construct sequence:
CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGGGCAAA
CTGAGCGAACAACTGAAGCATTGCAA
TGGCATTCTTAAAGAGCTCCTGAGTA
AAAAACACGCCGCCTATGCGTGGCCA
TTTTATAAACCGGTGGATGCCTCTGC
GCTGGGTCTGCATGATTATCACGATA
TCATTAAACATCCGATGGATCTCTCA
ACCGTTAAACGTAAAATGGAAAATCG
CGATTATCGTGATGCCCAGGAATTTG
CGGCGGATGTACGCCTCATGTTTTCG
AACTGCTACAAATATAACCCTCCAGA
TCACGATGTTGTGGCAATGGCACGAA
AGCTACAGGATGTATTTGAGTTCCGT
TATGCCAAGATGCCAGATTGACAGTA
AAGGTGGATACGGATCCGAA

Final protein sequence (Tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq^smGK
LSEQLKHCNGILKELLSKKHAAYAWP
FYKPVDASALGLHDYHDIIKHPMDLS
TVKRKMENRDYRDAQEFAADVRLMFS
NCYKYNPPDHDVVAMARKLQDVFEFR
YAKMPD

^ 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.
Lysis 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 17,000rpm for 60 minutes and the supernatant collected for purification.

Column 1: Ni-affinity. Ni-sepharose (Amersham), 5ml of 50% slurry in 1.5 x 10cm column, washed with binding buffer.
Column 1 Buffer: **Binding buffer:** 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 5 mM imidazole. **Wash buffer:** 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 30 mM Imidazole. **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 30ml 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: 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; 500 mM NaCl; 5% glycerol using an ÄKTA express system.
Mass spectrometry characterization: LC- ESI -MS TOF gave a measured mass of 13351Da for this construct as predicted from the sequence of this protein.
Protein concentration: Protein was concentrated to 8.2mg/ml using an Amicon 3kDa cut-off concentrator.
Crystallisation: Crystals were grown at 4°C in 150nl sitting drops from a 2:1 ratio of a protein solution (11.2mg/ml) to reservoir solution containing 30% Jeffamine 600, 0.05 M CsCl, 0.1 M MES pH 6.5.
Data Collection: Crystals were cryo-protected using the well solution supplemented by 20% ethylene glycol and flash frozen in liquid nitrogen. **X-ray source:** Diffraction data were collected from a single crystal on a Rigaku FRE at a single wavelength of 1.54 Å. The final structure was refined to 1.61 Å. **Phasing:** The structure was solved by molecular replacement using an ensemble of known bromodomain structure as a starting model.