Materials and Method

Note: To our best knowledge, this should represent an accurate description of the materials and methods required to reproduce our work. If any of the content on this page is difficult to interpret or should you have trouble repeating our work, do not hesitate to contact us as soon as possible in order for us to provide additional information and advice.

Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: PB1A-c023

GenBank GI number: gi|30794372

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

Amplified construct sequence:

```
CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGCAGCTGTATGATACCGTG
CGTAGCTGCCGCAACAACCAGGGCCAGCTG
ATTGCGGAACCGTTCTATCATCTGCCGAGC
AAAAAAAAATATCCGGATTATTATCAGCAG
ATTAAAATGCCGATCAGCCTGCAGCAGATT
CGCACCAAACTGAAAAATCAGGAATATGAA
ACCCTGGATCACCTGGAATGCGATCTGAAC
CTGATGTTTGAAAACGCGAAACGTTATAAC
GTGCCGAATAGCGCGATCTATAAACGTGTT
CTGAAACTGCAGCAGGTTATGCAGGCCAAA
AAAAAAGAACTGGCGCGTCGCGATGATATT
GAATGACAGTAAAGGTGGATACGGATCCGA
A
```

Final protein sequence (tag sequence in lowercase):

```
mhhhhhhssgvdlgtenlyfq^smQLYDTV
RSCRNNQGQLIAEPFYHLPSKKKYPDYYQQ
IKMPISLQQIRTKLKNQEYETLDHLECDLN
LMFENAKRYNVPNSAIYKRVLKLQQVMQAK
KKELARRDDIE
```

^ TEV cleave site

Tags and additions: Cleavable N-terminal His6 tag.

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

Growth medium, induction protocol: 5 ml from a 50 ml overnight culture containing 50 µg/ml kanamycin & 34 µg/ml chloramphenicol were used to inoculate each of two 1 litre cultures of LB containing 50 µg/ml kanamycin & 34 µg/ml chloramphenicol. Cultures were grown at 37 °C until the OD_{600} reached ~0.5 then the temperature was adjusted to 18 °C. Expression was induced overnight using 0.5 mM IPTG at an OD_{600} of 0.9. 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; 5 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 16,500 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, 50 to 250 mM Imidazole , 5% Glycerol (step elution).

Column 1 Procedure: The flow-through from column 1 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 and 250 mM); fractions were collected until essentially all protein was eluted.
Column 2: Size Exclusion Chromatography. Superdex S200 16/60 HiLoad
Column 2 Buffers: 25 mM HEPES, pH 7.5; 300 mM NaCl, 0.5 mM TCEP
Column 2 Procedure: The protein was concentrated and applied to an S200 16/60 HiLoad gel filtration column equilibrated in 25 mM HEPES, pH 7.5; 300 mM NaCl, 0.5 mM TCEP using an ÄKTAexpress system.
Mass spec characterization: LC- ESI -MS TOF gave a measured mass of 15593 for this construct as predicted from the sequence of this protein.
Protein concentration: Protein was concentrated to 9.3 mg/ml using an Amicon 3 kDa cut-off concentrator.
Crystallization: Crystals were grown at 4 °C in 300 nl sitting drops from a 1:1 ratio of protein to reservoir solution containing 0.1 M NaOAc.$3H_2O$; 1.1 M $(NH_4)_2SO_4$.
Data Collection: Crystals were cryo-protected using the well solution supplemented with 2M Li_2SO_4 and flash frozen in liquid nitrogen. **X-ray source:** Diffraction data were collected from a single crystal on Diamond beamline IO2 at a single wavelength of 0.9795 Å and the structure was refined to 2.2 Å. **Phasing:** The structure was solved by molecular replacement using an ensemble of known bromodomain structures as a starting model.

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