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-c044

GenBank GI number: gi|30794372

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

Amplified construct sequence:

```
CATATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACCTGT
ACTTCCAATCCATGAGCGGCATTAGCCCG
AAAAAAAGCAAATATATGACCCCGATGCA
GCAGAAACTGAACGAAGTGTATGAAGCGG
TGAAAAACTATACCGATAAACGCGGTCGC
CGTCTGAGCGCCATTTTTCTGCGCCTGCC
GAGCCGCAGCGAACTGCCGGATTATTATC
TGACCATTAAAAAACCGATGGATATGGAA
AAAATTCGCAGCCACATGATGGCGAATAA
ATATCAGGATATTGATAGCATGGTTGAAG
ATTTTGTTATGATGTTCAATAACGCGTGC
ACCTATAACGAACCGGAAAGCCTGATCTA
TAAAGATGCGCTGGTGCTGCATAAAGTGC
TGCTGGAAACCCGCCGCGATCTGGAAGGC
GATTGACAGTAAAGGTGGATACGGATCCG
AA
```

Tags and additions: Cleavable N-terminal His6 tag.

Final protein sequence:

```
MHHHHHHSSGVDLGTENLYFQ^SMSGISP
KKSKYMTPMQQKLNEVYEAVKNYTDKRGR
RLSAIFLRLPSRSELPDYYLTIKKPMDME
KIRSHMMANKYQDIDSMVEDFVMMFNNAC
TYNEPESLIYKDALVLHKVLLETRRDLEG
D
```

^ TEV cleave site

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:**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.

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).
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
Buffers: 10 mM HEPES, pH 7.5; 500 mM NaCl, 5% glycerol
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 14626 for this construct as predicted from the sequence of this protein.
Protein concentration: Protein was concentrated to 10.7 mg/ml using an Amicon 3 kDa cut-off concentrator.
Crystallization: NMP was added to the protein solution to a final concentration of 2 mM prior to crystallization. Crystals were grown at 4°C in 300 nl sitting drops from a 1:1 ratio of protein to reservoir solution containing 0.15 M sodium nitrate, 25% PEG 3350, 10% EtGly.
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 Superbright at a single wavelength of 1.5 Å and the structure was refined to 1.66 Å. **Phasing:** The structure was solved by molecular replacement using an ensemble of known bromodomain structures as a starting model.