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]

Entry clone accession/ sequence:
MHHHHHHSSGVDLGTENLYFQ^SMSGISPK
KSKYMTPMQQKLNEVYEAVKNYTDKRGRRL
SAIFLRLPSRSELPDYYLTIKKPMDMEKIR
SHMMANKYQDIDSMVEDFVMMFNNACTYNE
PESLIYKDALVLHKVLLETRRDLEGD
^ TEV cleave site

Tags and additions: Cleavable N-terminal His6 tag.

Amplified construct sequence:
TACTTCCAATCCATGAGCGGCATTAGCCCG
AAAAAAAGCAAATATATGACCCCGATGCAG
CAGAAACTGAACGAAGTGTATGAAGCGGTG
AAAAACTATACCGATAAACGCGGTCGCCGT
CTGAGCGCCATTTTTCTGCGCCTGCCGAGC
CGCAGCGAACTGCCGGATTATTATCTGACC
ATTAAAAAACCGATGGATATGGAAAAAATT
CGCAGCCACATGATGGCGAATAAATATCAG
GATATTGATAGCATGGTTGAAGATTTTGTT
ATGATGTTCAATAACGCGTGCACCTATAAC
GAACCGGAAAGCCTGATCTATAAAGATGCG
CTGGTGCTGCATAAAGTGCTGCTGGAAACC
CGCCGCGATCTGGAAGGCGATTGACAGTAA
AGGTGGATA

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

Growth medium, induction protocol: 5ml from a 50 ml overnight culture containing 50µg/ml kanamycin/34µg/ml chloramphenicol was used to inoculate each of two 1 litre cultures of LB containing 50 µg/ml kanamycin/34µg/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: 50mM HEPES pH 7.5; 500 mM NaCl; 5 mM imidazole, 5% glycerol.

Extraction buffer, extraction method: Frozen pellets were thawed and fresh 0.5mM TCEP, 1mM 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: Ion exchange - Nucleic acid removal. DEAE cellulose (DE52, Whatman), 10 g of resin in 2.5 x 20 cm column. The resin was hydrated in 2.5M NaCl, then washed with 20 ml binding buffer prior to loading the sample.

Buffers: 50mM HEPES pH 7.5; 500 mM NaCl; 5 mM imidazole, 5% glycerol

Procedure: Supernatant was applied by gravity flow, followed by a wash with 50 ml binding buffer. The column flow-through was collected.

Column 2: 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, 50 to 250 mM Imidazole, 5% Glycerol (step elution).

Procedure: The flowthrough 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. 10 mM DTT was added for overnight storage.

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

Column 3: Size Exclusion Chromatography. Superdex S200 16/60 HiLoad

Buffers: 25 mM HEPES, pH 7.5; 300 mM NaCl, 0.5 mM TCEP

Procedure: PB1 was concentrated and applied to an S200 16/60 HiLoad gel filtration column equilibrated in 25mM HEPES, pH 7.5; 300mM NaCl, 0.5 mM TCEP using an ÄKTAexpress system.

Column 4: Ni-affinity. Ni-sepharose (Amersham), 2 ml of 50% slurry in a Bio-rad poly-prep column, washed with binding buffer.

Buffers:
Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% Glycerol
Wash buffer: 25 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM TCEP
Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 250 mM Imidazole , 5% Glycerol

Procedure: Gel filtration fractions containing PB1 were pooled and loaded by gravity flow on the Ni-sepharose column. After loading a further 4 ml of binding buffer was added and the full flow through was collected. The column was then washed with 5 ml wash buffer at gravity flow. Finally, 6 ml of elution buffer was added. Flow through, wash and elution fractions were analysed by SDS PAGE. The TEV-cleaved PB1protein was mainly found in the wash fraction. 10 mM DTT was added for overnight storage.

Mass spec 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 11.49 mg/ml using an Amicon 3 kDa cut-off concentrator.

Crystallization: Crystals were grown at 4 °C in 600nl sitting drops from a 1:1 & 1:2 ratio of protein to reservoir solution 0.2M Na Nitrate; 25w/v PEG3350; 5v/v ethylene glycol.

Data Collection: Crystals were cryo-protected using the well solution supplemented with 20% ethylene glycol and flash frozen in liquid nitrogen.

X-ray source: Diffraction data were collected from a single crystal on a Rigaku FR-E SuperBright at a single wavelength of 1.5 Å and the structure was refined to 1.75 Å
Phasing: The structure was solved by molecular replacement using an ensemble of known bromodomain structures as a starting model.