Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: CREBBPA-c003

GenBank GI number: gi|4758056

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

Tags and additions: Tag sequence: mhhhhhhssgvdlgtenlyfq*sm, cleaved at the * with TEV protease.

Expressed sequence: (tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq*smRKKIF
KPEELRQALMPTLEALYRQDPESLPFRQP
VDPQLLGIPDYFDIVKNPMDLSTIKRKLD
TGQYQEPWQYVDDVWLMFNNAWLYNRKTS
RVYKFCSKLAEVFEQEIDPVMQSLG

Host: BL21(DE3)-R3: a phage resistant BL21(DE3) derivative

Growth medium, induction protocol
Host cells transformed with the expression plasmids were plated out onto LB-agar plates containing 50 mg/ml kanamycin. The next day several colonies were combined into 1 ml TB (Terrific Broth), 50 µg/ml kanamycin, which was then grown overnight and stored as glycerol stocks at -80°C.
The glycerol stock was used to inoculate a 10-ml starter culture in TB + kanamycin (50 µg/ml). This starter culture was grown overnight at 37°C and used to inoculate a 1 liter culture in the same medium. The culture was grown in baffled flasks at 37°C until the OD_{600}reached ~3.5. After that the temperature was lowered to 18°C. Protein production was induced with 0.1mM IPTG and the recombinant bromodomain was incubation continued at 18°C overnight. The next day cells were harvested by centrifugation at 4000 rpm for 30 minutes. The cell pellet was stored at -80°C degrees

Extraction buffer and extraction method: Lysis buffer: 500mM NaCl, 50mM pH8.0 KH_2PO_4, 0.5mM TCEP, Benzonase 1µl/15 ml buffer, Protease inhibitor (1 µl/ml); **Afinity binding buffer:** 10mM Imidazole, 500mM NaCl, 50mM pH8.0 KH_2PO_4, 0.5mM TCEP.

Procedure: The cell pellet (40g) from 4 L culture was re-suspended in one volume (40 ml) of lysis buffer. The re-suspended cells were lysed by one passage through a Constant Systems cell breaker and subsequent sonication; the cell breaker was washed with 1x extraction buffer, bringing the total volume to 120 ml. DNA was precipitation by addition of polyethyleneimine (PEI, pH 7.5) to a final concentration of 0.15 % during an incubation time of 30 min on ice, followed by a centrifugation at 17,000 rpm (4°C); The supernatant was further cleared by filtration through a 0.2 µm serum Acrodisc filter.

Column 1: Ni-affinity chromatography: HisTrap FF Crude, 5 ml (GE Healthcare).

Buffers: Binding Buffer: 50mM NaH_2PO_4, 500mM NaCl, 30mM Imidazole, pH 8.0, 0.5mM TCEP;
Elution Buffer: 50mM NaH_2PO_4, 500mM NaCl, 250mM Imidazole, pH 8.0, 0.5mM TCEP.

Procedure: All purification steps were carried out using an AKTAexpress system (GE Healthcare) at 7ºC. The lysate was loaded on a pre-equilibrated His-trap column at 0.8 ml/min. After loading, the column was washed at 0.8 ml/min with 50 ml binding buffer and the protein was eluted with 25 ml of elution buffer. The peak fraction was collected automatically according to A280.

Enzymatic treatment and Tag removal: TEV protease (1:20 w/w), was added to the sample after gel filtration. The sample was incubated at 4°C overnight. The sample was then passed over a column of Ni-sepharose (0.5 ml) to trap the cleaved tag and other Ni-binding proteins.

Column 2: Size exclusion chromatography HiLoad 16/60 Superdex 75

SEC-Buffers: 10mM Hepes, pH 7.4, 500 mM NaCl, 5% glycerol, 0.5mM TCEP
Procedure: Fractions containing the expressed bromo domain were collected after his6-tag cleavage and were loaded on a SEC column at 1.0 ml/min. Eluted fractions were >95% pure as judged by SDS-PAGE
Mass spec characterization (After tag cleavage): Expected MW: 14207 (cleaved protein); Measured MW: 14207
Protein concentration: The protein was concentrated to 10 mg/ml in SEC buffer using a centricon device with a 10kDa cut off
Crystallization: The protein (10 mg/ml) in SEC buffer was mixed with an equal volume (100 nl) of reservoir solution (0.2M Potassium thiocyanate; 25% (w/v) PEG3350; 5% (v/v) ethylene glycol and equilibrated as a sitting drop at 4°C.
Data Collection: Crystals were flash frozen in liquid nitrogen using the crystallization condition supplemented with 25% ethylene glycol. Diffraction data were collected to 1.98 Å at a RIGAKU FR-E+ SuperBright light source at a single wavelength of 1.54128 Å.