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]

Amplified construct sequence:

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CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGCGCAAAAAAATTTTTAAA
CCGGAAGAACTGCGTCAGGCGCTGATGCCG
ACCCTGGAAGCGCTGTATCGCCAGGATCCG
GAAAGCCTGCCGTTTCGTCAGCCGGTGGAT
CCGCAGCTGCTGGGTATCCCGGATTATTTT
GATATTGTGAAAAACCCGATGGATCTGAGC
ACCATCAAACGCAAACTGGATACCGGCCAG
TATCAGGAACCGTGGCAGTATGTGGATGAT
GTTTGGCTGATGTTTAATAATGCGTGGCTG
TATAACCGTAAAACCAGCCGTGTGTATAAA
TTCTGTAGCAAACTGGCGGAAGTTTTTGAA
CAGGAAATTGATCCGGTGATGCAGAGCCTG
GGCTGACAGTAAAGGTGGATACGGATCCGA
A
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Final protein sequence (tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq*smRKKIFK
PEELRQALMPTLEALYRQDPESLPFRQPVD
PQLLGIPDYFDIVKNPMDLSTIKRKLDTGQ
YQEPWQYVDDVWLMFNNAWLYNRKTSRVYK
FCSKLAEVFEQEIDPVMQSLG
^ TEV cleave site
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Tags and additions: MHHHHHHSSGVDLGTENLYFQ*SM, cleaved at the * with TEV protease.

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 50µg/ml kanamycin. The next day several colonies were combined into 1 ml TB (Terrific Broth), 50µg/ml kanamycin, which was then grown overnight and stored as glycerol stocks at -80°C. The glycerol stock was used to inoculate a 10ml starter culture in TB + kanamycin (50µg/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.
Binding buffer:
Lysis buffer: 500mM NaCl, 50mM pH8.0 KH2PO4, 0.5mM TCEP, Benzonase 1ml/15 ml buffer, Protease inhibitor (1 ml/ml).

Affinity binding buffer: 10mM Imidazole, 500mM NaCl, 50mM pH8.0 KH2PO4, 0.5mM TCEP.
Extraction buffer, extraction method: The cell pellet (40g) from 4L cultue was re-suspended in one volume (40ml) 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 120ml. 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 0.2µm serum Acrodisc filter.

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

Column 1 Buffers:
Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 0.5mM TCEP

Column 1 Procedure: The supernatant was loaded by gravity flow on the DAE-52 column. The column was then washed with 2 x 50 ml binding buffer at gravity flow.

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 (0.5ml) to trap the cleaved tag and other Ni-binding proteins.

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

SEC-Buffers: 10 mM Hepes, pH 7.4, 500 mM NaCl, 5% glycerol, 0.5mM TCEP.

Column 2 Procedure: Fractions containing the expressed bromodomain were collected after his6-tag cleavage and were loaded on a SEC column at 1.0ml/min. Eluted fractions were >95% pure as judged by SDS-PAGE.

Mass spectrometry characterization (After Tag cleavage):
Expected MW: 14207 (cleaved protein)
Measured MW: 14207

Protein concentration: The protein was concentrated to 10mg/ml in SEC buffer using a centricon device with a 10kDa cut off.

Crystallisation:
Acetylated lysine complex (3P1C): 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 in the presence of 5mM of acetylated lysine.
N-Methyl-2-pyrrolidone (3P1D): The protein (10 mg/ml) in SEC buffer was mixed with an equal volume (100 nl) of reservoir solution (0.2M Potassium thiocyanate; 20% (w/v) PEG3350; 5% (v/v) ethylene glycol and equilibrated as a sitting drop at 4°C in the presence of 5mM of N-Methyl-2-pyrrolidone.
Dimethyl-sulfoxide (3P1E): 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 in the presence of 5mM of dimethyl sulfoxide.
3-methyl-3,4-dihydroquinazolin-2(1H)-one (3P1F): The protein (10 mg/ml) in SEC buffer was mixed with an equal volume (100 nl) of reservoir solution (0.15M Potassium thiocyanate; 20% (w/v) PEG3350; 10% (v/v) ethylene glycol and equilibrated as a sitting drop at 4°C in the presence of 5mM of 3-methyl-3,4-dihydroquinazolin-2(1H)-one.

Data Collection: Crystals were flash frozen in liquid nitrogen using the crystallization condition supplemented with 25% ethylene glycol. Diffraction data were collected at a RIGAKU FR-E+ SuperBright light source at a single wavelength of 1.54128 Å.