BRD4A(1) (3UVX) Materials & Methods

Entry Clone Source: Synthetic

Entry Clone Accession: gi|19718731

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

Amplified construct sequence:

```
CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGAACCCC
CCGCCCCCAGAGACCTCCAACCCTAA
CAAGCCCAAGAGGCAGACCAACCAAC
TGCAATACCTGCTCAGAGTGGTGCTC
AAGACACTATGGAAACACCAGTTTGC
ATGGCCTTTCCAGCAGCCTGTGGATG
CCGTCAAGCTGAACCTCCCTGATTAC
TATAAGATCATTAAAACGCCTATGGA
TATGGGAACAATAAAGAAGCGCTTGG
AAAACAACTATTACTGGAATGCTCAG
GAATGTATCCAGGACTTCAACACTAT
GTTTACAAATTGTTACATCTACAACA
AGCCTGGAGATGACATAGTCTTAATG
GCAGAAGCTCTGGAAAAGCTCTTCTT
GCAAAAAATAAATGAGCTACCCACAG
AAGAATGACAGTAAAGGTGGATACGG
ATCCGAA
```

Final protein sequence (Tag sequence in lowercase):

```
mhhhhhhssgvdlgtenlyfq^smNP
PPPETSNPNKPKRQTNQLQYLLRVVL
KTLWKHQFAWPFQQPVDAVKLNLPDY
YKIIKTPMDMGTIKKRLENNYYWNAQ
ECIQDFNTMFTNCYIYNKPGDDIVLM
AEALEKLFLQKINELPTEE

^ TEV cleavage site
```

Tags and additions: Cleavable N-terminal His6 tag

Host: BL21(DE3)-R3-pRARE2

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 cell disruptor. 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.

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, 5% glycerol, 50 to 250 mM Imidazole (step elution).

Column 1 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 5ml 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

Column 2 Buffers:
Gel Filtration buffer: 10 mM HEPES, pH 7.5; 500 mM NaCl, 5% glycerol

Column 2 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 ÅKTAexpress system.

Mass spec characterization: LC-ESI-MS TOF gave a measured mass of 15083 for construct as predicted from the sequence of this protein.

Concentration: Protein was concentrated to 8.0 mg/ml using an Amicon 3kDa cut-off concentrator.

Crystallization: Prior to crystallization, protein buffer was exchanged to 50 mM HEPES pH7.5, 150 mM NaCl. Protein was mixed with the peptide at a ratio 50 µM protein for 1.5 mM peptide then concentrated to 8.0 mg/ml using an Amicon 3kDa cut-off concentrator. Crystals were grown at 4°C in 300 nl sitting drops from a 1:2 ratio of protein/peptide to reservoir solution containing 0.35 M sodium formate, 20 w/v PEG3350, 10 % ethylene glycol.

Data Collection:
Resolution: 1.91 Å **X-ray source**: Diamond IO4
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 in house on an Rigaku FRE-E equipped with an RAXIS IV detector at a single wavelength of 1.52 Å and the structure was refined to 1.91 Å.
Phasing: The structure was solved by molecular replacement using an ensemble of known bromodomain structures as a starting model.