Entry Clone Source: Volker Dötsch group

Entry Clone Accession: GI:31543818

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

Amplified DNA sequence:
GATGATGAACTGTTATACTTACCAGT
GAGGGGCCGTGAGACTTATGAAATGC
TGTTGAAGATCAAAGAGTCCCTGGAA
CTCATGCAGTACCTTCCTCAGCACAC
AATTGAAACGTACAGGCAACAGCAAC
AGCAGCAGCACCAGCACTTACTTCAG
AAACAGACCTCAATACAGTCT

Final protein sequence (Tag sequence in lowercase):
mghhhhhhdydipttenlyfq^gsDD
ELLYLPVRGRETYEMLLKIKESLELM
QYLPQHTIETYRQQQQQQHQHLLQKQ
TSIQS

^ TEV cleavage site

Tags and additions: Cleavable N-terminal His6 tag.

Host: BL21 (DE3)

Growth medium, induction protocol: 10 ml from a 50 ml overnight culture containing 100 µg/mL ampiciline were used to inoculate each of two 1 liter cultures of LB containing 100 µg/mL ampiciline. Cultures were grown at 37°C until the OD_{600} reached ~0.6 then the temperature was adjusted to 22°C. Expression was induced overnight using 1 mM IPTG at an OD_{600} of 0.8. The cells were collected by centrifugation and the pellet re-suspended in binding buffer and frozen.
Binding buffer: 50mM Tris buffer, pH 7.8; 500mM NaCl; 10 mM Imidazole
Extraction buffer, extraction method: Frozen pellets were thawed. Cells were lysed using sonication. The lysate was centrifuged at 17,000 rpm for 30 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: 50mM Tris buffer, pH 7.8; 500mM NaCl; 10 mM Imidazole
Wash buffer: 50mM Tris buffer, pH 7.8; 500mM NaCl; 30 mM Imidazole
Elution buffer: 50mM Tris buffer, pH 7.8; 500mM NaCl; 500 mM Imidazole.

Column 1 Procedure: The supernatant was loaded by gravity flow on the Ni-sepharose column. The column was washed first with 30 ml of binding buffer then with 30 ml of wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5ml portions of elution buffer; fractions were collected until essentially all protein was eluted.

Enzymatic treatment: The N-terminal His tag was cleaved by treatment with TEV protease, overnight. Cleaved protein was purified by rebinding on 3 ml pre-equilibrated 50% Ni-NTA beads. The flow through fractions as well as fractions eluted with binding buffer were collected.

Column 2: 5 ml HiTrap Q HP

Column 2 Buffers:
Buffer A: 20mM Tris buffer, pH 8.5
Buffer B: 20mM Tris buffer, pH 8.5; 1 M NaCl

Column 2 Procedure: The column was equilibrated with 5 column volumes of Buffer A. The sample was applied to the column and the column eluted with a 20 column volume gradient from 0 mM to 500 mM NaCl. 1 ml fractions were collected and analysed by SDS-PAGE. The most pure fractions were pooled.

Column 3: Size Exclusion Chromatography; Superdex S75 16/60 HiLoad

Column 3 Buffers:
Buffer: 20mM Tris buffer, pH 7.8.

Column 3 Procedure: The eluted fractions from the Anion Exchange column were concentrated to a final volume of 5 mL and applied to an S75 16/60 HiLoad gel filtration column equilibrated in 20mM Tris buffer, pH 7.8 using an ÄKTAexpress system. Eluted proteins were collected in 2 mL fractions and analysed on SDS-PAGE

Protein concentration: The protein was concentrated to 104.5 mg/ml using an Amicon 10 kDa cut-off concentrator and stored at 4°C. The protein concentration was determined spectrophotometrically using $\varepsilon_{280} = 5960$

Mass spectrometry characterization:
Measured mass:: 14535.8 Da
LC- ESI -MS TOF gave a measured mass of 14535.8 Da for the cleaved protein as predicted from the sequence.

Crystallisation: Crystals were grown by vapour diffusion at 20°C, in 150 nL sitting drops mixing 100 nL protein (15 mg/mL) and 50 nL mother liquor (35% w/v LMW PgSm) equilibrated against 20 µl reservoir containing mother liquor

Data collection:

Crystals were cryo-protected using the well solution and flash frozen in liquid nitrogen.

Phasing: The structure was solved by molecular replacement using an ensemble of p53 family tetramerization domain structures as a starting model