Materials and Method

Note: To our best knowledge, this should represent an accurate description of the materials and methods required to reproduce our work. If any of the content on this page is difficult to interpret or should you have trouble repeating our work, do not hesitate to contact us as soon as possible in order for us to provide additional information and advice.

Entry Clone Source: MGC

Entry Clone Accession: IMAGE:5185069

SGC Construct ID: CA7A-c007

GenBank GI number: gi|4885101

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

Amplified construct sequence:

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CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGCACGGCTGGGGCTACGGC
CAGGACGACGGCCCCTCGCATTGGCACAAG
CTGTATCCCATTGCCCAGGGAGATCGCCAA
TCACCCATCAATATCATCTCCAGCCAGGCT
GTGTACTCTCCCAGCCTGCAACCACTGGAG
CTTTCCTATGAGGCCTGCATGTCCCTCAGC
ATCACCAACAATGGCCACTCTGTCCAGGTA
GACTTCAATGACAGCGATGACCGAACCGTG
GTGACTGGGGGCCCCCTGGAAGGGCCCTAC
CGCCTCAAGCAGTTTCACTTCCACTGGGGC
AAGAAGCACGATGTGGGTTCTGAGCACACG
GTGGACGGCAAGTCCTTCCCCAGCGAGCTG
CATCTGGTTCACTGGAATGCCAAGAAGTAC
AGCACTTTTGGGGAGGCGGCCTCAGCACCT
GATGGCCTGGCTGTGGTTGGTGTTTTTTTG
GAGACAGGAGACGAGCACCCCAGCATGAAT
CGTCTGACAGATGCGCTCTACATGGTCCGG
TTCAAGGGCACCAAAGCCCAGTTCAGCTGC
TTCAACCCCAAGTGCCTCCTGCCTGCCAGC
CGGCACTACTGGACCTACCCGGGCTCTCTG
ACGACTCCCCCACTCAGTGAGAGTGTCACC
TGGATTGTGCTCCGGGAGCCCATCTGCATC
TCTGAAAGGCAGATGGGGAAGTTCCGGAGC
CTGCTTTTTACCTCGGAGGACGATGAGAGG
ATCCACATGGTGAACAACTTCCGGCCACCA
CAGCCACTGAAGGGCCGCGTGGTAAAGGCC
TCCTTCTGACAGTAAAGGTGGATACGGATC
CGAA
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Tags and additions: N-terminal Histidine-tag with TEV protease cleavage site

Final protein sequence (tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfqsMHGWGYGQ
DDGPSHWHKLYPIAQGDRQSPINIISSQAV
YSPSLQPLELSYEACMSLSITNNGHSVQVD
FNDSDDRTVVTGGPLEGPYRLKQFHFHWGK
KHDVGSEHTVDGKSFPSELHLVHWNAKKYS
TFGEAASAPDGLAVVGVFLETGDEHPSMNR
LTDALYMVRFKGTKAQFSCFNPKCLLPASR
HYWTYPGSLTTPPLSESVTWIVLREPICIS
ERQMGKFRSLLFTSEDDERIHMVNNFRPPQ
PLKGRVVKASF
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Growth medium, induction protocol: 10ul of a glycerol stock was inoculated into 5ml of TB medium (supplemented with 50ug/ml Kanamycin, 34ug/ml Chloramphenicol) and cultured at 37°C o/n in a

shaking incubator (250 rpm). Next day 0.75 ml of o/n culture was used to inoculate 1 litre of TB medium (6 x) and grown at 37°C with vigorous shaking (160 rpm) until the culture reaches an OD_{600} of 1.6. Temperature was reduced to 18°C, and cells were induced with IPTG at a concentration of 0.3 mM, and further cultivated for 16 hrs. Cells were harvested by centrifugation at 6500 rpm for 10 min, and the cell pellet was stored at -20°C until further use.

Extraction buffer, extraction method:
Lysis buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol + 1mM PMSF.
The thawed cells were broken by 5 passes at 16.000 psi through a high pressure homogeniser followed by centrifugation for 45 min at 15.000rpm.

Column 1: Ni-affinity, His-Trap, 5 ml (Amersham)
Column 2: Superdex 200, HiPrep 16/60 (Amersham)

Buffers:
Start buffer: 50mM HEPES pH 7.5, 500mM NaCl, 20mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP
Washing buffer: 50mM HEPES pH 7.5, 500mM NaCl, 40mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP
Elution buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5% glycerol, 250mM Imidazole, 0.5mM TCEP
GF buffer: 10mM HEPES pH 7.5, 500mM NaCl, 5% glycerol, 0.5mM TCEP

Procedure: The cell extract was loaded on the AKTA Express system The extinction at 280nm was monitored and fractions were collected and analyzed by SDS-PAGE. Positive fractions were pooled reductive methylation.

Reductive methylation: Protein sample was diluted to a concentration of 1-2 mg/ml in 50mM HEPES pH 7.5, 300mM NaCl and 20ul of fresh 1M Dimethylamine borane complex (DMAB) & 40ul 1M formaldehyde was added per ml protein solution. Sample was mixed by rotation at 4rpm at 277K (4°C) for 2 hours and then further 20ul of fresh 1M DMAB & 40ul 1M formaldehyde was added per ml protein solution and reaction was continued for 2 more hours. Following addition of further 10ul fresh1M DMAB per ml protein solution, reaction was incubated overnight. Next morning methylation reaction was terminated by adding 100mM Tris buffer pH 7.5 and DMAB was removed from sample by 3 times centrifugal buffer exchange.
Later sample was loaded on the gel filtration column in 50mM HEPES pH 7.5, 300mM buffer at 1.0 ml/min on an AKTA Purifier system. Eluted proteins were collected in 1 ml fractions.

Enzymatic treatment: None

Protein concentration: The target protein was concentrated to 12 mg/ml using Vivaspin 10K concentrators and stored at -80°C.

Mass spectrometry characterization: Corresponds to theoretical mass, as determined by ESI-TOF MS.

Crystallization: Crystals were grown by vapour diffusion in sitting drop at 20°C. Before setting up the experiment 6-Ethoxy-2-benzothiazolesulfonamide was added to the protein to a final concentration of 2mM. A sitting drop consisting of 100 nl protein and 50 nl well solution was equilibrated against well solution containing 0.2 M Na_K_tartrate, 0.2 M Ammonium sulphate, 0.1 M Tri-Nacit pH 4.5. Crystals were mounted in the presence of 30% (v/v) glycerol and flash-cooled in liquid nitrogen.

Data Collection: Resolution: 2.32 Å **, X-ray source:** FRE superbright, single wavelength