Entry Clone Source: MGC

SGC Construct ID: AMPHA-c010

Entry Clone Accession: gi|4502081

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

Amplified DNA sequence:

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ATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACC
TGTACTTCCAATCCATGGCCGACATC
AAGACGGGCATCTTCGCCAAGAACGT
CCAGAAGCGACTCAACCGCGCGCAGG
AAAAGGTCCTCCAAAAGCTGGGGAAA
GCTGATGAGACAAAAGACGAACAGTT
CGAAGAATATGTCCAGAACTTCAAAC
GGCAAGAAGCAGAGGGTACCAGACTT
CAGCGAGAACTCCGAGGATATTTAGC
AGCAATCAAAGGCATGCAGGAGGCCT
CCATGAAGCTCACAGAGTCGCTGCAT
GAAGTCTATGAGCCTGACTGGTATGG
GCGGGAAGATGTGAAAATGGTTGGTG
AGAAATGTGATGTGCTGTGGGAAGAC
TTCCATCAAAAACTCGTGGATGGGTC
CTTGCTAACACTGGATACCTACCTGG
GGCAATTTCCTGACATAAAGAATCGC
ATCGCCAAGCGCAGCAGGAAGCTAGT
GGACTATGACAGTGCCCGCCACCATC
TGGAAGCTCTGCAGAGCTCCAAGAGG
AAGGATGAGAGTCGAATCTCTAAGGC
AGAAGAAGAATTTCAGAAAGCACAGA
AAGTGTTTGAAGAGTTTAACGTTGAC
TTACAAGAAGAGTTACCATCATTATG
GTCAAGACGAGTTGGATTTTATGTTA
ATACTTTCAAAAACGTCTCCAGCCTT
GAAGCCAAGTTTCATAAGGAAATTGC
GGTGCTTTGCCACAAACTGTATGAAG
TGATGACAAAACTGGGTGACCAGCAC
GCCGACAAGGCCTGA
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smAD
IKTGIFAKNVQKRLNRAQEKVLQKLG
KADETKDEQFEEYVQNFKRQEAEGTR
LQRELRGYLAAIKGMQEASMKLTESL
HEVYEPDWYGREDVKMVGEKCDVLWE
DFHQKLVDGSLLTLDTYLGQFPDIKN
RIAKRSRKLVDYDSARHHLEALQSSK
RKDESRISKAEEEFQKAQKVFEEFNV
DLQEELPSLWSRRVGFYVNTFKNVSS
LEAKFHKEIAVLCHKLYEVMTKLGDQ
HADKA
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^ TEV cleavage site

Tags and additions: Cleavable N-terminal His6 tag. This construct includes amino acids 1 - 233 of AMPH.

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain)

Growth medium, induction protocol: The expression plasmid was transformed into the host strain and plated on LB-agar containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. Several colonies were combined to inoculate a 1ml culture in TB (+ 50 µg/ml kanamycin, 34 µg/ml chloramphenicol). The culture was grown overnight, glycerol was added to 15% v/v (from a 60% stock), and the resulting glycerol stock was frozen at -80°C in 100 µl aliquots. A loopful of cells from the glycerol stock was inoculated into 20ml of TB medium containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol and grown overnight at 37°C. 2x 1L TB medium (containing 50 µg/ml kanamycin) were each inoculated with 10 ml of the overnight culture and grown in 2.5L UltraYield baffled flasks until OD_{600} of 3.0. Cells were cooled to 18°C, IPTG added to 0.1mM and growth continued at 18°C overnight. The cells were collected by centrifugation then the pellets were scraped out and transferred to 50ml Falcon tubes and frozen at -80°C.
Lysis buffer: 40mM Tris, pH 8, 250 mM KCl, 30 mM imidazole, 1 mM TCEP, 10% glycerol, 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution), Benzonase (3 µl per 25 ml, 30 units/ml).
PEI (polyethyleneimine) stock: 5% (w/v) pH 7.5, made from 50% solution (sigma P3143), pH adjusted with HCl.
Extraction method: Frozen cell pellets were thawed briefly in a bath of warm water (20 - 37°C) then transferred to ice. One volume (i.e. 1 ml for every gram of cells) of 2x lysis buffer was added, followed by 1x lysis buffer to a total volume of 300ml. The cells were resuspended by agitating and disrupted by sonication. Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine) from a 5% (w/v, pH 7.5) stock, followed by centrifugation for 1 hour at 25,000 xg.

Column 1: Histrap FF 5ml (GE Healthcare).

Column 1 Buffers:
Affinity buffer: 40mM Tris, pH 8, 250 mM KCl, 30 mM imidazole, 1 mM TCEP, 10% glycerol
Wash buffer: 40mM Tris, pH 8, 250 mM KCl, 30 mM imidazole, 1 mM TCEP, 10% glycerol
Elution buffer: 40mM Tris, pH 8, 250 mM KCl, 300 mM imidazole, 1 mM TCEP, 10% glycerol

Column 1 Procedure: The cell extract was loaded onto the column at 5 ml/minute on an ÄKTA-express system (GE Healthcare). The column was then washed with 10 volumes of loading buffer, 10 volumes of wash buffer, then eluted with elution buffer at 4 ml/min. The eluted peak of A280 was automatically collected. The eluted AMPH protein was treated with TEV protease (His6-tagged) overnight at 4°C, while being dialysed in 40mM Tris pH 8.0, 250 mM KCl, 30 mM imidazole, 1 mM TCEP, 10% glycerol, using 3000MWCO dialysis tubing. The protease and other impurities were removed by passing through a 5mL NiNTA column

Column 2: Superdex 75 16/60 Gel Filtration 120ml

Column 2 Buffers:
GF Buffer: 25 mM Tris, pH 8, 100 mM KCl, 10% glycerol, 1 mM TCEP.

Column 2 Procedure: The eluted fraction was loaded and fractionated on the gel filtration column in GF buffer at 1.2 ml/min. 2ml fractions were collected at the A280 peaks. The fractions were analysed by SDS-PAGE and relevant fractions were pooled. This column resulted in a monodisperse peak of the AMPH. .

Protein concentration: The protein was then concentrated to 14.5 mg/ml (concentrations estimated by A280, using an extinction coefficient of 28480).

Mass spectrometry characterization:
Measured mass::29584.9 Da
Expected mass::29582.8 Da

Crystallisation: Crystals were grown by vapour diffusion at 4°C in 150nl sitting drops. A crystal was grown by mixing 50nl of protein solution (14 mg/mL) and 100nl of precipitant consisting of 20% PEG 3350 and 100mM citrate buffer pH 5.5 (JCSG condition A2). Crystals appeared in about a week at 4°C. Crystals were cryo-protected by bathing the crystal in mother liquor supplemented with 25% ethylene glycol and flash-freezing in liquid nitrogen. This crystal was used to collect a native dataset.

Data collection:
X-ray source: Diamond I04
Resolution: 1.8Å
The structure was solved by Molecular Replacement using 3SOG as a search model in the PHENIX suite. The structure was also refined in PHENIX and deposited with the PDB ID 4A3A.