SNX24A (4AZ9) Materials & Methods

Entry clone source: MGC

SGC Construct ID: SNX24A-c003

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

DNA sequence:

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ATGGAGGTCTACATCCCGTCCTTTCG
CTATGAAGAGAGCGACCTGGAGCGGG
GATACACGGTGTTTAAGATAGAAGTG
CTAATGAATGGAAGAAAACATTTTGT
TGAAAAGAGATACAGCGAATTTCATG
CTTTGCACAAAAAGCTTAAGAAATGT
ATAAAAACTCCAGAAATCCCTTCTAA
ACATGTTAGGAACTGGGTCCCCAAAG
TCTTGGAACAGCGACGACAAGGCTTG
GAAACATACTTACAGGCTGTCATTTT
AGAAAATGAAGAACTTCCCAAACTGT
TTCTTGATTTCCTAAATGTGCGACAC
TTGCCCTCTCTACCAAAGGCAGAAAG
TTGTGGATCTTTTGATGAAACAGAGT
CTGAAGAGTCAAGCAAACTGTCCCAC
CAGCCTGTGCTGCTGTTCCTCAGGGA
TCCATATGTCTTGCCTGCAGCCAGCG
ATTTTCCAAATGTGGTTATTGAAGGA
GTCCTCCATGGGATATTTTACCCTCA
TCTACAGCCCAGGTAG
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smeV
YIPSFRYEESDLERGYTVFKIEVLMN
GRKHFVEKRYSEFHALHKKLKKCIKT
PEIPSKHVRNWVPKVLEQRRQGLETY
LQAVILENEELPKLFLDFLNVRHLPS

^ TEV protease recognition site
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Tags and additions: Cleavable N-terminal His6 tag

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

Growth Medium & Induction Protocol: A glycerol stock was used to inoculate 2X60 ml of TB media containing 50 µg/ml kanamycin and 50 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day this starter culture was used to inoculate 12L of TB media (10 ml starter culture used per 1L) containing 50 µg/ml kanamycin. When the OD_{600} reached approximately 1.0 the temperature was reduced to 18°C and after a further 30 minutes the cells were induced by the addition of 0.1 mM IPTG. The expression was continued overnight. Cells were harvested by centrifugation at 16,000 RPM after which the supernatant was poured out and the cell pellet either placed in a -80°C freezer or used directly for purification.
Binding buffer: 50 mM HEPES pH 7.5; 500 mM NaCl; 10 mM imidazole, 5% glycerol.

Cell Lysis: Cell pellets were dissolved in approximately 50ml lysis buffer and broken by passing through the homogeniser (x6) at a constant pressure of 15KPa. The cell debris was pelleted at 16,000 RPM and the supernatant used for further purification
Lysis buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.5, 1 tablet per 50 ml protease inhibitor cocktail EDTA-free (Roche)

Column 1: Ni-NTA (5.0 ml volume in a gravity-flow column).

Column 1 Buffers:
Binding buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.5
Wash buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.5
Elution buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.5

Column 1 Procedure: The clarified cell extract was incubated with 5.0 ml pre-equilibriated 50% Ni-NTA bead solution for 1 hour at 4°C with rotation after which it was passed through a glass column. The column was then washed with 50ml Binding Buffer (2 x 25ml) and 50 ml Wash Buffer (2 x25 ml). The protein was eluted with 50 ml of Elution Buffer in 5 x 5 ml fractions.

Column 2: Superdex s200 16/60 Gel Filtration

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

Column 2 Procedure: Two separate pools from the Ni-NTA column were created; elution 1 and the combined (BB1+WB1+WB2). Both pools were then concentrated to 5ml and applied separately to the GF column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.0 ml fractions were collected.

Enzymatic Treatment and Purification A test cleavage for both pools showed that the protein is not completely cleaved therefore it was decided not to carry out a TEV-cut overnight and so correspondingly, His-tags were not removed by the Ni-rebind step.

Column 3: HIGH TRAP -SP

Column 3 Buffers:
Zero Salt Buffer: 25mM HEPES, 5% Glycerol pH 7.5
Low Salt Buffer: 25mM HEPES, 50mM NaCl, 5% Glycerol pH 7.5
High Salt Buffer: 25mM HEPES, 2M NaCl, 5% Glycerol pH 7.5

Column 3 Procedure: Two separate pools from the Ni-NTA column were created; elution 1 and the combined (BB1+WB1+WB2). Both pools were then concentrated to 5ml and applied

separately to the GF column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.0 ml fractions were collected.

Concentration: To set up plates the sample was concentrated to 18.1 mg/ml using a 10 kDa mwco concentrator

Mass spec characterization:
Expected mass: 15479.8 Da, Measured mass: 15480.67 Da

Crystallization: Crystals were grown by vapour diffusion in sitting drop at 20°C. A sitting drop consisting of 100 nl protein and 50 nl well solution was equilibrated against well solution containing 0.1M tris pH 8.5, 0.2M Magnesium Chloride, 30%(w/v) PEG 4000. Crystals were mounted in the presence of 25% (v/v) glycerol and flash-cooled in liquid nitrogen.

Data Collection: Resolution: 1.8 Å
X-ray source: Diamond Light Source beamline IO3