SNX27A (4HAS) Materials & Methods

Entry clone source: MGC

Entry clone accession: gi| 8069330

SGC Construct ID: SNX27A-c006

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

DNA sequence:

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CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGGATTAC
ACAGAAAAGCAAGCAGTGCCCATATC
GGTCCCCAGATACAAACATGTGGAGC
AGAATGGTGAGAAGTTTGTGGTATAT
AATGTTTACATGGCAGGGAGGCAGCT
GTGTTCTAAGCGGTACCGGGAGTTTG
CTATCCTACACCAGAACCTGAAGAGA
GAGTTTGCCAACTTTACATTTCCTCG
ACTCCCAGGGAAGTGGCCATTTTCAT
TATCAGAACAACAATTAGATGCCCGA
CGTCGGGGATTGGAAGAATATCTAGA
AAAAGTGTGTTCAATACGAGTAATTG
GTGAGAGTGACATCATGCAGGAATTC
CTATCAGAATCCTGACAGTAAAGGTG
GATACGGATCCGAA
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Final protein sequence (His$_6$ affinity Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smDY
TEKQAVPISVPRYKHVEQNGEKFVVY
NVYMAGRQLCSKRYREFAILHQNLKR
EFANFTFPRLPGKWPFSLSEQQLDAR
RRGLEEYLEKVCSIRVIGESDIMQEF
LSES

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

Host: BL21(DE3)-R3-pRARE2.

Transformation and Glycerol stock preparation: The construct DNA was transformed into competent cells of the expression strain by a standard heat shock procedure. One colony from the transformation was used to inoculate 1 ml of TB media containing 50 ìg/ml kanamycin and 34 ìg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture.

Expression: 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 ìg/ml kanamycin. When the OD600 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. Expression was continued overnight.

Cell harvest: Cells were harvested by centrifugation at 6000 x g after which the supernatant was poured out and the cell pellet placed in a -80°C freezer.

Cell Lysis: Cell pellets were dissolved in approximately 50ml lysis buffer and broken by homogenization by 5 passes at 12,000 psi. The cell debris was pelleted at 40,000 x g and the supernatant used for further purification.
Lysis buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 0.5 mM TCEP, 1 tablet per 50 ml protease inhibitor cocktail EDTA-free (Roche)

Column 1: Ni-NTA (1.5 ml volume in a gravity-flow column).

Column 1 Buffers:
Binding buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 0.5 mM TCEP
Elution buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.4, 0.5 mM TCEP
Elution buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4, 0.5 mM TCEP

Column 1 Procedure: The clarified cell extract was incubated with 1.5 ml of Ni-NTA pre-equilibrated with lysis buffer for 1 hour at 4°C with rotation after which it was passed through a glass column. The column was then washed with Binding Buffer (2 x 20 ml) and Wash Buffer (2 x 15 ml). The protein was eluted with 25 ml of Elution Buffer in 5 x 5 ml fractions.

Column 2: Superdex 200 16/60 Gel Filtration.

Column 2 Buffers:
Gel Filtration buffer: 10 mM Hepes pH 7.4, 500 mM NaCl, 0.5 mM TCEP, 5% Glycerol.

Column 2 Procedure: The two wash buffer fractions from column 1 were pooled and concentrated to 5 ml with a 10 kDa mwco spin concentrator and injected into an s200 16/60 column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.0 ml fractions were collected. The protein eluted at between 100 ml and 110 ml volume.

Column 3: TEV cleavage/ Ni-NTA rebind

Column 3 Procedure: Protein from fractions eluted at 100-110 ml from s200 gel filtration were pooled and incubated with 1:20 mol:mol TEV protease overnight at 4°C. Then protein plus TEV was passed through a column containing 0.25 ml Ni-NTA pre-equilibrated with GF Buffer. Column was washed 2 x 500 µl of GF Buffer, 2 x 500 µl of Binding Buffer, 2 x 500 µl of Wash Buffer and 2 x 500 µl of Elution Buffer. Flow-through and GF Buffer were pooled.

Concentration: Pooled protein fractions were concentrated to 16 mg/ml using a 10 kDa mwco concentrator.

Mass spec characterization (after TEV protease digestion):
Expected mass: 13284.2 Da, Measured mass: 13284.24 Da

Crystallization: Crystals were grown by vapour diffusion in sitting drop at 4°C. A sitting drop consisting of 50 nl protein and 100 nl well solution was equilibrated against well solution containing 0.1 M citrate pH 5.5 and 18% PEG 3350 . Crystals were mounted in the presence of 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data Collection: 1.80 Å **X-ray source:** FREL