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

Tags and additions: N-terminal His-tag with a TEV protease cleavage site. **Tag sequence:** mhhhhhhssgvdlgtenlyfq(*)sm.

Expressed sequence (tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfqsmGLSRVR
AVFFDLDNTLIDTAGASRRGMLEVIKLLQ
SKYHYKEEAEIICDKVQVKLSKECFHPYN
TCITDLRTSHWEEAIQETKGGAANRKLAE
ECYFLWKSTRLQHMTLAEDVKAMLTELRK
EVRLLLLTNGDRQTQREKIEACACQSYFD
AVVVGGEQREEKPAPSIFYYCCNLLGVQP
GDCVMVGDTLETDIQGGLNAGLKATVWIN
KNGIVPLKSSPVPHYMVSSVLELPALLQS
IDCKVSMST

Tag removed: no

Host: BL21(DE3)-R3-pRARE2

Expression protocol: Transformed 50 µl competent BL-21 (DE3)-R3-pRARE2 phage resistant cells with 6 µl of the plasmid DNA and plated out onto LB plate plus 50 µg/ml kanamycin and 35 µg/ml chloramphenicol. The next day colonies were picked out into fresh deep well blocks containing 1 ml TB + 50 µg/ml kanamycin and 35 µg/ml chloramphenicol which were grown overnight and glycerol stocks were prepared by adding 333 µl of 60 % glycerol to 1 ml of cell suspension, which were stored at -80°C to be used for future scale up preparations. The glycerol stock was used to inoculate 10 ml of TB (terrific Broth) supplemented with 50 µg/ml kanamycin and 35 µg/ml chloramphenicol. This starter culture was grown overnight at 37°C and used to inoculate a 1 liter culture in the same medium. The culture was grown at 37°C until the OD_{600}reached ~3.0. After that the temperature was lowered to 18°C. Protein production was induced with 0.1 mM IPTG and recombinant NANPA was expressed at that temperature overnight. The next day cells were harvested by centrifugation at 5000 rpm for 20 minutes then the supernatant was discarded and pellets resuspended in 70mls of 2x lysis buffer. Once resuspended, transfer to two 50 ml falcon tubes. Place these in a bag and then put in -80°C freezer until required.

Cell extraction: 2x Lysis buffer: 100 mM K-phosphate, pH 7.5, 1M NaCl, 20% glycerol 1 mM TCEP, 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution), and 15 units/ml Benzonase. **Lysis buffer:** 50 mM K-phosphate, pH 7.5, 0.5M NaCl, 1 mM TCEP.

Procedure: Frozen resuspended cell pellets were thawed briefly in a bath of warm water (37°C) then transferred to ice. Supplements were added thawed cells in lysis buffer: TCEP, Benzonase and protease inhibitors. Lysed cells using the sonicator at 30% amplititde on for 5 seconds and off for 5 seconds for a total of 15 minutes for each falcon tube. Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine) from a 5% (w/v, pH 7.5) stock, stirring for 30 minutes, then centrifugation for 30 minutes at 17,000RPM. The supernatant was then further clarified by filtration (Acrodisc filters, 0.2 µm).

Column 1: Ni-affinity, HisTrap Crude FF, 5 ml (GE Healthcare)

Buffers: Affinity buffer: 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 5 mM imidazole, 10% glycerol 0.5 mM TCEP; **Wash buffer:** 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 30 mM imidazole, 10% glycerol 0.5 mM TCEP; **Elution buffer:** 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 300 mM imidazole, 10% glycerol 0.5 mM TCEP.

Procedure: The cell extract was loaded on the column at 4 ml/minute on an AKTA-express system (GE Healthcare). The column was washed with 10 volumes of lysis buffer, 10 volumes of wash buffer, and then eluted with elution buffer at 4 ml/min. The eluted peak of A280 was automatically collected.

Column 2: Gel filtration, Hiload 16/60 Superdex S75 prep grade, 120 ml (GE Healthcare)

Gel Filtration Buffer: 50mM HEPES, 250mM NaCl, 5% glycerol, 0.5 mM TCEP
Procedure: The eluted fraction from the Ni-affinity Histrap column was loaded on the gel filtration column in GF buffer at 0.80 ml/min. Eluted proteins were collected in 2-ml fractions and analyzed on SDS-PAGE.
Mass spectrometry characterization: ESI-MS revealed that the protein had a mass of 30366.0639 Da (Expected mass 30366).
Compound: none
Protein concentration: Buffer was changed to 15 mM HEPES pH7.5, 165 mM NaCl, 1.5% Glycerol, 5mM DTT. The protein was concentrated to 12.9 mg/ml using a centricon with a 5kDa cut off. The protein concentration was determined spectrophotometrically using e280= 29910.
Crystallization: Crystals were grown at 20°C by vapour diffusion in sitting drops mixing protein (12.9 mg/mL) and well solution containing 0.8M Na_phosphate_(NaH_2PO_4)-4M; 0.16M K_phosphate_(K_2HPO_4)-4M at respective ratios of protein to precipitant 3:1, 2:1. Crystals were cryo-protected using 25% (v/v) ethylene glycol and flash cooled in liquid nitrogen.
Data Collection: Resolution: 2.6Å; **X-ray source:** SLS-X10