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

Coding DNA sequence:

```
CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGCTGCCAGTGCTGACCCTG
CAGCACTTTCAGCATATGCACATCAAAGTT
GGAGACCGCGCTGAACTTAGGAGGGCCTTC
ACACAGACTGATGTGGCTACCTTCTCAGAA
TTAACAGGGGATGTCAATCCTTTGCATTTG
AATGAAGACTTTGCAAAACACACCAAGTTT
GGAAATACAATTGTACATGGAGTTTTGATC
AACGGACTTATCTCAGCTCTCCTAGGAACT
AAAATGCCAGGGCCAGGCTGTGTATTTCTT
TCCCAGGAAATTAGCTTTCCAGCCCCTTTA
TATATTGGAGAAGTTGTTTTAGCTTCTGCA
GAAGTGAAAAAGCTGAAGCGGTTCATTGCT
ATTATTGCAGTGTCATGTTCTGTAATAGAA
AGTAAAAAGACTGTTATGGAAGGCTGGGTT
AAAGTTATGGTTCCAGAAGCTTCCAAATCC
TGACAGTAAAGGTGGATACGGATCCGAA
```

Final protein sequence (tag sequence in lowercase):

```
mhhhhhhssgvdlgtenlyfq(*)smLPVL
TLQHFQHMHIKVGDRAELRRAFTQTDVATF
SELTGDVNPLHLNEDFAKHTKFGNTIVHGV
LINGLISALLGTKMPGPGCVFLSQEISFPA
PLYIGEVVLASAEVKKLKRFIAIIAVSCSV
IESKKTVMEGWVKVMVPEASKS
```

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

Host: BL21(DE3)-R3-pRARE2

Expression protocol: : The glycerol stock of host strain BL21(DE3)-R3-pRARE2 was used to inoculate 10 ml of TB (terrific Broth) supplemented with 50 µg/ml kanamycin and 35 g/ml chloramphenicol. This starter culture was grown overnight at 37°C and used to inoculate a 1 liter culture in TB supplemented with 50 µg/ml kanamycin only. 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 HTD2A 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 re-suspended in 70ml of 2x lysis buffer. Stored at-80°C.

Cell extraction: 2x Lysis buffer: 100 mM K-phosphate, pH 7.5, 1 M NaCl, 20% glycerol 0.5 mM TCEP; **Procedure:** Frozen cells, previously re-suspended, were thawed, and supplemented with: TCEP, Benzonase and protease inhibitors. Cells were lysed by sonication. Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine), 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)

Column 1 Buffers: 2 x Lysis buffer: 100 mM K-phosphate, pH 7.5, 1 M NaCl, 20% 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.

Column 1 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)

Column 2 Buffers: **Gel Filtration Buffer** - 20mM HEPES pH 7.5, 250mM NaCl, 5% glycerol and 0.5 mM TCEP

Column 2 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.

Column 3: Ni-NTA qiagen

Column 3 Buffers: 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.

Enzymatic treatment: The amount of TEV enzyme used, in mgs, was calculated by using a 1:20 TEV to protein ratio in mgs. It was left incubating overnight at 4°C. The following day ran protein sample through 1ml slurry Nickel in a 10mm gravity column. Collected this as flow-through. Washed column 3 times with 1 ml of wash buffer each time. Finally eluted in 10mls of elution buffer and collected this as eluant. Pooled wash fractions and buffer exchanged in GF buffer using PD10 column.

Mass spectrometry characterization: ESI-MS revealed that the protein had a mass of 16171.0 Da (Expected mass 16171.1)

Compound: none

Protein concentration: Protein was stored in 20 mM HEPES pH7.5, 250 mM NaCl and 5% Glycerol at -80°C. The protein was concentrated to 7.2 mg/ml using a centricon with a 5 KDa cut off. The protein concentration was determined spectrophotometrically using ε^{280}= 8480.

Crystallization: Crystals were grown at 4°C by vapour diffusion in sitting drops mixing protein (7.2 mg/ml) and well solution containing 0.5% jeff2001; 1.1M Na (malonate); 0.1M HEPES pH 7.0 at a protein to precipitant ratio of 1:2.
Crystals were cryo-protected using Malonate 7.0 and flash cooled in liquid nitrogen.

Data Collection: Resolution (estimated): 2 Å; **Resolution (scaled):** 1.99 Å; **X-ray source:** Dmnd I04