Entry Clone Source: MGC

Entry Clone Accession: IMAGE:3630659

SGC Construct ID: NMRAL1A-c004

GenBank GI number: gi|10190720

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

Host: BL21(DE3)-R3-pRARE2

Amplified construct sequence:

```
CATATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACCTGT
ACTTCCAATCCATGGTGGACAAGAAACTG
GTGGTGGTTTTCGGAGGCACAGGTGCCCA
GGGTGGCTCCGTGGCCCGCACACTCCTGG
AAGATGGGACATTCAAGGTTCGAGTGGTG
ACCCGAAACCCTAGGAAGAAGGCAGCAAA
GGAGCTGAGGCTGCAAGGTGCAGAAGTAG
TGCAGGGAGACCAAGATGACCAGGTCATC
ATGGAGCTGGCCCTGAATGGGGCTTACGC
CACCTTCATCGTGACCAATTACTGGGAGA
GCTGCAGCCAGGAGCAGGAGGTCAAGCAG
GGGAAGCTGCTCGCTGATCTGGCCAGGCG
CCTGGGCCTCCACTATGTGGTCTACAGCG
GCCTGGAGAACATCAAGAAGCTGACGGCA
GGGAGATTGGCCGCCGCGCACTTTGACGG
CAAAGGGGAGGTGGAGGAATATTTCCGGG
ACATTGGCGTTCCCATGACCAGTGTGCGG
CTGCCCTGCTATTTTGAGAACCTCCTCTC
CCACTTCTTGCCCCAGAAAGCCCCAGACG
GAAAGAGCTACTTGCTGAGCTTGCCCACA
GGTGACGTTCCCATGGATGGCATGTCCGT
GTCTGACCTGGGTCCTGTGGTGCTCAGCC
TTTTGAAGATGCCAGAAAAATACGTCGGC
CAGAACATCGGGCTGAGCACTTGCAGGCA
CACGGCCGAGGAGTACGCTGCCCTGCTCA
CCAAGCACACCCGCAAGGTCGTGCACGAT
GCCAAGATGACTCCTGAGGACTACGAAAA
GCTTGGCTTTCCCGGTGCCCGGGACCTGG
CCAACATGTTCCGTTTCTATGCCCTGAGA
CCCGACCGTGACATCGAGCTGACCCTGAG
ACTCAACCCCAAGGCCCTGACGCTGGACC
AGTGGCTGGAACAGCACAAAGGGGACTTC
AACCTGTGACAGTAAAGGTGGATACGGAT
CCGAA
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Tags and additions: N-terminal His-tag with TEV protease cleavage site

Expressed sequence(Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfqsMVDKKLV
VVFGGTGAQGGSVARTLLEDGTFKVRVVT
RNPRKKAAKELRLQGAEVVQGDQDDQVIM
ELALNGAYATFIVTNYWESCSQEQEVKQG
KLLADLARRLGLHYVVYSGLENIKKLTAG
RLAAAHFDGKGEVEEYFRDIGVPMTSVRL
PCYFENLLSHFLPQKAPDGKSYLLSLPTG
DVPMDGMSVSDLGPVVLSLLKMPEKYVGQ
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NIGLSTCRHTAEEYAALLTKHTRKVVHDA
KMTPEDYEKLGFPGARDLANMFRFYALRP
DRDIELTLRLNPKALTLDQWLEQHKGDFN
L

Expression: BL21(DE3)-R3 glycerol stock harbouring the NMRAL1 pNIC28-Bsa4 were inoculated into 50ml of TB with 100µg/ml of ampicilin and 34µg/ml chloramphenicol and grown overnight at 37°C, 200 rpm. 10ml of overnight culture were added to 1L X 4 of TB with 100µg/ml ampicilin and incubated at 37°C, 160rpm. After the OD_{600} reached 1.0, the temperature was dropped to 18°C and 1ml of 0.2M IPTG was added to the final concentration of ~0.2mM. The culture was then incubated with shaking overnight at 18°C, 160rpm. The following morning the 4L culture was harvested and centrifuged for 10min at 6000rpm. Supernatant was discarded and cell pellets were resuspended in 70ml of a lysis buffer and frozen at -80°C.

Extraction: The thawed cells were broken by a high pressure homogenizer. 5 µl Benzonase and PEI (polyethyleneimine) (0.15 %) was added to the lysate. The lysate was centrifuged at 16,000 rpm for 45 minutes and the supernatant collected for purification.

Extraction buffer: Lysis Buffer 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 10mM Imidazole 0.5 mM TCEP, protease inhibitors.

Purification: Column 1: Ni-affinity. Ni-NTA, 5 ml of 50% slurry in column, washed with binding buffer. **Column 2:** Size Exclusion Chromatography. Superdex S200 16/60 HiLoad , equilibrated with in GF buffer

Buffers: Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 250 mM Imidazole , 5% Glycerol, 0.5 mM TCEP; **Gel filtration (GF) buffer:** 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP.

Procedure: The cleared lysate was loaded by gravity flow on the Ni-NTA column. The column was then washed with 25 ml binding buffer. The protein was eluted by gravity flow by applying 20ml of elution buffer and collected in 2ml fractions. The fractions were analyzed on a SDS gel, pooled together, concentrated and applied to the gel filtration column. The absorbance at 280nm was monitored and fractions were collected and analyzed by SDS-PAGE. Protein fractions were pooled for TEV cleavage.

TEV cleavage: The His-tag was cleaved with 1mg TEV per 40mg target protein at 4°C overnight. The protein was purified on Ni-NTA equilibrated with GF buffer using buffers as above.

Concentration and buffer exchange: Using Amicon Ultra-15 concentrators with 10kDa cutoff the protein was concentrated to 12.7mg/ml.

Mass spectrometry characterization: The calculated mass of the TEV cleaved protein was calculated to be 33,318 Da and experimentally this was confirmed by mass spec analysis.

Crystallization:
Crystallization with NADP and Niflumic: Protein was supplemented with 1.5 mM NADP and 1.mM Niflumic acid. Crystal were grown at 20°C in 150nl sitting drops mixing NMRAL1 (9.6mg/ml) with and reservoir solution containing 2.1 M NaMalate pH 7.0 in a 1:1 ratio.
Crystallization with NADP and 2-(4-chloro-phenylamino)-nicotinic acid: Protein was supplemented with 1.9 mM NADP and 1.5 mM 2-(4-chloro-phenylamino)-nicotinic acid. Crystal were grown at 20°C in 150nl sitting drops mixing NMRAL1 (12.7mg/ml) with and reservoir solution containing 2M ammonium sulphate and 0.1% BIS-TRI pH 5.5 in a 1:2 ratio.

Data Collection: Resolution: 1.9 Å(2WM3) and 2.2 Å(2WMD); **X-ray source:** SLS-X10 (2WM3) and Diamond I02 (2WMD).