Entry Clone Source: Origene

Entry Clone Accession: NM_001204 variant

SGC Construct ID: BMPR2A-c019

GenBank GI number: gi|15451916

Vector: pNIC-CH. Details [PDF]; Sequence [FASTA] or [GenBank]

Construct DNA sequence:

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CTTAAGAAGGAGATATACTATGGAGGCAG
CAGCATCCGAACCCTCTCTTGATCTAGAT
AATCTGAAACTGTTGGAGCTGATTGGCCG
AGGTCGATATGGAGCAGTATATAAAGGCT
CCTTGGATGAGCGTCCAGTTGCTGTAAAA
GTGTTTTCCTTTGCAAACCGTCAGAATTT
TATCAACGAAAAGAACATTTACAGAGTGC
CTTTGATGGAACATGACAACATTGCCCGC
TTTATAGTTGGAGATGAGAGAGTCACTGC
AGATGGACGCATGGAATATTTGCTTGTGA
TGGAGTACTATCCCAATGGATCTTTATGC
AAGTATTTAAGTCTCCACACAAGTGACTG
GGTAAGCTCTTGCCGTCTTGCTCATTCTG
TTACTAGAGGACTGGCTTATCTTCACACA
GAATTACCACGAGGAGATCATTATAAACC
TGCAATTTCCCATCGAGATTTAAACAGCA
GAAATGTCCTAGTGAAAAATGATGGAACC
TGTGTTATTAGTGACTTTGGACTGTCCAT
GAGGCTGACTGGAAATAGACTGGTGCGCC
CAGGGGAGGAAGATAATGCAGCCATAAGC
GAGGTTGGCACTATCAGATATATGGCACC
AGAAGTGCTAGAAGGAGCTGTGAACTTGA
GGGACTGTGAATCAGCTTTGAAACAAGTA
GACATGTATGCTCTTGGACTAATCTATTG
GGAGATATTTATGAGATGTACAGACCTCT
TCCCAGGGGAATCCGTACCAGAGTACCAG
ATGGCTTTTCAGACAGAGGTTGGAAACCA
TCCCACTTTTGAGGATATGCAGGTTCTCG
TGTCTAGGGAAAAACAGAGACCCAAGTTC
CCAGAAGCCTGGAAAGAAAATAGCCTGGC
AGTGAGGTCACTCAAGGAGACAATCGAAG
ACTGTTGGGACCAGGATGCAGAGGCTCGG
CTTACTGCACAGTGTGCTGAGGAAAGGAT
GGCTGAACTTATGATGATTTGGGAAAGAA
ACAAATCTGTGAGCCCAACAGCGCACCAT
CATCACCACCATTGAGGATCC
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Tags and additions: ahhhhhh. Non-cleavable C-terminal hexahistidine tag.

Expressed protein sequence (tag sequence in lowercase):

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MEAAASEPSLDLDNLKLLELIGRGRYGAV
YKGSLDERPVAVKVFSFANRQNFINEKNI
YRVPLMEHDNIARFIVGDERVTADGRMEY
LLVMEYYPNGSLCKYLSLHTSDWVSSCRL
AHSVTRGLAYLHTELPRGDHYKPAISHRD
LNSRNVLVKNDGTCVISDFGLSMRLTGNR
LVRPGEEDNAAISEVGTIRYMAPEVLEGA
VNLRDCESALKQVDMYALGLIYWEIFMRC
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TDLFPGESVPEYQMAFQTEVGNHPTFEDM
QVLVSREKQRPKFPEAWKENSLAVRSLKE
TIEDCWDQDAEARLTAQCAEERMAELMMI
WERNKSVSPTahhhhhh

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

Growth medium, induction protocol: A glycerol stock was used to inoculate a 50 ml starter culture containing TB media and 34 µg/ml chloramphenicol and 50 µg/ml kanamycin. The starter culture was grown overnight at 37°C with shaking at 250 rpm. The following morning six flasks containing 500 ml TB media with 34 µg/ml chloramphenicol and 50 µg/ml kanamycin were each inoculated with 5 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an $OD_{600} \geq 0.5$ was reached. The flasks were then cooled down to 18°C and 0.5 mM IPTG added to induce protein expression overnight. Cells were harvested by centrifugation at 4500 rpm at 4°C for 15 min. Cell pellets from each flask were resuspended in 30 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole), transferred to 50 ml tubes, and stored at -20°C.

Extraction buffer, extraction method: The frozen cells were thawed and 0.5 mM TCEP, 1 mM PMSF, and 10 mM L-arginine/10 mM L-glutamate mix were added to the cell suspension. The cells were lysed by sonication over 12 min with the sonicator pulsing ON for 5 sec and OFF for 15 sec. The cell lysate was spun down by centrifugation at 16.5k rpm and 4°C for 1 h. The supernatant was recovered for purification.

Column 1: Anion-exchange for Nucleic acid removal with DEAE cellulose (DE52, Whatmann). 10 g of resin was suspended in 100 ml 2.5 M NaCl, and then applied onto a 2.5 x 20 cm column. The resin was then equilibrated with 100 ml binding buffer prior to loading the sample.

Buffers: Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole; **Wash buffer:** 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 30 mM imidazole.

Procedure: The supernatant was first applied onto the column by gravity flow, which was followed by a wash with 100 ml wash buffer. The column flow-through and wash was directly applied onto a Ni-IDA column.

Column 2: Ni-Affinity Chromatography. 5 ml of 50 % Ni-IDA slurry (Genscript) was applied onto a 1.5 x 10 cm column. The column was first washed deionised destilled H2O, and then equilibrated with binding buffer.

Buffers: Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole; **Wash buffer:** 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 30 mM imidazole; **Elution buffer:** 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 50 to 250 mM imidazole.

Procedure: The flow-through from column 1 (DE52) was applied by gravity flow onto the Ni-IDA column. The bound protein was eluted by applying a step gradient of imidazole – using 7 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 250 mM). 10 mM DTT was added to each fraction collected for overnight storage at 4°C.

Enzymatic treatment: No treatment was required as the protein was expressed with a non-cleavable C-terminal His6 tag.

Column 3: Size Exclusion Chromatography – S200 HiLoad 16/60 Superdex run on **ÄKTA-Express**

Buffer: Gel Filtration buffer: 300 mM NaCl, 25 mM Hepes pH 7.5, 1mM DTT

Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. Eluted protein from the Ni-IDA column was pooled and concentrated to 3 ml using an Amicon Ultra-15 filter with a 10 kDa cut-off. The concentrated protein was directly applied onto the equilibrated S200 16/60 column, and run at a flow-rate of 1 ml/min. The protein was eluted at 80 – 95 ml. Fractions containing the protein were pooled together, and 10 mM DTT was added for overnight storage at 4°C.

Column 4: Anion-exchange - MonoQ 5/50 GL column on **ÄKTA-Purifier**
Buffers: Buffer A (low salt): 50 mM Hepes pH 7.4; **Buffer B (high salt):** 50 mM Hepes, pH 7.4; 1 M NaCl.
Procedure: Prior to applying the protein, the MonoQ column was washed with buffer B before equilibration with buffer A. The eluted protein from gel filtration was diluted with buffer A to 50 ml then applied to a MonoQ column, and run at a flow-rate of 1 ml/min with a linear gradient. The protein was eluted at 13-14% buffer B. Fractions containing the protein were pooled and the buffer adjusted to 50 mM Hepes pH 7.4, 300 mM NaCl, 10% glycerol, 10 mM DTT, 50 mM L-arginine/50 mM L-glutamate. Protein was stored at 4°C.
Concentration: The protein was concentrated in an Amicon Ultra-4 filter with a 10 kDa cut-off.
Mass spectrometry characterization: The purified protein was homogeneous and had an experimental mass of 38.413 kDa, as expected from its primary structure. Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% acetonitrile in water with 0.1% formic acid.
Crystallization: Protein was buffered in 50 mM HEPES, pH 7.4, 300 mM NaCl, 10% glycerol, 10 mM DTT, 50 mM L-arginine/50 mM L-glutamate. To this 10 mM ADP was added and the protein concentrated to 5.8 mg/ml (calculated using an extinction co-efficient of 46870). Crystals were grown at 4°C in 150 nl sitting drops mixing 75 nl protein solution with 75 nl of a reservoir solution containing 25% PEG8000, 0.3 M $(NH_4)_2SO_4$, 0.1M Na-cacodylate pH 6.0, 50 mM $MgCl_2$. On mounting crystals were cryo-protected with an additional 20% ethylene glycol.
Data Collection: Resolution: 2.35Å, **X-ray source:** Swiss Light Source PX10