CDC42BPAA (4AW2) Materials & Methods

SGC Construct ID: CDC42BPAA-c005

Vector: pNIC-CTH0. Details [PDF]; Sequence [FASTA] or [GenBank].

DNA sequence:

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ATGTCTGGAGAAGTGCGTTTGAGGCA
GTTGGAACAGTTTATTTTAGATGGGC
CAGCTCAGACCAATGGGCAGTGCTTC
AGTGTGGAGACGTTACTGGACATACT
CATCTGTCTTTATGATGAATGCAATA
ATTCTCCATTGAGAAGAGAGAAGAAC
ATTCTTGAATATCTAGAATGGGCTAA
ACCATTCACTTCTAAAGTGAAGCAAA
TGCGCTTACATAGAGAAGACTTCGAA
ATATTAAAGGTGATTGGTCGAGGAGC
TTTTGGTGAGGTTGCTGTAGTTAAAC
TGAAAAATGCAGATAAAGTATTTGCC
ATGAAAATTCTGAACAAGTGGGAAAT
GCTGAAAAGAGCTGAGACAGCGTGCT
TTCGTGAAGAGAGGGATGTGTTGGTA
AATGGAGACAGTAAGTGGATAACCAC
TCTGCACTATGCTTTTCAGGATGACA
ATAACTTATACCTGGTTATGGATTAT
TATGTTGGTGGGGATTTACTTACTCT
GCTCAGCAAATTTGAAGATCGATTGC
CAGAAGAGATGGCTCGGTTTTACTTG
GCTGAGATGGTGATAGCCATTGACTC
AGTTCACCAGCTCCACTATGTTCACA
GAGACATCAAACCTGACAATATATTG
ATGGATATGAATGGACATATTCGTTT
AGCAGATTTTGGTTCTTGTCTGAAGC
TGATGGAAGATGGAACGGTCCAGTCC
TCAGTGGCAGTTGGAACTCCAGACTA
CATTTCCCCGGAAATCCTTCAGGCTA
TGGAGGGCGGGAAAGGCAGATATGGA
CCAGAGTGTGACTGGTGGTCCTTGGG
AGTCTGCATGTATGAGATGCTTTACG
GAGAAACACCATTCTATGCGGAATCT
CTGGTGGAGACATATGGAAAAATTAT
GAATCATAAAGAGAGGTTTCAGTTTC
CAACCCAAGTGACTGATGTGTCTGAA
AATGCTAAAGATCTTATTCGAAGACT
CATTTGTAGCAGAGAACATCGACTTG
GCCAGAATGGAATAGAAGACTTTAAG
AAACATCCGTTTTTCAGTGGAATTGA
TTGGGATAACATCCGAAACTGTGAAG
CACCTTACATTCCAGAAGTTAGTAGT
CCAACAGACACGTCAAATTTCGATGT
GGATGACGACTGTTTAAAAAACTCTG
AAACAATGCCCCCACCAACACATACT
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GCATTTTCTGGCCATCATCTACCATT
TGTTGGGTTTACATATACTAGTAGCT
GTGTTCTTTCTGATCGGAGCTGTTTA
AGAGTCACAGCAGGTCCCACCTCAGC
AGAGAACCTCTACTTCCAATCGCACC
ATCATCACCACCATTGA

Final protein sequence (Tag sequence in lowercase):
MSGEVRLRQLEQFILDGPAQTNGQCF
SVETLLDILICLYDECNNSPLRREKN
ILEYLEWAKPFTSKVKQMRLHREDFE
ILKVIGRGAFGEVAVVKLKNADKVFA
MKILNKWEMLKRAETACFREERDVLV
NGDSKWITTLHYAFQDDNNLYLVMDY
YVGGDLLTLLSKFEDRLPEEMARFYL
AEMVIAIDSVHQLHYVHRDIKPDNIL
MDMNGHIRLADFGSCLKLMEDGTVQS
SVAVGTPDYISPEILQAMEGGKGRYG
PECDWWSLGVCMYEMLYGETPFYAES
LVETYGKIMNHKERFQFPTQVTDVSE
NAKDLIRRLICSREHRLGQNGIEDFK
KHPFFSGIDWDNIRNCEAPYIPEVSS
PTDTSNFDVDDDCLKNSETMPPPTHT
AFSGHHLPFVGFTYTSSCVLSDRSCL
RVTAGPTSAenlyfq^shhhhhh

(Met1 to Ser424, D254G mutant)

^ TEV protease recognition site

Tags and additions: Cleavable C-terminal His6 tag

Expression strain: BL-21(DE3)-R3-lambda-ppase (A homemade phage resistant version of BL21(DE3) that carries a plasmid for co-expression of lambda phosphatase).

Transformation: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure. The colonies were grown overnight in LB media and used to prepare a glycerol stock.

Expression: A scraping of frozen glycerol stock was used to inoculate 35 ml of LB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol in a 250 ml baffled shaker flask, which was placed in a 37°C shaker overnight. The next day 3x 10 ml of this starter culture was used to inoculate 3x 1L of LB media containing 40 µg/ml kanamycin in 2L baffled shaker flasks. When the OD_{600} was approximately 0.8, the temperature was reduced to 20°C and the cells were induced by the addition of 0.4 mM IPTG. The expression was continued overnight.

Cell Harvest: Cells were collected by centrifugation and the pellets resuspended in Lysis Buffer and then frozen at -20°C.

Cell lysis: The resuspended cell pellet was thawed and lysed by sonication. PEI (polyethyleneimine) was added to a final concentration of 0.15 %. The cell debris and precipitated DNA were spun down.
Lysis buffer: 50 mM Tris pH 7.8, 200 mM NaCl, 20 mM Imidazole, 5% Glycerol, 50 mM Arginine/Glutamate, 0.5 mM TCEP, 0.2 mM PMSF.

Column 1: 5 ml of Ni-Sepharose in a 2 cm diameter gravity flow column.
Column 1 Buffers: **Binding buffer:** 50 mM Tris pH 7.8, 200 mM NaCl, 20 mM Imidazole, 5% Glycerol, 50 mM Arginine/Glutamate, 0.5 mM TCEP. **Wash buffer 1:** As Binding Buffer except 1 M NaCl and 40 mM imidazole. **Wash buffer 1:** As Binding Buffer except 60 mM imidazole. **Elution buffer:** As Binding Buffer except 250 mM imidazole.
Column 1 Procedure: The clarified supernatant was passed through the column. The column was washed with 75 ml of Binding Buffer and 50 ml each of Wash Buffer 1 and 2. 25 ml of Elute Buffer was passed through to elute the protein.
Column 2: S200 16/60 Gel Filtration (GE Healthcare)
Column 2 Buffers: **Gel Filtration buffer:** 20 mM Tris pH 7.8, 200 mM NaCl, 5% Glycerol, 50 mM Arginine/Glutamate, 0.5 mM TCEP
Column 2 Procedure: The eluted protein was concentrated to 5 ml volume and injected onto the column.
Concentration: The CDC42BPAA was concentrated to 43 mg/ml (measured by 280 nm absorbance).
Mass spec characterization: Expected: 49883.9 Observed: 49698.3 (Matches expected mass minus N-terminal Methionine and with the D254G mutation)
Crystallization: The compound was added to the protein to a concentration of 1 mM. Crystals grew from a 1:2 ratio of protein:compound and precipitant solution (0.2 M KSCN, 0.1 M BisTrisPropane pH 8.5, 20.0% PEG 3350, 10.0% Ethylene Glycol), using the vapour diffusion method.
Data Collection: Crystals were cryo-protected by equilibration into precipitant solution containing 25% ethylene glycol, and then flash frozen in liquid nitrogen. Data was collected at Diamond, beamline IO4-1.