Entry Clone Source: Complex

Entry Clone Accession: n/a

SGC Construct ID: XX02CAMK2DA-c001

GenBank GI number: n/a

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

Amplified DNA sequences:

CAMK2D:

TACTTCCAATCCATGACGGACGAGTATCA
GCTTTTCGAGGAGCTTGGAAAGGGGGCAT
TCTCAGTGGTGAGAAGATGTATGAAAATT
CCTACTGGACAAGAATATGCTGCCAAAAT
TATCAACACCAAAAAGCTTTCTGCTAGGG
ATCATCAGAAACTAGAAAGAGAAGCTAGA
ATCTGCCGTCTTTTGAAGCACCCTAATAT
TGTGCGACTTCATGATAGCATATCAGAAG
AGGGCTTTCACTACTTGGTGTTTGATTTA
GTTACTGGAGGTGAACTGTTTGAAGACAT
AGTGGCAAGAGAATACTACAGTGAAGCTG
ATGCCAGTCATTGTATACAGCAGATTCTA
GAAAGTGTTAATCATTGTCACCTAAATGG
CATAGTTCACAGGGACCTGAAGCCTGAGA
ATTTGCTTTTAGCTAGCAAATCCAAGGGA
GCAGCTGTGAAATTGGCAGACTTTGGCTT
AGCCATAGAAGTTCAAGGGGACCAGCAGG
CGTGGTTTGGTTTTGCTGGCACACCTGGA
TATCTTTCTCCAGAAGTTTTACGTAAAGA
TCCTTATGGAAAGCCAGTGGATATGTGGG
CATGTGGTGTCATTCTCTATATTCTACTT
GTGGGGTATCCACCCTTCTGGGATGAAGA
CCAACACAGACTCTATCAGCAGATCAAGG
CTGGAGCTTATGATTTTCCATCACCAGAA
TGGGACACGGTGACTCCTGAAGCCAAAGA
CCTCATCAATAAAATGCTTACTATCAACC
CTGCCAAACGCATCACAGCCTCAGAGGCA
CTGAAGCACCCATGGATCTGTCAACGTTC
TACTGTTGCTTCCATGATGCACAGACAGG
AGACTGTAGACTGCTTGAAGAAATTTAAT
GCTAGAAGAAAACTAAAGGGTGCCATCTT
GACAACTATGCTGGCTACAAGGAATTTCT
CAGCAGCCAAGAGTTTGTTGAAGAAACCA
GATGGAGTAAAGGAGTCAACTGAGAGTTC
AAATTGACAGTAAAGGTGGATA

Calmodulin:

TACTTCCAATCCATGGCTGATCAGCTGAC
CGAAGAACAGATTGCTGAATTCAAGGAAG
CCTTCTCCCTATTTGATAAAGATGGCGAT
GGCACCATCACAACAAAGGAACTTGGAAC
TGTCATGAGGTCACTGGGTCAGAACCCAA
CAGAAGCTGAATTGCAGGATATGATCAAT
GAAGTGGATGCTGATGGTAATGGCACCAT
TGACTTCCCCGAATTTTTGACTATGATGG
CTAGAAAAATGAAAGATACAGATAGTGAA

GAAGAAATCCGTGAGGCATTCCGAGTCTT
TGACAAGGATGGCAATGGTTATATCAGTG
CAGCAGAACTACGTCACGTCATGACAAAC
TTAGGAGAAAAACTAACAGATGAAGAAGT
AGATGAAATGATCAGAGAAGCAGATATTG
ATGGAGACGGACAAGTCAACTATGAAGAA
TTCGTACAGATGATGACTGCAAAATGACA
GTAAAGGTGGATA

Tags and additions: Tag sequence: Cleavable N-terminal His6 tag.

Final protein sequence: (tag sequence in lowercase)

CAMK2D:
mhhhhhhssgvdlgtenlyfq^sMTDEYQ
LFEELGKGAFSVVRRCMKIPTGQEYAAKI
INTKKLSARDHQKLEREARICRLLKHPNI
VRLHDSISEEGFHYLVFDLVTGGELFEDI
VAREYYSEADASHCIQQILESVNHCHLNG
IVHRDLKPENLLLASKSKGAAVKLADFGL
AIEVQGDQQAWFGFAGTPGYLSPEVLRKD
PYGKPVDMWACGVILYILLVGYPPFWDED
QHRLYQQIKAGAYDFPSPEWDTVTPEAKD
LINKMLTINPAKRITASEALKHPWICQRS
TVASMMHRQETVDCLKKFNARRKLKGAIL
TTMLATRNFSAAKSLLKKPDGVKESTESS
N

Calmodulin:
mhhhhhhssgvdlgtenlyfq^sMADQLT
EEQIAEFKEAFSLFDKDGDGTITTKELGT
VMRSLGQNPTEAELQDMINEVDADGNGTI
DFPEFLTMMARKMKDTDSEEEIREAFRVF
DKDGNGYISAAELRHVMTNLGEKLTDEEV
DEMIREADIDGDGQVNYEEFVQMMTAK

^TEV cleavage site

Host: BL21 (DE3) Rosetta-*Phage resistant*

Expression protocol CAMK2D: Transformed 50 µl competent BL-21 (DE3) phage resistant cells with 10 µl of the plasmid DNA and plated out onto LB plate plus 50 mg/ml kanamycin and 35 µg/ml chloramphenicol. The next day colonies were picked into fresh deep well blocks containing 1 ml LB + 50 mg/ml kanamycin and 35 µg/ml chloramphenicol. Cultures were grown overnight and glycerol stocks were prepared by adding 333 ml of 60 % glycerol to 1 ml of cell suspension, which were stored at -80°C and used for future scale up preparations. The glycerol stock was used to inoculate 10 ml of LB supplemented with 50 mg/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 ~0.5. After that the temperature was lowered to 18°C. Protein production was induced with 1mM IPTG and recombinant CAMK2D was expressed at that temperature over night. The next day cells were harvested by centrifugation at 4000 rpm for 15 minutes. The cell pellet was stored at -80°C degrees.

Lysis andNi-affinity chromatography: Buffers: Binding buffer: 50 mM HEPES pH 7.5, 300mM NaCl,, 20 mM Imidazole. **Wash buffer 1:** 50 mM HEPES pH 7.5, 1M NaCl, 20mM Imidazole.**Wash buffer 2**:**as for lysis buffer. Elution buffer:** 50mM HEPES pH 7.5, 300mM NaCl, 150 mM Imidazole.

Procedure: The cell pellet (38 g) was re-suspended in one volume (38 ml) of binding buffer. The re-suspended cells were lysed by sonication. The lysate was cleared by a centrifugation at 20,000 rpm (4°C). 5 ml of 50% Ni-NTA slurry (Qiagen) was applied to a 1.5 x 10 cm gravity column. The column was equilibrated with 100 ml binding buffer. The lysate was applied to the column and was subsequently washed with 50 ml wash buffer 1 and 2. CAMK2D was eluted with 25 mls of elution buffer. The eluted protein was collected and analyzed by SDS-PAGE. DTT was added to the protein sample to a final concentration of 10mM. The protein was dephosphorylated with l-phosphatase (and the addition of $MnCl_2$ to 50 mM). The N-terminal his6-tag was cleaved by incubating CAMK2D overnight with TEV protease.

Column 2: Size exclusion chromatography (Superdex S75, 60 x 1cm)
SEC-Buffers: 50 mM Hepes, pH 7.5, 300 mM NaCl, 5 mM DTT.
The fractions eluted of the Ni-affinity chromatography were concentrated to about 4 ml using Centricon concentrators (10kDa cut off). The concentrated protein was applied to a Superdex S75 column equilibrated in SEC buffer at a flow rate of 0.8 ml/min. Eluted fractions were 95% pure as judged by SDS-PAGE.

Column 3: hiTrap Q Sepharose chromatography (***Calmodulin only***)
Q cation exchange Buffers: Buffer A 50 mM Tris pH 8.8, 100 mM NaCl; Buffer B 50 mM Tris pH 8.8, 1 M NaCl.
The Calmodulin containing fractions from the size exclusion chromatography were pooled and diluted to a final concentration of 100 mM NaCl with the addition of 50 mM Tris pH 8.8. The protein was bound onto a 5 ml hiTrap Q Sepharose column. The protein was eluted using a NaCl gradient. $CaCl_2$ was added to a final concentration of 10 mM.

Mass spectrometry characterization: ESI-MS revealed the expected mass for the TEV-cleaved unphosphorylated CAMK2DA protein (36955 Da) and his-tagged Calmodulin (16925 Da).

Protein concentration: CAMK2D kinase was concentrated in SEC buffer using a centricon with a 10kDa cut off to 12.8 mg/ml. Calmodulin was concentrated to 5.6mg/ml.

Generation of the calmodulin complex: The two proteins were combined to a final 1:1 molar ratio. $CaCl_2$ was added to a final concentration of 1 mM. The complex had an estimated final concentration of 8mg/ml for crystallization trials.

Crystallization: CAMK2D/Calmodulin complex was crystallized at 4°C using the sitting-drop vapor diffusion method at 8mg/ml in the presence of 1 mM SU6656 (Calbiochem) added from a 50 mM DMSO stock. Diffraction quality crystals were obtained by mixing 200 nl of protein solution with 100 nl of reservoir solution containing 0.10M Na/KPO4, 20% PEG 3350, 10% ethylene glycol.

Data Collection: Crystals were flash frozen in liquid nitrogen after being cryo protected in reservoir solution supplemented with 15% ethylene glycol (25% (v/v) final). Diffraction data were collected to 1.9 Å at the Swiss light source beam-line X10SA at a single wavelength.