Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: DYRK1AA-c004

GenBank GI number: gi|18765758

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

Amplified construct sequence:

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CATATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACCTGT
ACTTCCAATCCATGAGCTCCCATAAGAAG
GAACGTAAGGTGTACAACGATGGTTATGA
CGACGATAATTACGACTACATCGTTAAGA
ACGGCGAGAAGTGGATGGATCGCTATGAA
ATTGACTCGCTGATCGGTAAAGGCTCTTT
CGGTCAGGTCGTAAAAGCCTATGATCGTG
TGGAGCAAGAATGGGTCGCAATAAAGATC
ATTAAGAACAAGAAGGCGTTCTTGAACCA
GGCACAGATCGAGGTCCGCCTGTTGGAAC
TGATGAATAAGCACGATACCGAGATGAAA
TACTATATCGTGCATCTGAAACGTCACTT
CATGTTTCGCAATCACCTGTGTTTGGTAT
TCGAGATGCTGTCATACAACCTGTATGAC
CTGTTGCGTAATACTAACTTCCGCGGCGT
TAGTTTGAATCTGACACGCAAATTCGCCC
AACAAATGTGCACCGCTTTGTTGTTTCTG
GCGACTCCAGAATTGAGCATTATTCATTG
TGATCTGAAACCTGAGAACATCCTGTTGT
GCAACCCGAAACGCTCCGCAATCAAAATT
GTCGACTTTGGTTCTTCATGTCAGCTGGG
CCAGCGTATCTACCAATACATTCAGAGTC
GCTTCTATCGTTCGCCCGAAGTGTTGCTG
GGTATGCCATACGATTTGGCCATCGACAT
GTGGAGCCTGGGCTGCATTCTGGTAGAAA
TGCATACGGGAGAACCTTTGTTTTCCGGA
GCAAACGAAGTGGATCAAATGAACAAGAT
CGTTGAGGTCCTGGGTATTCCGCCCGCGC
ACATCTTGGACCAGGCCCCAAAAGCACGC
AAATTCTTTGAAAAGCTGCCTGATGGCAC
CTGGAACCTCAAAAAAACGAAGGACGGTA
AACGTGAATATAAGCCCCCCGGCACACGC
AAATTGCATAACATCCTGGGTGTAGAAAC
CGGCGGTCCAGGCGGTCGTCGCGCTGGCG
AATCTGGTCATACGGTAGCCGATTACTTG
AAGTTCAAAGACCTGATTTTGCGTATGCT
GGATTATGACCCTAAAACCCGCATCCAAC
CGTACTATGCGCTGCAGCATTCATTCTTT
AAGAAAACAGCAGATGAGTGACAGTAAAG
GTGGATACGGATCCGAA
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Tags and additions: Tag sequence: mhhhhhhssgvdlgtenlyfq*s(m) TEV-cleavable (*) N-terminal his6 tag.

Expressed sequence (tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smSSHKK
ERKVYNDGYDDDNYDYIVKNGEKWMDRYE
IDSLIGKGSFGQVVKAYDRVEQEWVAIKI
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IKNKKAFLNQAQIEVRLLELMNKHDTEMK
YYIVHLKRHFMFRNHLCLVFEMLSYNLYD
LLRNTNFRGVSLNLTRKFAQQMCTALLFL
ATPELSIIHCDLKPENILLCNPKRSAIKI
VDFGSSCQLGQRIYQYIQSRFYRSPEVLL
GMPYDLAIDMWSLGCILVEMHTGEPLFSG
ANEVDQMNKIVEVLGIPPAHILDQAPKAR
KFFEKLPDGTWNLKKTKDGKREYKPPGTR
KLHNILGVETGGPGGRRAGESGHTVADYL
KFKDLILRMLDYDPKTRIQPYYALQHSFF
KKTADE

Host:BL21 (DE3) phage resistant Rosetta strain

Growth medium, induction protocol: 5ml from a 50 ml overnight culture containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol was used to inoculate 1 liter of LB media containing the same concentration of antibiotics. Cultures were grown at 37°C until the OD600 reached ~0.3. After that the temperature was adjusted to 20°C. Expression was induced over night using 1mM IPTG at an OD_{600} of 0.6. The cells were collected by centrifugation and the pellet resuspended in binding buffer and frozen.
Binding buffer: 50mM HEPES pH 7.5; 500 mM NaCl; 5% glycerol, 5 mM imidazole and 0.5mM TCEP.

Extraction method: Cell pellets were lysed by C5 high pressure homogenizer (Avestin). The lysate was centrifuged at 21,000 rpm for 60 minutes and the supernatant collected for purification.

Column 1: Ni-affinity chromatography.
Buffers: Binding buffer: Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl , 5% Glycerol, 5mM Imidazole. Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl , 30 mM Imidazole , 5% glycerol. Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl , 50 to 250 mM imidazole, 5% Glycerol .

Procedure:5 ml of 50% Ni-NTA slurry (Qiagen) was applied to a 1.5 x 10 cm gravity column. The column was equilibrated with 50 ml binding buffer. The lysate was applied to the column which was subsequently washed with 50 ml wash buffer 1 and 2. The protein was eluted by gravity flow by applying 5 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150mM, 250 mM); fractions were collected until essentially all protein was eluted. The eluted protein was analyzed by SDS-PAGE. DTT was added to the protein sample to a final concentration of 5mM.

Column 2: Size exclusion chromatography (Superdex S200, 16/60)
SEC-Buffers: 25 mM Hepes, pH 7.5, 500 mM NaCl, 5 mM DTT.
The fractions eluted of the Ni-affinity chromatography were concentrated to about 15 mls using Centricon concentrators (10 kDa cut off). The concentrated protein was applied to a Superdex S200 column equilibrated in SEC buffer at a flow rate of 1 ml/min. Eluted fractions were 95% pure as judged by SDS-PAGE.

Protein concentration: Centricon with a 30 kDa cut off in SEC-buffer.

Mass spectrometry characterization : The mass of the protein was calculated to be 44403 Da and experimentally determined mass was 40403.4 Da for the His tag containing protein. However, up to three phosphorylation sites very evident in the ESI-MS spectrum from which one had been identified to be localized at the activation segment tyrosine Y321. There was no evidence for additional sites in the electron density and we assume that two additional partially occupied sites are present in disordered regions of the protein that have not been included in this model. The identity of the protein was reconfirmed to be correct by DNA sequencing both DNA strands of this expression construct.

Crystallization: DYRK1A substrate peptide complex crystallisation: DYRK1A was concentrated to 15 mg/ml. The protein was mixed with 1mM of inhibitor and 1.5 mM substrate peptide (RYRPGTPALRE). The protein was crystallized by a sitting drop,vapour diffusion method at 4°C, mixing 75 nl protein solution with a 75nl well solution containing 0.2M sodium formate, 20% PEG3350, 10% Ethylene Glycol.

Data Collection: Resolution: Crystals were cryo-protected using the well solution supplemented with 30% ethylene glycol and flash frozen in liquid nitrogen. Diffraction data were collected at Diamond beam

line I02. **Resolution**: 2.5 Å.