XX03CAB39LA (3ZHP) Materials & Methods

Sequence - STK24A

Entry Clone Source: Synthetic DNA

SGC Construct ID: STK24A-c010

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

DNA sequence:

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ATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACC
TGTACTTCCAATCCATGGACCCAGAA
GAGCTTTTTACAAAACTAGAGAAAAT
TGGGAAGGGCTCCTTTGGAGAGGTGT
TCAAAGGCATTGACAATCGGACTCAG
AAAGTGGTTGCCATAAAGATCATTGA
TCTGGAAGAAGCTGAAGATGAGATAG
AGGACATTCAACAAGAAATCACAGTG
CTGAGTCAGTGTGACAGTCCATATGT
AACCAAATATTATGGATCCTATCTGA
AGGATACAAAATTATGGATAATAATG
GAATATCTTGGTGGAGGCTCCGCACT
AGATCTATTAGAACCTGGCCCATTAG
ATGAAACCCAGATCGCTACTATATTA
AGAGAAATACTGAAAGGACTCGATTA
TCTCCATTCGGAGAAGAAAATCCACA
GAGACATTAAAGCGGCCAACGTCCTG
CTGTCTGAGCATGGCGAGGTGAAGCT
GGCGGACTTTGGCGTGGCTGGCCAGC
TGACAGACACCCAGATCAAAAGGAAC
ACCTTCGTGGGCACCCCATTCTGGAT
GGCACCCGAGGTCATCAAACAGTCGG
CCTATGACTCGAAGGCAGACATCTGG
TCCCTGGGCATAACAGCTATTGAACT
TGCAAGAGGGGAACCACCTCATTCCG
AGCTGCACCCCATGAAAGTTTTATTC
CTCATTCCAAAGAACAACCCACCGAC
GTTGGAAGGAAACTACAGTAAACCCC
TCAAGGAGTTTGTGGAGGCCTGTTTG
AATAAGGAGCCGAGCTTTAGACCCAC
TGCTAAGGAGTTATTGAAGCACAAGT
TTATACTACGCAATGCAAAGAAAACT
TCCTACTTGACCGAGCTCATCGACTG
A
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Final protein sequence (Tag sequence in lowercase):

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smDPEELFTKLEKIGKGSFGEVFKGI
DNRTQKVVAIKIIDLEEAEDEIEDIQ
QEITVLSQCDSPYVTKYYGSYLKDTK
LWIIMEYLGGGSALDLLEPGPLDETQ
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IATILREILKGLDYLHSEKKIHRDIK
AANVLLSEHGEVKLADFGVAGQLTDT
QIKRNTFVGTPFWMAPEVIKQSAYDS
KADIWSLGITAIELARGEPPHSELHP
MKVLFLIPKNNPPTLEGNYSKPLKEF
VEACLNKEPSFRPTAKELLKHKFILR
NAKKTSYLTELID

Tags and additions: Cleavable N-terminal His6 tag

Host: BL21(DE3)-R3-pRARE2.

Growth Medium & Induction Protocol: A number of colonies were used to inoculate 50 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 0.4-0.5, the temperature was reduced to 20°C. When the OD_{600} was 0.6 the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight.

Lysis buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 20mM Imidazole, +0.5 mM TCEP, +0.2 mM PMSF

Sequence - CAB39LA

Construct Source: Dario Alessi

Final protein sequence (Tag sequence in lowercase):
gplgsMLPLFSKSHKNPAEIVKILKD
NLAILEKQDKKTDKASEEVSKSLQAM
KEILCGTNEKEPPTEAVAQLAQELYS
SGLLVTLIADLQLIDFEGKKDVTQIF
NNILRRQIGTRSPTVEYISAHPHILF
MLLKGYEAPQIALRCGIMLRECIRHE
PLAKIILFSNQFRDFFKYVELSTFDI
ASDAFATFKDLLTRHKVLVADFLEQN
YDTIFEDYEKLLQSENYVTKRQSLKL
LGELILDRHNFAIMTKYISKPENLKL
MMNLLRDKSPNIQFEAFHVFKVFVAS
PHKTQPIVEILLKNQPKLIEFLSSFQ
KERTDDEQFADEKNYLIKQIRDLKKT
AP

Tags and additions: N-terminal, Prescission protease cleavable glutathione S-transferase tag. The N-terminal residues, GPLGS, derive from the vector following Prescission protease digestion to remove the expression tag.

Host: BL21(DE3)-R3-pRARE2.

Growth Medium & Induction Protocol: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure. A number of colonies were used to inoculate 50 ml of LB media containing 50 µg/ml ampicillin 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 80 µg/ml ampicillin in 2L baffled shaker flasks.

When the OD_{600} was approximately 0.4-0.5, the temperature was reduced to 20°C. When the OD_{600} was 0.6 the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight. Cells were spun at 5000rpm for 10 mins and the pellets resuspended in Lysis Buffer and then frozen at -20°C.

Lysis buffer: 50mM Tris-HCl pH 8.0; 1mM EDTA; 1mM EGTA; 1mM sodium orthovanadate; 25mM NaF; 5mM DTT; 1:2000 Protease Inhibitor Cocktail.

Purification - STK24A

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.

Column 1: 5 ml of Ni-Sepharose in a 2 cm diameter gravity flow column

Column 1 Buffers:
Binding buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 20 mM Imidazole, 5% Glycerol, 0.5 mM TCEP.
Wash buffer 1: As Binding Buffer except 1 M NaCl and 40 mM imidazole.
Wash buffer 2: 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 50 ml of Binding Buffer and 50 ml each of Wash Buffer 1 and 2. 25 ml of Elution Buffer was passed through to elute the protein.

Column 2: S200 16/60 Gel Filtration (GE Healthcare)

Column 2 Buffer:
Gel Filtration buffer: 20 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM TCEP

Column 2 Procedure: The STK24A protein was concentrated to 5 ml volume and injected onto the gel filtration column

Purification - CAB39LA

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.

Column 1: 5 ml of Glutatione-Sepharose in a 2 cm diameter gravity flow column.

Column 1 Buffers:
Buffer 1: 50mM Tris-HCl pH 8.0, 1mM EDTA, 1mM EGTA, 1mM sodium orthovanadate, 25mM NaF, 5mM DTT, 1:2000 Protease Inhibitor Cocktail
Buffer 2: 50mM Tris-HCl pH 8.0, 200 mM NaCl, 0.1mM EGTA, 5mM DTT

Column 1 Procedure: The clarified supernatant was passed through the column. The column was washed six times with 10 ml of Buffer 1, then six times with 10 ml of Buffer 2. The N-terminal GST tag was removed by incubating the beads with PreScission protease (40mg protease per 1ml of beads) at room temperature for 30 min and subsequently at 4°C for 16 hours. The protein was eluted with 50 ml of Buffer 2.

Gel Filtration Purification - STK24A:CAB39LA complex

Column 3: S200 16/60 Gel Filtration (GE Healthcare)

Column 3 Buffers:
Gel Filtration Buffer: 20 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM TCEP

Column 3 Procedure: The CAB39LA protein was concentrated to 7.8 mg/ml. The STK24A protein was concentrated to 2.4 mg/ml. Equimolar amounts of each protein were mixed and injected onto the gel filtration column.

Concentration: The protein complex was concentrated to 9.7 mg/ml (measured by 280 nm absorbance).

Mass spec characterization:
STK24A Expected: 33257
STK24A Observed: 33257
CAB39LA Expected: 39224.2
CAB39LA Observed: 39224.8

Crystallization: Crystals grew from a 1:1 ratio of protein and precipitant solution (0.2M Na/KPO_4, 10% PEG 3350, 10% Ethylene Glycol), using the vapour diffusion method.

Data Collection:
Resolution: 3.1Å **X-ray source**: Diamond IO4-1
Crystals were cryo-protected by equilibration into precipitant solution containing 25% ethylene glycol, and then flash frozen in liquid nitrogen.