MOCS2B (4AP8) Materials & Methods

Entry Clone Source: MGC

Entry Clone Accession: AT76-G3

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

DNA sequence:

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CGGACTCGTTCTGAAGGCGCCTCCAC
CTTTTATGACCACCTCTTTCCCAGAT
TATTCGTTTTGATGAAGCTAAAATTT
TAATCTAAAAAGAAATGCACCTCATG
GAGAATTCTTGTGAAGAACTGTGCTT
CATCTGTGGATTTCTACACCCTTGAT
CATTTGCAAACCTGTAATTATTTCGT
AAAGAGTTGTTTGCACGGAGTGACAG
GTTGAAGTATTGTATTTTGCAAAAAG
TGCTGAAATAACAGGAGTTCGTTCAG
AGACCATTTCTGTGCCTCAAGAAATA
AAAGCGTTGCAGCTGTGGAAGGAGAT
AGAAACTCGACATCCTGGATTGGCTG
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GTTCGTCAAGAATATGTCGAGCTTGG
AGATCAGCTCCTCGTGCTTCAGCCTG
GAGACGAAATTGCCGTTATCCCCCCC
ATTAGTGGAGGATAGTGCTTTTGAGC
CATCTAGGAAAGATATGGATGAAGTT
GAAGAGAAATCTAAAGATGTTATAAA
CTTTACTGCCGAGAAACTTTCAGTAG
ATGAAGTCTCACAGTTGGTGATTTCT
CCGCTCTGTGGTGCAATATCCCTATT
TGTAGGGACTACAAGAAATAACTTTG
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CTATGCCATTGATACTTTAAAAGCCA
AGGTGCCCATATGGAAAAAGGAAATA
TACGAAGAGTCATCAACTTGGAAAGG
AAACAAAGAGTGCTTTTGGGCATCCA
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CATGCAATCTTAACTTTGTTAAACTA
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ACAAAAGGGAGAAAAAGGAAGCAAGA
TAAATGGGTATGTAGGATGAAGGGTT
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ATTTAAAATGGAACTAAAGATAGAAG
GAGGACTGTAGGAAGAAATGGAATAA
TTTAAATGTGAGGAAAGATATCTGTG
GTAGACATGTCCTTCCATGACTAATT
TCTAATTGTAACTCAACACACATTGA
GGTATGGGCCCTCCTCAGTGACTTTA
ACTAGCTCAGAAACGTACTCCCCCAC
CAACCCCACCTCACCGCCCCCCATCC
CGGTTCTGGGAGAGCATTGTTATTAA
GGATGCATGACAGGAATGTTGGCAGA
ACTGGAAAGTATTAAAAAAGCATTAT
CAGACAGTCTTGATATTATACATTTT
CAGAAATATATTAAAAATAATAAACT
AAAACCCATGATTTCAAAAGTTTTAA
AAAAAAAAAAAAA

Final protein sequence (Tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq^smSA
FEPSRKDMDEVEEKSKDVINFTAEKL
SVDEVSQLVISPLCGAISLFVGTTRN
NFEGKKVISLEYEAYLPMAENEVRKI
CSDIRQKWPVKHIAVFHRLGLVPVSE
ASIIIAVSSAHRAASLEAVSYAIDTL
KAKVPIWKKEIYEESSTWKGN

^ TEV protease recognition site

Tags and additions: Cleavable N-terminal His6 tag

Host: BL21(DE3)-R3-pRARE2

Growth Medium & Induction Protocol: The construct DNA was transformed into competent cells of the expression strain by a standard heat shock procedure. One colony from the transformation was used to inoculate 1.5 ml of TB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture. A glycerol stock was used to inoculate 60 ml of TB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day this starter culture was used to inoculate 6L of TB media (9 ml starter culture used per 1L) containing 50 µg/ml kanamycin. When the OD_{600} reached approximately 1.0 the temperature was reduced to 18°C and after a further 30 minutes the cells were induced by the addition of 0.1 mM IPTG. The expression was continued overnight.

Extraction buffer, extraction method: Cell pellets were dissolved in approximately 50ml lysis buffer and broken by passing through the homogeniser (x6) at a constant pressure of 15KPa. The cell debris was pelleted at 16,000 RPM and the supernatant used for further purification.
Lysis buffer: 10 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 1 tablet per 50 ml protease inhibitor cocktail EDTA-free (Roche)

Column 1: Ni-NTA (5.0 ml volume in a gravity-flow column).

Column 1 Buffers:
Binding buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.4

Wash buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.4
Elution buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4

Column 1 Procedure: The clarified cell extract was incubated with 5.0 ml pre-equilibriated 50% Ni-NTA bead solution for 1 hour at 4°C with rotation after which it was passed through a glass column. The column was then washed with 50ml Binding Buffer (2 x 25ml) and 50 ml Wash Buffer (2 x25 ml). The protein was eluted with 50 ml of Elution Buffer in 8 x 5 ml fractions.

Column 2: Superdex s75 16/60 Gel Filtration

Column 2 Buffers:
Gel Filtration buffer: 10 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol

Column 2 Procedure: Only Elution Fractions 1-8 were pooled from the Ni-NTA column. This pool was then concentrate to 5ml and applied directly to the GF column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.0 ml fractions were collected. During this concentration step, the protein began to crash. It was decided to halt the process and carry out a buffer exchange swaping the protein from Elution buffer into Gel filtration buffer. A second attempt to concentrate in the GF buffer was then carried out which had no effect. Concentration was nevertheless proceeded with but with careful monitoring of protein stability. The protein eluted at a volume of between 90 ml and 100 ml.

Enzymatic treatment: The N-terminal His6-tag was cleaved by incubating the protein overnight with TEV protease (20°C). Cleaved protein was purified by batch binding on 1ml pre-equilibriated 50% Ni-NTA bead solution. The column was then washed with 2 ml Gel Filtration Buffer (2x1ml) and 2 ml Binding Buffer (2x1 ml). The protein was eluted with 2 ml of Elution Buffer (2x1ml).

Gel Filtration buffer: 10 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol
Binding buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.4
Elution buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4

Mass spec characterization:
Expected: ?
Observed: ?

Concentration: To set up plates the sample was concentrated to 8.62 mg/ml using a 10 kDa mwco concentrator

Crystallization: Crystals were grown by vapour diffusion in sitting drop at 4°C. A sitting drop consisting of 50 nl protein and 100 nl well solution was equilibrated against well solution containing 0.1M HEPES pH 7.3, 0.8M potassium/sodium tartrate. Crystals were mounted in the presence of 20% (v/v) glycerol and flash-cooled in liquid nitrogen.

Data Collection:Data Collection:
Resolution: 2.78Å **X-ray source**: Diamond IO3