

ACVR2A (4ASX) Materials & Methods

Entry clone source: Origene

Entry clone accession: GI:65508448

SGC Construct ID: ACVR2A-c046

Vector: pFB-LIC-Bse. Details [[PDF](#)]; Sequence [[FASTA](#)] or [[GenBank](#)].

DNA sequence:

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TACTTCCAATCCATGCCACTGCAGTT
ATTAGAAGTGAAAGCAAGGGGAAGAT
TTGGTTGTGTCTGGAAAGCCCAGTTG
CTTAACGAATATGTGGCTGTCAAAAT
ATTCACAAATACAGGACAAACAGTCAT
GGCAAAATGAATACGAAGTCTACAGT
TTGCCTGGAATGAAGCATGAGAACAT
ATTACAGTTCATGGTGCAGAAAAAC
GAGGCACCACTGTTGATGTGGATCTT
TGGCTGATCACAGCATTGATGAAAGA
GGGTTCACTATCAGACTTCTTAAGG
CTAATGTGGTCTCTGGAATGAAC TG
TGTCAATTGCAGAAACCATGGCTAG
AGGATTGGCATATTACATGAGGATA
TACCTGGCTAAAGATGGCCACAAA
CCTGCCATATCTCACAGGGACATCAA
AAGTAAAAATGTGCTGTTGAAAAACA
ACCTGACAGCTTGCATTGCTGACTTT
GGGTTGGCCTAAATTTGAGGCTGG
CAAGTCTGCAGGCGATAACCATGGAC
AGGTTGGTACCCGGAGGTACATGGCT
CCAGAGGTATTAGAGGGTGTATAAA
CTTCCAAAGGGATGCATTTTGAGGA
TAGATATGTATGCCATGGATTAGTC
CTATGGGAACTGGCTTCTGCTGTAC
TGCTGCAGATGGACCTGTAGATGAAT
ACATGTTGCCATTGAGGAGGAATT
GGCCAGCATCCATCTTGAAGACAT
GCAGGAAGTTGTTAAGAGATTATTGG
CAGAAACATGCTGGAATGGCAATGCT
CTGTGAAACCATTGAAGAATGTTGGG
ATCACGACGCAGAAGCCAGGTTATCA
GCTGGATGTGTAGGTGAAAGAATTAC
CCAGATGCAGAGACTAACAAATATT
GACAGTAAAGGTGGATA
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Expressed protein sequence (His₆ affinity Tag sequence in lowercase):

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mghhhhhssgvdlgtenlyfq^SMP
LQLLEVKARGRFGCVWKAQLLNEYVA
VKIFPIQDKQSWQNEYEVYSLPGMKH
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ENILQFIGAEKRGTSVDV р DLWLITAF
HEKGSLSDFLKANVVSWNELCHIAET
MARGLAYLHEDIPLGLKDGHKPAISHR
DIKSKNVLLKNNLTACIADFGLALKF
EAGKSAGDTHGQVGTRRYMAPEVLEG
AINFQRDAFLRIDMYAMGLVLWELAS
RCTAADGPVDEYMLPFEETIGQHPSL
EDMQEVVVHKKRPVLRDYWQKHAGM
AMLCETIEECWDHDAEARLSAGCVGE
RITQMQLTNI

^ TEV protease recognition site

Tags and additions: Tev-cleavable N-terminal His6 tag

Host: SF9 Spodoptera frugiperda Insect cells.

Growth medium, induction protocol: 2L of SF9 cells at a density of 2 million/ml were infected with 10 ml of Virus/L. Cells were incubated at 27°C in the shaker incubator and harvested after 48 hours. Cells were harvested by centrifugation at 900g at 4°C for 20 min. Cell pellets from each flask (11 volume) were resuspended in 20 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole) in the presence of protease inhibitor SET V (Calbiochem) added to the cell suspension at 1:100 dilution, transferred to 50 ml tubes, and stored at 20°C.

Extraction buffer, extraction method: The frozen cells were thawed and 0.15% PEI (pH7.4) was added. The cells were lysed by passage 4-5x through an Emulsiflex C5 homogenizer. The cell lysate was spun down by centrifugation at 21.5K rpm at 4°C for 1 h. The supernatant was recovered for purification

Column 1: Ni-Affinity Chromatography. 4 ml of 50 % Ni-sepharose slurry was applied onto a 1.5 x 10 cm column. The column was equilibrated with binding buffer (25ml).

Column 1 Buffers:

Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole 0.5 mM TCEP

Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole 0.1 mM TCEP

Elution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 50 to 250 mM imidazole 0.5 mM TCEP

Column 1 Procedure: The supernatant following centrifugation was applied by gravity flow onto the Ni-sepharose column. The bound protein was then washed with 50ml binding buffer and subsequently with 30 ml wash buffer. ACVR2A protein was then eluted by applying a step gradient of imidazole □ using 5 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 250 mM). Elution fractions were analyzed by SDS PAGE and the 100, 150 and 250 mM imidazole fractions were kept and pooled. 10 mM DTT was added for overnight storage at 4°C.

Enzymatic treatment: The tag was not cleaved

Column 2: Size Exclusion Chromatography □ S200 HiLoad 16/60 Superdex run on ÄKTA-Express

Column 2 Buffers:

Gel Filtration buffer: 300 mM NaCl, 50 mM HEPES pH 7.5, 0.5 mM TCEP, pH 7.5

Column 2 Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. The protein was concentrated to 3 ml using an Amicon Ultra-15 filter with a 30 kDa cut-off. The concentrated protein was directly applied onto the equilibrated S200 16/60 column, and run at a flow-rate of 1 ml/min. Fractions containing the protein were pooled together.

Mass spec characterization:

The purified protein was homogeneous and had an experimental mass of 36410.6 Da. The theoretical expected mass from the construct primary sequence is 36497.9. The difference corresponds to loss of the N-terminal methionine and acetylation of the resulting N-terminus. Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% methanol in water with 0.1% formic acid.

Concentration: Protein was concentrated to 12.4 mg/ml using an Amicon 30 kDa cut-off concentrator.

Crystallization: Protein was concentrated to 12.4 mg/ml in gel filtration buffer. The oxo-beta-carboline inhibitor Dihydro-Bauerine C (K01764) was added to the final sample at a concentration of 1mM. Crystals were grown at 4°C in 150 nl sitting drops mixing 100 nl protein solution with 50 nl of a reservoir solution containing 20% PEG 3350; 0.20M Na(malonate). On mounting crystals were cryo-protected with an additional 20% ethylene glycol.

Data Collection: Resolution: 2.05 Å

X-ray source: Diamond Light Source, station I04, using monochromatic radiation at wavelength 0.9611 Å