C14ORF124A (4B4O) Materials & Methods

Entry clone source: MGC

Entry clone accession: IMAGE 2900140

SGC Construct ID: C14ORF124A-c105

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

Protein sequence (His_6 affinity Tag sequence in lowercase):

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MRVLVGGGTGFIGTALTQLLNARGHE
VTLVSRKPGPGRITWDELAASGLPSC
DAAVNLAGENILNPLRRWNETFQKEV
LGSRLETTQLLAKAITKAPQPPKAWV
LVTGVAYYQPSLTAEYDEDSPGGDFD
FFSNLVTKWEAAARLPGDSTRQVVVR
SGVVLGRGGGAMGHMLLPFRLGLGGP
IGSGHQFFPWIHIGDLAGILTHALEA
NHVHGVLNGVAPSSATNAEFAQTFGA
ALGRRAFIPLPSAVVQAVFGRQRAIM
LLEGQKVIPRRTLATGYQYSFPELGA
ALKEIaenlyfq^shhhhhhdykddd
dk

^ TEV protease recognition site
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Tags and additions: Cleavable C-terminal His6 tag

Host: BL21(DE3)-R3-pRARE2.

Growth medium, induction protocol: A glycerol stock was used to inoculate a 5 ml overnight culture of BL21(DE3)-R3-pRARE2 /C14ORF124A in LB supplemented with 50 µg/ml Kan and 34 µg/ml Chloramphenicol. This culture was then used to inoculate 1400 ml of LB supplemented with 50 µg/ml Kan and 34 µg/ml Chloramphenicol, and this culture was grown overnight at 37oC with vigorous shaking (200 rpm). The next morning the cells were pelleted, washed 3 times with minimal media and finally resuspended in 140 ml of minimal media that was used to innoculate 14x1 litre prewarmed minimal media supplemented 50 µg/ml Kan and 34 µg/ml Chloramphenicol and the cells grown at 37°C. At an of OD 0.5 amino acids and selenomethionine was added (100 mg/L of lysine, threonine and phenylalanin, 50 mg/L of leucine, isoleucine and valine and 25 mg/L Selenomethionine) and the culture grown to and OD of 1.0. Then the temperature was decreased to 18°C and additional Selenomethionine added to a final concentration of 75 mg/L and the protein expression induced with 0.5 mM IPTG over night. The cells were collected by centrifugation and frozen at -80°C.

Column 1: Ni-Sepharose FF, 2.5 ml (GE/Amersham Biosciences).

Column 1 Buffers:
Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5% glycerol, Complete® protease inhibitors (Roche, 1 tbl/50 ml).
Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 45 mM imidazole.
Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 250 mM imidazole

Column 1 Procedure: Frozen cell pellets were thawed and resuspended in a total volume of 40 ml of lysis buffer . The cells were disrupted by sonication and cell debris removed by centrifugation for 45 minutes at 40 000xg. The cell lysate was applied onto a 2.5 ml Ni-Sepharose FF column equilibrated with binding buffer. The column was subsequently washed with 50 ml of wash buffer and the protein eluted using 10 ml elution buffer. All fractions were collected and analysed by SDS-PAGE.

Column 2: SuperDex 200 16/60 HiLoad (GE/Amersham)

Column 2 Buffers:
Gel Filtration buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5 % glycerol.

Column 2 Procedure: The eluted protein from the Ni-affinity column was loaded on the gel filtration column at 1.0 ml/min on an AKTA Purifier system. Eluted protein was collected in 1 ml fractions and analysed by SDS-PAGE.

Column 3: HP Q column

Column 3 Buffers:
Buffer A: 10 mM Tris-Cl pH 8.5, 50mM NaCl
Buffer B: 10 mM Tris-Cl pH 8.5, 2 M NaCl

Column 3 Procedure: The buffer of C14ORF124A was exchanged to10 mM Tris-Cl pH 8.5, 50mM NaCl before it was applied onto a 5ml HP Q column. The protein was eluted from the column using a linear gradient with buffer B.

Concentration: The protein was concentrated in Amicon (3 K) to 6.4 mg/ml. The protein concentration was determined spectrophotometrically using the predicted molar extinction coefficient 37930 (M^{-1} cm^{-1}).

Mass spec characterization:
Expected mass: 13284.2 Da, Measured mass: 13284.24 Da The mass of the SeMet labelled protein was determined with ESI-MS to 33926 Da, in agreement with the predicted mass of the protein.

Crystallization: C14ORF124A was crystallised by vapor diffusion at 4°C from a sitting drop consisting of 100 nl protein (6.4 mg/ml) with 5 mM NADPH and 50 nl 0.2 M Na formate, 15 % PEG3350. The crystal was transferred to a cryo protectant composed of 25 % glycerol before flash-cooling in liquid nitrogen

Data Collection: 2.70 Å **X-ray source:** Diamond light source I02