Entry Clone Source: MGC

Entry Clone Accession: IMAGE:3910208

SGC Construct ID: H2AFY2A-c020

GenBank GI number: gi|8923920

Vector: pNH-TrxT. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

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ATGCACCATCATCATCATCATTCTTCTGGT
ATGAGCGATAAAATTATTCACCTGACTGAC
GACAGTTTTGACACGGATGTACTCAAAGCG
GACGGGGCGATCCTCGTCGATTTCTGGGCA
GAGTGGTGCGGTCCGTGCAAAATGATCGCC
CCGATTCTGGATGAAATCGCTGACGAATAT
CAGGGCAAACTGACCGTTGCAAAACTGAAC
ATCGATCAAAACCCTGGCACTGCGCCGAAA
TATGGCATCCGTGGTATCCCGACTCTGCTG
CTGTTCAAAAACGGTGAAGTGGCGGCAACC
AAAGTGGGCGCACTGTCTAAAGGTCAGTTG
AAAGAGTTCCTCGACGCTAACCTGGCCGGT
ACCGAGAACTTGTACTTCCAATCCATGAAA
GAAGGAACTTCAAATTCCACCTCTGAAGAT
GGGCCAGGGGATGGATTCACCATTCTGTCT
TCTAAGAGCCTTGTTCTGGGACAGAAGCTG
TCCTTAACCCAGAGTGACATCAGCCATATT
GGCTCCATGAGAGTGGAGGGCATTGTCCAC
CCAACCACAGCCGAAATTGACCTCAAAGAA
GATATAGGTAAAGCCTTGGAAAAGGCTGGG
GGAAAAGAGTTCTTGGAAACGGTAAAGGAG
CTTCGCAAATCCCAAGGCCCTTTGGAAGTC
GCCGAAGCCGCCGTCAGCCAATCCAGTGGA
CTCGCAGCCAAATTTGTCATCCACTGTCAC
ATCCCTCAGTGGGGCTCCGACAAATGTGAA
GAACAGCTTGAAGAGACCATCAAAAACTGC
CTGTCAGCGGCGGAGGACAAGAAGCTAAAG
TCCGTCGCGTTCCCGCCTTTCCCCAGCGGC
AGAAACTGCTTTCCCAAACAGACTGCGGCC
CAGGTGACCCTCAAAGCCATCTCAGCCCAC
TTTGATGACTCGAGCGCGTCCTCGCTGAAG
AACGTGTACTTCCTGCTCTTCGACAGCGAG
AGCATCGGCATCTACGTGCAGGAGATGGCC
AAGCTCGACGCCAAGTGA
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Protein sequence without tag: (aa 167-372 of H2AFY2, encompassing the Macro domain)

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SMKEGTSNSTSEDGPGDGFTILSSKSLVLG
QKLSLTQSDISHIGSMRVEGIVHPTTAEID
LKEDIGKALEKAGGKEFLETVKELRKSQGP
LEVAEAAVSQSSGLAAKFVIHCHIPQWGSD
KCEEQLEETIKNCLSAAEDKKLKSVAFPPF
PSGRNCFPKQTAAQVTLKAISAHFDDSSAS
SLKNVYFLLFDSESIGIYVQEMAKLDAK
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Tags and additions: N-terminal, TEV cleavable His6 and thioredoxin tag
MHHHHHHSSGMSDKIIHLTDDSFDTDVLKA
DGAILVDFWAEWCGPCKMIAPILDEIADEY
QGKLTVALNIDQNPGTAPKYGIRGIPTLLL
FKNGEVAATKVGALSKGQLKEFLDANLAGT
ENLYFQ*SM

Host cells: BL21 (DE3)-R3-pRARE2 (a phage-resistant derivative of Rosetta2 [Novagen])

Growth medium, induction protocol:
The expression plasmid was transformed into the host strain and plated on LB-agar containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol. Several colonies were combined to inoculate a 1-ml culture in LB (+ 50 µg/ml kanamycin, 35 µg/ml chloramphenicol). The culture was grown overnight, glycerol was added to 15% v/v (from a 60% stock), and the resulting glycerol stock was frozen at -80°C in 100 µl aliquots.
A loop full of cells from the glycerol stock was inoculated into 60ml of LB medium containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol and grown overnight at 37°C. Four 1-L batches of TB medium (+50 µg/ml kanamycin) in 2.5L UltraYield baffled flasks were inoculated with 15 ml each of the overnight culture. The cultures were grown at 37°C for 4 hours to an OD_{600} of 1. The cultures were then transferred to 20°C, and after 45 minutes IPTG was added to 0. 5 mM. Growth was continued overnight. The cells were collected by centrifugation, the pellets were resuspended in 105 ml of Lysis Buffer with the addition of a 1:5000 dilution of Calbiochem. Protease inhibitor set VII. The resuspended cell pellet was placed in a -80°C freezer.
Lysis Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole pH 7.4, 0.5 mM TCEP

Cell Lysis: The re-suspended cell pellet was lysed by sonication. PEI (polyethyleneimine) was added to a final concentration of 0.15 % from a 5% (w/v, pH 7.5) stock, and the cell debris and precipitated DNA were spun down (17000 rpm, JA17 rotor, 45 min).

Column 1: Histrap FF 5 ml (GE Healthcare)

Column 1 Buffers:
Binding Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole pH 7.4, 1.0 mM TCEP
Wash Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 30 mM Imidazole pH 7.4, 1.0mM TCEP
Elution Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 300 mM Imidazole pH 7.4, 1.0 mM TCEP

Column 1 Procedure: The cell extract was loaded onto the column at 5 ml/minute on an ÄKTA-express system (GE Healthcare). The column was then washed with 10 volumes of binding buffer, 10 volumes of wash buffer, and eluted with elution buffer at 4 ml/min. The eluted peak of A_{280} was automatically collected.

Column 2: Gel Filtration, HiLoad 16/60 Superdex 75 (120 ml)

Column 2 Buffers: GF Buffer: 20 mM HEPES pH 7.5, 250 mM NaCl, 5% glycerol; 1.0 mM TCEP

Column 2 Procedure: The eluted fraction was loaded and fractionated on the gel filtration column in GF buffer at 1.2 ml/min. Fractions of 1.75ml were collected at the A_{280} peaks. The

fractions were analyzed by SDS-PAGE and relevant fractions were further analyzed by Mass spectrometry (ESI-TOF).

Tag cleavage: The His-Trx tag was designed to be cleavable by Tobacco Etch Virus (TEV) protease. Fractions containing H2AFY2 were pooled and treated with recombinant His-tagged TEV at a ratio of 1:30 (w/w). Removal of the tag, the protease and contaminating proteins was achieved by batch binding with 5ml bed volume of Ni-NTA. The unbound fractions were analyzed by SDS-PAGE and MS.

Mass spectrometry characterization: predicted 22364.4 Da, measured: 22365.7.

Protein concentration: The final fraction was concentrated to 30.9 mg/ml using a 10kDa MW CO spin concentrator (measured by 280 nm absorbance). Fresh (unfrozen) protein was used for crystallization.

Crystallisation: Crystals were grown by vapour diffusion in a 1:1 ratio of protein solution and crystallisation buffer. The crystallisation buffer was composed of 50 mM $MgCl_2$, 100 mM HEPES (pH 7.5) and 30% PEG550 at 20°C. The crystal was flash frozen in liquid nitrogen without using a cryo buffer.

Data collection and structure determination: A data set was collected at Diamond IO3. After integration the data set turned out to be twinned in space group C2 with a resolution of 2.1 Å. PHASER and an ensemble of super positioned PDB structures 1YD9, 1ZR3, 1ZR5, 3IID was used for phasing. Intensity based twin-refinement in REFMAC5 was used during building and refinement.