AURKBA (4AF3) Materials & Methods

Entry Clone Source: Mammalian Gene Collection (IMAGE collection Clone ID 2819846)

SGC Construct ID: AURKBA-c004

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

AURKBA DNA sequence:

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ATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACC
TGTACTTCCAATCCATGCAGAAGGTG
ATGGAGAATAGCAGTGGGACACCCGA
CATCTTAACGCGGCACTTCACAATTG
ATGACTTTGAGATTGGGCGTCCTCTG
GGCAAAGGCAAGTTTGGAAACGTGTA
CTTGGCTCGGGAGAAGAAAAGCCATT
TCATCGTGGCGCTCAAGGTCCTCTTC
AAGTCCCAGATAGAGAAGGAGGGCGT
GGAGCATCAGCTGCGCAGAGAGATCG
AAATCCAGGCCCACCTGCACCATCCC
AACATCCTGCGTCTCTACAACTATTT
TTATGACCGGAGGAGGATCTACTTGA
TTCTAGAGTATGCCCCCCGCGGGGAG
CTCTACAAGGAGCTGCAGAAGAGCTG
CACATTTGACGAGCAGCGAACAGCCA
CGATCATGGAGGAGTTGGCAGATGCT
CTAATGTACTGCCATGGGAAGAAGGT
GATTCACAGAGACATAAAGCCAGAAA
ATCTGCTCTTAGGGCTCAAGGGAGAG
CTGAAGATTGCTGACTTCGGCTGGTC
TGTGCATGCGCCCTCCCTGAGGAGGA
AGACAATGTGTGGCACCCTGGACTAC
CTGCCCCCAGAGATGATTGAGGGGCG
CATGCACAATGAGAAGGTGGATCTGT
GGTGCATTGGAGTGCTTTGCTATGAG
CTGCTGGTGGGGAACCCACCCTTTGA
GAGTGCATCACACAACGAGACCTATC
GCCGCATCGTCAAGGTGGACCTAAAG
TTCCCCGCTTCTGTGCCCACGGGAGC
CCAGGACCTCATCTCCAAACTGCTCA
GGCATAACCCCTCGGAACGGCTGCCC
CTGGCCCAGGTCTCAGCCCACCCTTG
GGTCCGGGCCAACTCTCGGAGGGTGC
TGCCTCCCTCTGCCCTTCAATCTGTC
GCCTGA
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AURKBA Final protein sequence (Tag sequence in lowercase):

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smQKVMENSSGTPDILTRHFTIDDFE
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IGRPLGKGKFGNVYLAREKKSHFIVA
LKVLFKSQIEKEGVEHQLRREIEIQA
HLHHPNILRLYNYFYDRRRIYLILEY
APRGELYKELQKSCTFDEQRTATIME
ELADALMYCHGKKVIHRDIKPENLLL
GLKGELKIADFGWSVHAPSLRRKTMC
GTLDYLPPEMIEGRMHNEKVDLWCIG
VLCYELLVGNPPFESASHNETYRRIV
KVDLKFPASVPTGAQDLISKLLRHNP
SERLPLAQVSAHPWVRANSRRVLPPS
ALQSVA

(Gln55 to Ala344)
The N-terminal residues, sm, derive from the vector following TEV protease digestion to remove the expression tag.

Entry Clone Source: Andrea Musacchio

SGC Construct ID: INCENPA-c002

Vector: pGTvL1-SGC. Details [PDF]; Sequence [FASTA] or [GenBank].

INCENPA DNA sequence:
ATGTCCCCTATACTAGGTTATTGGAA
AATTAAGGGCCTTGTGCAACCCACTC
GACTTCTTTTGGAATATCTTGAAGAA
AAATATGAAGAGCATTTGTATGAGCG
CGATGAAGGTGATAAATGGCGAAACA
AAAAGTTTGAATTGGGTTTGGAGTTT
CCCAATCTTCCTTATTATATTGATGG
TGATGTTAAATTAACACAGTCTATGG
CCATCATACGTTATATAGCTGACAAG
CACAACATGTTGGGTGGTTGTCCAAA
AGAGCGTGCAGAGATTTCAATGCTTG
AAGGAGCGGTTTTGGATATTAGATAC
GGTGTTTCGAGAATTGCATATAGTAA
AGACTTTGAAACTCTCAAAGTTGATT
TTCTTAGCAAGCTACCTGAAATGCTG
AAAATGTTCGAAGATCGTTTATGTCA
TAAAACATATTTAAATGGTGATCATG
TAACCCATCCTGACTTCATGTTGTAT
GACGCTCTTGATGTTGTTTTATACAT
GGACCCAATGTGCCTGGATGCGTTCC
CAAAATTAGTTTGTTTTAAAAAACGT
ATTGAAGCTATCCCACAAATTGATAA
GTACTTGAAATCCAGCAAGTATATAG
CATGGCCTTTGCAGGGCTGGCAAGCC
ACGTTTGGTGGTGGCGACCATCCTCC
AAAATCGAGCTCAGAGAACCTGTACT
TCCAATCCATGGAGGCCCATCCCCGG
AAGCCCATCCCCACCTGGGCCCGAGG
CACCCCGCTCAGCCAGGCTATCATTC
ACCAGTACTACCACCCACCGAACCTT
CTGGAGCTCTTTGGAACCATTCTCCC
ACTGGACTTGGAGGATATCTTCAAGA

AGAGCAAGCCCCGCTATCACAAGCGC
ACCAGCTCTGCTGTCTGGAACTCACC
GCCCCTGCAGTGACAGTAAAGGTGGA
TACTCGAGCGGCCGCATCGTGACTGA
CTGA

INCENPA Final protein sequence (Tag sequence in lowercase):
smEAHPRKPIPTWARGTPLSQAIIHQ
YYHPPNLLELFGTILPLDLEDIFKKS
KPRYHKRTSSAVWNSPPLQ

(Glu835 to Gln903)
The N-terminal residues, sm, derive from the vector following TEV protease digestion to remove the expression tag.

AURKBA expression

Host: BL21(DE3)-R3-pRARE2.

Transformation: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure.

Growth Medium & Induction Protocol: A number of colonies were used to inoculate 70 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 4x 15 ml of this starter culture was used to inoculate 4x 1L of TB media containing 40 µg/ml kanamycin in 2L baffled shaker flasks. When the OD_{600} was approximately 1.2, the temperature was reduced to 20°C. After a further 25 minutes the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight.

Cell Harvest: Cells were spun at 5500rpm for 10 mins and the pellets resuspended in Lysis Buffer and then frozen at -20°C.

INCENPA expression

Host: BL21(DE3)-R3-pRARE2.

Transformation: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure.

Growth Medium & Induction Protocol: A number of colonies were used to inoculate 100 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 6x 15 ml of this starter culture was used to inoculate 6x 1L of TB media containing 80 µg/ml ampicillin in 2L baffled shaker flasks. When the OD_{600} was approximately 0.5, the temperature was reduced to 20°C. After a further 25 minutes the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight.

Cell Harvest: Cells were spun at 5500rpm for 10 mins and the pellets resuspended in Lysis Buffer and then frozen at -20°C.

Extraction buffer, extraction method: The resuspended cell pellets for AURKBA and INCENPA were thawed, combined, and lysed by high-pressure homogenization. PEI (polyethyleneimine) was added to a final concentration of 0.15 %. The cell debris and precipitated DNA were spun down.

Lysis Buffer: 50 mM Tris pH 7.8, 200 mM NaCl, 20 mM Imidazole, 0.5 mM TCEP, Sigma protease inhibitor cocktail.

Column 1: 10 ml of Ni-Sepharose divided in 2x 2.5cm diameter gravity flow columns.

Column 1 Buffers:
Binding buffer: As Lysis Buffer
Wash buffer: As Lysis Buffer except 40 mM Imidazole.
Elution buffer: As Lysis Buffer except 250 mM Imidazole.

Column 1 Procedure: The supernatant was applied by gravity flow onto the Ni column. The Ni column was washed with Wash buffer and the bound protein was eluted by applying a step gradient of Imidazole (5 ml fractions of Elution buffer supplemented with 50mM, 100mM, 150mM and 250mM Imidazole). Collected fractions were pooled and stored at 4°C.

Growth Medium & Induction Protocol: The clarified supernatant was passed through the column. The column was washed with Binding Buffer and Wash Buffer. 50 ml of Elute Buffer was passed through to elute the protein.

Column 2: 10 ml Glutathione Sepharose in a gravity flow column

Column 2 Procedure: The eluted protein from column 1 was passed through column 2. The column was washed with Binding Buffer and then eluted with 50 ml of Binding Buffer containing 10 mM reduced L-glutathione

TEV protease digestion: TEV protease was added. The sample was left at 4°C overnight.

Column 3: S75 16/60 Gel Filtration

Column 3 Buffers:
Gel Filtration buffer: 25 mM Hepes pH 7.5, 200 mM NaCl, 0.5 mM TCEP

Column 3 Procedure: The protein was concentrated to 5 ml volume and injected onto an S75 16/60 GF column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.75 ml fractions were collected.

Column 4: Glutathione Sepharose

Column 4 Procedure: Pooled fractions from the gel filtration were passed through 1 ml of glutathione sepharose.

Column 5: Ni-Sepharose

Column 5 Procedure: The flow-through from the glutathione sepharose was loaded onto 0.7 ml of Ni-sepharose. The column was eluted with 5 ml of GF Buffer containing 20, 40, 60, 80, 100, 120 mM imidazole. The desired protein complex appeared in the 40-120 mM fractions.

Concentration: Compound VX-680 was added to the pooled fractions from Column 5. The sample was twice concentrated to 0.25 ml and diluted to 4 ml with GF Buffer before being concentrated to 0.2 ml volume at which the protein concentration was 6 mg/ml (measured by 280 nm absorbance).

Mass spec characterization (before TEV protease digestions):

	Expected	Observed
AURKBA	36116.3	36204.3, 36284.3, 36359.5, 36439.6 (1-4 phosphorylations)

	Expected	Observed
INCENPA	34753.4	34761.0

Masses are +8 from the expected value, for both proteins (miscalibration of mass spectrometer).

After TEV protease digestion the AURKBA protein was monophosphorylated indicating that the additional phosphorylations were on the purification tag.

Crystallization: The complex was crystallised at 20°C in 300 nl drops from a 2:1 ratio of AuroraB:INCENP:VX-680 (6 mg/ml protein) and reservoir solution (10% w/v PEG3350, 0.2M KSCN, 10% Ethylene Glycol, 0.1M BisTrisPropane pH 6.15).

Data Collection:
The crystals were cryo-protected in reservoir solution with 25% (v/v) ethylene glycol and flash-frozen in liquid nitrogen. X-ray diffraction data was collected at 100 K on beam line I04 at DIAMOND.