**Entry Clone Source:** MGC

**Entry Clone Accession:** IMAGE:3837777

**SGC Construct ID:** CLK2A-c015

**GenBank GI number:** gi|4502883

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

**Amplified construct sequence:**

```
CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGCGCTCATCTTCGCACAGC
AGCCGGAGAGCCAAGAGTGTAGAGGACGAC
GCTGAGGGCCACCTCATCTACCACGTCGGG
GACTGGCTACAAGAGCGATATGAAATCGTT
AGCACCTTAGGAGAGGGGACCTTCGGCCGA
GTTGTACAATGTGTTGACCATCGCAGGGGT
GGGGCTCGAGTTGCCCTGAAGATCATTAAG
AATGTGGAGAAGTACAAGGAAGCAGCTCGA
CTTGAGATCAACGTGCTAGAGAAAATCAAT
GAGAAAGACCCTGACAACAAGAACCTCTGT
GTCCAGATGTTTGACTGGTTTGACTACCAT
GGCCACATGTGTATCTCCTTTGAGCTTCTG
GGCCTTAGCACCTTCGATTTCCTCAAAGAC
AACAACTACCTGCCCTACCCCATCCACCAA
GTGCGCCACATGGCCTTCCAGCTGTGCCAG
GCTGTCAAGTTCCTCCATGATAACAAGCTG
ACACATACAGACCTCAAGCCTGAAAATATT
CTGTTTGTGAATTCAGACTATGAGCTCACC
TACAACCTAGAGAAGAAGCGAGATGAGCGC
AGTGTGAAGAGCACAGCTGTGCGGGTGGTA
GACTTTGGCAGTGCCACCTTTGACCATGAG
CACCATAGCACCATTGTCTCCACTCGCCAT
TACCGAGCACCAGAAGTCATCCTTGAGTTG
GGCTGGTCACAGCCTTGTGATGTGTGGAGT
ATAGGCTGCATCATCTTTGAATACTATGTG
GGATTCACCCTCTTCCAGACCCATGACAAC
AGAGAGCATCTAGCCATGATGGAAAGGATC
TTGGGTCCTATCCCTTCCCGGATGATCCGA
AAGACAAGAAAGCAGAAATATTTTTACCGG
GGTCGCCTGGATTGGGATGAGAACACATCA
GCTGGGCGCTATGTTCGTGAGAACTGCAAA
CCGCTGCGGCGGTATCTGACCTCAGAGGCA
GAGGAACACCACCAGCTCTTCGATCTGATT
GAAAGCATGCTAGAGTATGAACCAGCTAAG
CGGCTGACCTTGGGTGAAGCCCTTCAGCAT
CCTTTCTTCGCCCGCCTTCGGGCTGAGCCG
CCCAACAAGTTGTGGGACTCCAGTCGGGAT
TGACAGTAAAGGTGGATACGGATCCGAA
```

**Final protein sequence (tag sequence in lowercase)**

```
mhhhhhhssgvdlgtenlyfqs^MRSSSHS
```

```
SRRAKSVEDDAEGHLIYHVGDWLQERYEIV
STLGEGTFGRVVQCVDHRRGGARVALKIIK
NVEKYKEAARLEINVLEKINEKDPDNKNLC
VQMFDWFDYHGHMCISFELLGLSTFDFLKD
NNYLPYPIHQVRHMAFQLCQAVKFLHDNKL
THTDLKPENILFVNSDYELTYNLEKKRDER
SVKSTAVRVVDFGSATFDHEHHSTIVSTRH
YRAPEVILELGWSQPCDVWSIGCIIFEYYV
GFTLFQTHDNREHLAMMERILGPIPSRMIR
KTRKQKYFYRGRLDWDENTSAGRYVRENCK
PLRRYLTSEAEEHHQLFDLIESMLEYEPAK
RLTLGEALQHPFFARLRAEPPNKLWDSSRD
^ TEV cleave site
```

**Tags and additions:** mhhhhhhssgvdlgtenlyfq*s(m) TEV-cleavable (*) N-terminal his6 tag.

**Host:** BL21(DE3)-R3-lambda-PPase

**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 TB (+ 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 loopful of cells from the glycerol stock was inoculated into 5-ml of LB medium containing 100 µg/ml kanamycin and 35 µg/ml chloramphenicol and grown overnight at 37°C. Overnight culture was used to inoculate 1 liter of LB containing 50 µg/ml kanamycin & 34 µg/ml chloramphenicol. Culture was grown at 37 °C until the $OD_{600}$ reached ~3 and then cooled to 18°C for 1 hour. IPTG was added to 0.1 mM, and growth continued at 18°C overnight. The cells were collected by centrifugation then pellet re-suspended in 2x lysis buffer and frozen. **Lysis buffer:** 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP, 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution), and 15 units/ml Benzonase. **2x Lysis buffer** contains the same components at double concentration.

**Extraction buffer, extraction method:** Frozen pellets were thawed and fresh 0.5 mM TCEP added to the lysate. Cells were lysed by high pressure homogenization (20 kpsi). The lysate was centrifuged at 16,500 rpm for 60 minutes and the supernatant collected for purification.

**Column 1:** Ni-affinity, HisTrap Crude FF, 5 ml (GE Healthcare).

**Column 1 Buffers:**
**Affinity buffer:** 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% Glycerol, 0.5 mM TCEP.
**Wash buffer:** 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 30 mM imidazole, 5% Glycerol, 0.5 mM TCEP
**Elution buffer:** 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 300 mM imidazole, 5% Glycerol, 0.5 mM TCEP.

**Column 1 Procedure:** The cell extract was loaded on the column at 4 ml/minute on an ÄKTA-express system (GE Healthcare). The column was washed with 10 volumes of lysis buffer, 10 volumes of wash buffer, and then eluted with elution buffer at 4 ml/min. The eluted peak of A280 was automatically collected.

**Column 2:** Gel filtration, Hiload 16/60 Superdex S75 prep grade, 120 ml (GE Healthcare)

**Column 2 Buffers:** 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP.

**Column 2 Procedure:** The eluted fractions from the Ni-affinity Histrap column were loaded on the gel filtration column in GF buffer at 1.2 ml/min. Eluted proteins were collected in 2-ml fractions and analyzed on SDS-PAGE.

**TEV protease digestion:** Peak fractions containing CLK2 were pooled and TEV protease was added at a molar ratio of 1:30. The digestion was left overnight at 4 °C. His-TEV and contaminating proteins were removed by passing to 300µl Ni resin pre-equilibrated in GF buffer. The flow through containing TEV-cleaved protein was collected, concentrated to 30 mg/ml using a centricon centrifugal device with a 30kDa MWCO, frozen at -80°C in 500µl aliquots.

**Mass spectrometry characterization:** Measured: 43319; Expected: 43318

**Protein concentration:** 2.5 mg of CLK2 was diluted into 15 ml of low salt buffer (5mM HEPES. pH 7.5, 200 mM NaCl, 1% glycerol). 2 molar excess of ligand was added to diluted CLK2 and protein-ligand complex was concentrated to 4 mg/ml using an Amicon 30 kDa cut-off concentrator.

**Crystallisation:** Prior to crystallization, pure protein at 4 mg/ml was incubated with 1mM K00750 compound. Optimized condition contained 25% MPD and 0.1M Bicine, pH 9.3. Viable crystals were obtained at 4 degree in a sitting drop mixing protein and reservoir solution at 2:1 volume ratio.

**Data collection:**
**Resolution:** The crystals were cryo-protected with the mother liquor containing 15% ethylene glycol and flash frozen in liquid nitrogen. Diffraction data were collected at Diamond Beamline I24 using monochromatic radiation at wavelength 0.9790 Å .
**Phasing**: Structure of CLK2 was solved by molecular replacement method using the human CLK3 model as a search model.