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### Title

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## Detecting KaiC phosphorylation rhythms of the cyanobacterial circadian oscillator *in vitro* and *in vivo*

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### Abstract

The central oscillator of the cyanobacterial circadian clock is unique in the biochemical simplicity of its components and the robustness of the oscillation. The oscillator is composed of three cyanobacterial proteins, KaiA, KaiB, and KaiC. If very pure preparations of these three proteins are mixed in a test tube in the right proportions and with ATP and MgCl<sub>2</sub>, the phosphorylation states of KaiC will oscillate with a circadian period and these states can be analyzed simply by SDS-PAGE. The purity of the proteins is critical for obtaining robust oscillation. Contaminating proteases will destroy oscillation by degradation of Kai proteins, and ATPases will attenuate robustness by consumption of ATP. Here, we provide a detailed protocol to obtain pure recombinant proteins from *Escherichia coli* to construct a robust cyanobacterial circadian oscillator *in vitro*. In addition, we present a protocol that facilitates analysis of phosphorylation states of KaiC and other phosphorylated proteins from *in vivo* samples.

### 1. Theory

Circadian clocks are complicated biochemical mechanisms that temporally regulate biological processes and the expression of many genes. Many circadian clock components have been discovered in an array of organisms by *in vivo* experiments. Because of the complexity of circadian systems in eukaryotic organisms, the relatively simple prokaryotic clock of cyanobacteria has been developed as a paradigm for circadian biology, and its molecular mechanism can be studied in exquisite detail. The three-dimensional structures of the key protein components of the central oscillator, KaiA (Ye, Vakonakis, Ioerger, LiWang, & Sacchettini, 2004), KaiB (Villarreal, Pattanayek, Williams, Mori, Qin, Johnson, 2013), and KaiC (Pattanayek, Wang, Mori, Xu, Johnson, & Egli, 2004), have been solved at high resolution. Importantly, the 24-hour rhythm of KaiC phosphorylation can be reconstituted by mixing purified KaiA, KaiB, and KaiC with adenosine triphosphate (ATP) *in vitro* (Nakajima, Imai, Ito, Nishiwaki, Murayama, Iwasaki, 2005).

Before the *in vitro* oscillator was developed, the expected model for circadian oscillation in cyanobacteria was that of a transcription-translation feedback loop mechanism, as is

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typically observed in eukaryotic systems. Takao Kondo and co-workers (Nagoya University) then discovered that the oscillation of KaiC phosphorylation continues in cyanobacteria even when translation or transcription is blocked (Tomita, Nakajima, Kondo, & Iwasaki, 2005); this finding led them to reconstitute the oscillator *in vitro*. Rhythms of KaiC phosphorylation are readily detected, both *in vivo* and *in vitro*, via SDS-PAGE analysis. Use of the *in vitro* oscillator allows researchers to identify specific steps in the biochemical mechanism of the circadian clock at the molecular level.

The purification of the three oscillator proteins is a crucial technique to construct a successful *in vitro* oscillator. Tiny amounts of impurities, such as proteases and ATPases, will abolish the oscillatory phosphorylation of KaiC. By applying protein-specific purification methods, those three proteins can be purified with sufficiently high purity and yield.

## 2. Equipment

Temperature-controlled shaking incubator

Refrigerated centrifuge

French press

Fast protein liquid chromatography apparatus (FPLC)

Nickel (Ni) affinity column (such as 5 ml HiTrap Chelating HP prepacked column from GE Healthcare)

Glutathione-S-transferase (GST) affinity column (such as 5 ml GSTrap HP prepacked column from GE Healthcare)

Anion exchange column (such as 5 ml HiTrap Q HP prepacked column from GE Healthcare)

Desalting column (such as HiPrap 26/10 desalting column from GE Healthcare)

Membrane protein concentrator (such as an Amicon centrifugal filter, 10kDa and 30kDa pore sizes)

Visible spectrophotometer

Sample changer

Heat block

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system (such as Mini-PROTEAN from BioRad)

Gel documentation system

### 3. Materials

Sodium chloride (NaCl)

Magnesium chloride (MgCl<sub>2</sub>)

Hydrogen chloride (HCl)

Sodium hydroxide (NaOH)

Ethylenediaminetetraacetic acid (EDTA)

Adenosine triphosphate (ATP)

Dithiothreitol (DTT)

Imidazole

PreScission™ protease (from GE Healthcare)

Ubiquitin-like-specific protease 1 (Ulp1)

Bradford reagent

Tris

Glycine

Sodium dodecyl sulfate (SDS)

Ammonium persulfate (APS)

Acrylamide (such as 30% acrylamide:bis solution, 29:1 from BioRad)

Bromophenol blue

Glycerol

Guanidinium chloride

Kanamycin

Isopropyl β-D-thiogalactopyranoside (IPTG)

Luria-Bertani broth (LB)

*Escherichia coli* (BL21DE3)

InstantBlue™ coomassie stain (From Expedeon)

#### 3.1. Solutions & buffers

Ni buffer A

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.0	20 mM	1 M	10 ml
NaCl	500 mM	5 M	50 ml
Imidazole	500 mM	5 M	50 ml

Add water up to 500 ml final volume and pass through a 0.45- $\mu$ m filter

#### Ni buffer B

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.0	20 mM	1 M	10 ml
NaCl	500 mM	5 M	50 ml
Imidazole	5 mM	5 M	5 ml

Add water up to final volume 500 ml and pass through a 0.45- $\mu$ m filter

#### Desalting buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.0	20 mM	1 M	10 ml
NaCl	150 mM	5 M	15 ml

Add water up to final volume 500 ml and pass through a 0.45- $\mu$ m filter

#### Anion exchange buffer A

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.0	20 mM	1 M	10 ml
NaCl	20 mM	5 M	2 ml

Add water up to final volume 500 ml and pass through a 0.45- $\mu$ m filter

#### Anion exchange buffer B

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.0	20 mM	1 M	10 ml
NaCl	1 M	5 M	100 ml

Add water up to final volume 500 ml and pass through a 0.45- $\mu$ m filter

*In vitro* reaction buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.0	20 mM	1 M	5 ml
NaCl	150 mM	5 M	7.5 ml
MgCl <sub>2</sub>	5 mM	1 M	1.25 ml
EDTA	0.5 mM	500 mM	0.25 ml
ATP	1 mM	10 mM	25 ml

Add water up to final volume 250 ml and pass through a 0.45- $\mu$ m filter

## GST buffer A

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.3	50 mM	1 M	10 ml
NaCl	150 mM	5 M	6 ml
MgCl <sub>2</sub>	5 mM	1 M	1ml
EDTA	1 mM	500 mM	0.4 ml
ATP	5 mM	10 mM	100 ml

Add water up to final volume 200 ml and pass through a 0.45- $\mu$ m filter

## GST buffer B

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.3	50 mM	1 M	2.5 ml
NaCl	150 mM	5 M	1.5 ml
MgCl <sub>2</sub>	5 mM	1 M	0.25ml
EDTA	1 mM	500 mM	0.1 ml
ATP	1 mM	10 mM	5 ml
Glutathione	6 mM	100 mM	3 ml

Add water up to final volume 50 ml and pass through a 0.45- $\mu$ m filter

## GST buffer C

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.3	50 mM	1 M	10 ml
NaCl	150 mM	5 M	6 ml
MgCl <sub>2</sub>	5 mM	1 M	1 ml
EDTA	1 mM	500 mM	0.4 ml

Component	Final concentration	Stock	Amount
ATP	1 mM	10 mM	20 ml

Add water up to final volume 200 ml and pass through a 0.45- $\mu$ m filter

10 $\times$  loading dye

Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.5	100 mM	1 M	1 ml
DTT	200 mM	1 M	2 ml
SDS	4 %	40 %	1 ml
Bromophenol Blue	0.2 %	2 %	1 ml
Glycerol	30%	60%	5 ml

Add water to final volume 10 ml

Tris-Glycine gel buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.5	25 mM	1 M	25 ml
Glycine	200 mM	2 M	100 ml
SDS	1%	40%	25 ml

Add water to final volume 1 L

Acrylamide running gel

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.8	400 mM	1.5 M	8 ml
Acrylamide	6.5 %	30 %	6.5 ml
SDS	0.1 %	40 %	0.075 ml
APS	0.1 %	10 %	0.3 ml
TEMED	0.03 %	100 %	0.009 ml

Add water to final volume 30 ml and pass through a 0.45- $\mu$ m filter

Acrylamide stacking gel

Component	Final concentration	Stock	Amount
Tris-HCl, pH 6.8	60 mM	500 mM	1.8 ml

Component	Final concentration	Stock	Amount
Acrylamide	5%	30%	2.5 ml
SDS	0.1 %	40 %	0.038 ml
APS	0.05 %	10 %	0.075 ml
TEMED	0.1 %	100 %	0.015 ml

Add water to final volume 15 ml and pass through a 0.45- $\mu$ m filter

## 4-11. Protocol

### 4.1. Duration

Preparation	3-5 days
Protocol	7-8 days

### 4.2. Preparation

Kai protein overexpression plasmids were prepared with some modification of pET28a(+) and pET41a(+) vectors. For KaiA and KaiB, sequences that encode SUMO-KaiA or SUMO-KaiB were inserted in between NdeI and HindIII restriction sites on pET28a(+). For KaiC, the PreScission<sup>TM</sup> protease cutting sequence (LEVLFEQGP) was encoded before the start codon of KaiC and inserted in between the NcoI and HindIII sites on pET41a(+). Each plasmid was confirmed by sequencing and introduced into *E. coli* (BL21DE3) for protein overexpression.

## 5. Step 1 Express KaiA or KaiB in *E. coli*

### 5.1 Overview

The overexpressed KaiA and KaiB will be prepared for further purification.

### 5.2. Duration

12 h

1.1 Inoculate 1L LB (containing the appropriate antibiotic) with transformed *E. coli* (BL21DE3) and grow the culture with vigorous shaking (~ 250 RPM) at 37°C until the absorbance reaches  $A_{600\text{nm}} = 0.7$ .

1.2 Add 1 ml of 1 M IPTG to induce overexpression of recombinant KaiA or KaiB and incubate an additional 6 h under the same conditions.

1.3 Harvest the cells by centrifugation for 10 min in a refrigerated centrifuge at  $5,500 \times g$

1.4 Remove the supernatant fraction and store the pellet at  $-80^{\circ}\text{C}$ .

### 5.3. Tip

Add 3-5 drops of Y-30 (Sigma) anti-foam to help aeration by preventing foam.



## 6. Step 2 Express KaiC in *E. coli*.

### 6.1 Overview

KaiC requires slower induction than KaiA or KaiB to obtain abundant folded, soluble protein. The overexpressed KaiC will be prepared for further purification.

### 6.2. Duration

24 h

- 2.1 Inoculate 1L LB (containing the appropriate antibiotic) with transformed *E. coli* (BL21DE3) and grow the culture at 37°C until the absorbance reaches  $A_{600\text{nm}} = 0.6$ .
- 2.2 Cool the culture down to 25°C.
- 2.3 Add 1 ml of 100 mM IPTG to induce overexpression of recombinant KaiC and incubate the culture with shaking (~ 150 RPM) at 25°C for an additional 16 h.
- 2.4 Harvest the cells by centrifugation for 10 min in a refrigerated centrifuge at  $5,500 \times g$
- 2.5 Remove the supernatant fraction and store the pellet at -80°C.

### 6.3. Tip

Room temperature incubation (~23°C) can be used if temperature control is unavailable. Cool down completely before inducing with IPTG. Incomplete cooling results in a lower yield.

## 7. Step 3 Purification of KaiA or KaiB

### 7.1 Overview

Purify KaiA and KaiB with high purity and yield by using a series of FPLC columns.

### 7.2. Duration

36 h

- 3.1 Resuspend KaiA or KaiB pellets (saved in Step 1.4) in 60 ml of Ni buffer A.
- 3.2 Pass the resuspended cells twice through a chilled French press cell at a rate that maintains 16,000 PSI.
- 3.3 Spin the lysates for 60 min in a refrigerated centrifuge at  $20,000 \times g$ .
- 3.4 Filter the supernatant fraction through a 0.45- $\mu\text{m}$  filter.
- 3.5 Inject the filtered supernatant into a Ni affinity column using FPLC with a 2 ml/min flow rate.
- 3.6 Wash the column with 25 mL Ni buffer A.
- 3.7 Elute His-tagged KaiA or KaiB with a 0 – 80% Ni buffer B gradient over 30 min.

- 3.8 Identify the protein-positive fractions from step 3.7 by Absorbance at 280 nm and combine positive fractions. Run the combined protein sample through a desalting column using Desalting buffer with 4 ml/min flow rate.
- 3.9 Identify the protein-positive fractions from step 3.8 by Absorbance at 280 nm and combine positive fractions. Inject the combined protein sample into an anion exchange column using FPLC with a 2 ml/min flow rate.
- 3.10 Wash the column with 25 ml Anion exchange buffer A.
- 3.11 Elute His-tagged KaiA or KaiB with a 0 – 80% Anion exchange buffer B gradient over 30 min.
- 3.12 Identify the protein-positive fractions from step 3.11 by Absorbance at 280 nm and combine positive fractions. Add 1 ml of 1 M NaCl and 0.1 ml of 100  $\mu$ M Ulp1 per each 10 ml of combined protein-positive sample.
- 3.13 Incubate the mixture for 16 h at 4°C.
- 3.14 Inject the incubated mixture into a Ni affinity column using FPLC with a 2 ml/min flow rate and collect the flow-through. This step will retain the tags on the column and allow untagged KaiA and KaiB to pass through.
- 3.15 Wash the column with 5 ml Ni buffer A and continue to collect the flow-through.
- 3.16 Inject the flow-through into a desalting column and wash out continuously using Anion exchange buffer A with a 4 ml/min flow rate until all protein-positive fractions are collected. Identify the protein-positive fractions by Absorbance at 280 nm and combine positive fractions.
- 3.17 Inject the combined positive fraction into an anion exchange column using FPLC with 2 ml/min flow rate.
- 3.18 Wash the column with 25 ml Anion exchange buffer A.
- 3.19 Elute KaiA or KaiB with a 0 – 80% Anion exchange buffer B gradient for 30 min.
- 3.20 Identify the protein-positive fractions from step 3.19 by Absorbance at 280 nm and combine positive fractions. Inject the combined fraction into a desalting column and wash out continuously using *In vitro* reaction buffer with a 4 ml/min flow rate until all protein-positive fractions are collected.
- 3.21 Concentrate the combined protein-positive fraction from step 3.20 using a membrane protein concentrator (such as an Amicon 10kDa centrifugal filter). KaiA and KaiB can be concentrated up to 150  $\mu$ M and 50  $\mu$ M, respectively.
- 3.22 Pass the concentrated protein through a 0.25  $\mu$ m syringe filter.
- 3.23 Measure the concentration by Bradford assay.
- 3.24 Store protein at –80°C.

### 7.3. Tip

The purification procedure should be performed at room temperature to obtain high purity.

#### 7.4. Tip

Dialysis can be used instead of a desalting column.

#### 7.5. Tip

The second affinity column (Step 3.14 - 3.15) also helps to remove impurities and is essential for obtaining the purity needed for the *in vitro* oscillator; this step should not be skipped.

### 8. Step 4 Purification of KaiC

#### 8.1 Overview

Purify KaiC with high purity and yield by using a series of FPLC columns.

#### 8.2. Duration

36 h

- 4.1 Resuspend KaiC pellets (saved in Step 2.5) in 60 ml of GST buffer A.
- 4.2 Follow Steps 3.2 to 3.4 as for KaiA and KaiB purification.
- 4.3 Inject the filtered supernatant into a GST affinity column using FPLC with a 1 ml/min flow rate.
- 4.4 Wash the column with 90 ml GST buffer A.
- 4.5 Elute GST-tagged KaiC with 12 ml GST buffer B and collect the entire elution volume.
- 4.6 Add 10  $\mu$ l (20 U) of PreScission™ protease per each 12  $\mu$ l of the collected eluent.
- 4.7 Incubate the KaiC mixture for 16 h at 4°C.
- 4.8 Inject the incubated KaiC mixture into a desalting column and wash out using GST buffer C with a 4 ml/min flow rate until all protein-positive fractions are collected.
- 4.9 Inject the protein-positive fractions into a GST affinity column with a 1 ml/min flow rate and collect the flow-through. This step will retain the tag on the column and allow untagged KaiC to pass through.
- 4.10 Wash the GST affinity column with 5 ml GST buffer C at a rate of 1 ml/min and continue to collect the flow-through. Combine the flow-through from Steps 4.9 and 4.10.
- 4.11 Inject the combined flow-through from the GST affinity column into a desalting column and wash out using *In vitro* reaction buffer with a 4 ml/min flow rate until all protein-positive fractions are collected.
- 4.12 Concentrate the combined protein-positive fraction from step 4.11 using a membrane protein concentrator (such as an Amicon 30 kDa centrifugal filter). KaiC can be concentrated to ~ 15  $\mu$ M.
- 4.13 Follow from Step 3.22 to 3.24 as for KaiA and KaiB purification.

### 8.3. Tip

The purification procedure should be performed at room temperature to obtain high purity.

### 8.4. Tip

Using a desalting column instead of dialysis may increase yield by preventing precipitation.

## 9. Step 5 *In vitro* oscillation reaction

### 9.1 Overview

The phosphorylation states of KaiC can be observed to oscillate with a 24-hour period by mixing KaiA, KaiB, KaiC, and ATP at 30°C.

### 9.2. Duration

76 h

5.1 Mix 1.2  $\mu\text{M}$  KaiA, 3.5  $\mu\text{M}$  KaiB, 3.5  $\mu\text{M}$  KaiC, and Kanamycin (10  $\mu\text{g}/\text{ml}$  final concentration) in *In vitro* reaction buffer.

5.2 Incubate the reaction mixture at 30°C.

5.3 Take 20  $\mu\text{l}$  aliquots of reaction mixture every 2 h for 3 days and add 2  $\mu\text{l}$  10 $\times$  loading dye. Each SDS-PAGE gel will require at least 5  $\mu\text{l}$  of sample, so 20  $\mu\text{l}$  aliquots allow for multiple gels to be run for each experiment.

5.4 Freeze each sample at  $-20^{\circ}\text{C}$  as it is collected until the reaction is complete.

5.5 Keep all samples at  $-20^{\circ}\text{C}$  prior to SDS-PAGE analysis.

### 9.3. Tip

The total reaction volume can be modified to provide the number of samples desired in Step 5.3 to run at least one gel of the timecourse.

## 10. Step 6 SDS-PAGE

### 10.1 Overview

SDS-PAGE (6.5 % polyacrylamide gel) is able to separate phosphorylated and unphosphorylated forms of KaiC.

### 10.2. Duration

2 h

6.1 Prepare eight 15-well polyacrylamide gels with Acrylamide running gel and Acrylamide stacking gel.

6.2 Mount the gel in the apparatus, place the entire apparatus into a water-tight high-sided tray, and fill the reservoir with Tris-Glycine buffer. Remove all bubbles at the bottom of the gel.

6.3 Take out comb and remove all bubbles in the wells. Load the samples (saved in Step 5.4) onto the gel.

6.4 Pack ice around the outside of the SDS-PAGE apparatus.

6.5 Run the gel at 60 V for 30 min and at 140 V for 100 min.

6.6 Put the gel in InstantBlue™ coomassie stain solution and shake gently on a platform shaker overnight.

6.7 Destain with water until bands are clearly visible.

### 10.3. Tip

Load 4 to 5 µl of sample in each of the 13 interior wells. Gel “smiling” can be avoided by loading dye only, rather than sample, in the outside wells on either end. The resulting straight line of bands will facilitate quantification.

### 10.4. Tip

The number of gels on Step 6.1 and the time on Step 6.5 calculated based on Mini-PROTEAN from BioRad.

## 11. Step 7 Densitometry

### 11.1 Overview

This is an easy quantification method for determining the phosphorylation level of KaiC in order to graph the oscillation.

### 11.2. Duration

1 h

7.1 Capture a high-resolution image of the stained gel.

7.2 Measure the density of each band with National Institutes of Health (NIH) ImageJ.

7.3 Refer to Figure 2 to identify KaiC phosphoforms. Combine values for bands that represent phosphoforms and calculate % phosphorylated KaiC (P-KaiC).

7.4 Draw a graph that plots % P-KaiC vs. time.

### 11.3. Tip

Follow ImageJ user guide on NIH website (<http://imagej.nih.gov/ij/docs/guide/index.html>) to measure the density of each band.

### 11.4. Tip

PeakFit software can be used instead of NIH ImageJ.

## 12-17. Detection of protein phosphorylation forms from *in vivo* cell extracts

Separation of the phosphoforms of KaiC, which is phosphorylated on Ser/Thr residues, from total soluble cell protein extracts can be facilitated by use of Phos-tag™ reagent in a

modified immunoblotting protocol. Phos-tag™ SDS-PAGE allows separation of phospho-states by reagent binding to phosphate groups and retardation of phosphorylated proteins during electrophoresis. This technique can also be applied to phosphorylated histidine kinases and response regulators, for which the inherent lability of phospho-His and phospho-Asp and the lack of phospho-specific antibodies to these residues has hindered *in vivo* detection of the phosphorylated states (Barbieri & Stock, 2008; Moronta-Barrios, Espinosa, & Contreras, 2012). The requirement for specific antibodies can be circumvented by the addition of epitope tags, although this approach has not yet been reported in Phos-tag™ assays. Phos-tag™ acrylamide separations require optimization of gel composition for each protein studied. Ranges of 25 to 50 μM of Phos-tag™ acrylamide reagent and 50 μM Mn<sup>2+</sup> are good starting references, but optimal concentrations must be determined empirically to obtain sharp banding. However, even with substantially distorted bands, if sufficient separation of unphosphorylated and phosphorylated protein bands is achieved, quantification of the fraction of phosphorylated proteins can be accurately estimated using image analysis software (Gutu & O'Shea, 2013).

The following protocol has been used to analyze the *in vivo* phosphorylation of KaiC and RpaA in *S. elongatus* cells subjected to 12:12 LD cycles (Fig. 3). It should form a suitable starting protocol that can be optimized for the protein under study.

### 13. Equipment

- Temperature-controlled lighted incubator
- Refrigerated centrifuge
- Cell homogenizer or benchtop vortexer
- Rotary shaker
- Power supply
- Electrophoretic apparatus for SDS-PAGE
- Protein blotting (Wet transfer) apparatus
- Standard blotting accessories (filter paper, blotting membrane)
- Imaging equipment and software

### 14. Materials

- 29:1 acrylamide: N, N'-methylene-bis-acrylamide
- deionized distilled water
- 1.5 M Tris-Cl pH 8.8
- 0.5 M Tris-Cl pH 6.8

sodium dodecyl sulfate (SDS)

100% isopropanol or saturated butanol

ammonium persulfate (APS)

TEMED

Phos-tag™ acrylamide AAL-007 (Wako Chemicals)

10 mM MnCl<sub>2</sub>

DTT

bromphenol blue

glass beads (100-300 μm diameter)

Tris

Glycine

Methanol

Bradford reagent

EDTA (0.5 M)

Antibodies to proteins of interest

Secondary antibodies (for example horseradish peroxidase, HRP-linked) and chemiluminescent substrate for detection

#### 14.1. Solutions and Buffers

TBS-T:

100 mL 10X Tris-buffered saline (TBS)

1 mL Tween-20

water to 1 L

Lysis Buffer:

25 mM Tris pH 8.0

0.5 mM EDTA

1 mM DTT and bacterial protease inhibitor cocktail (Sigma)

Transfer Buffer:

2.2 g CAPS

8.33  $\mu$ L 10N NaOH

100 mL methanol deionized water to 1 L

## 15. Protocol

### 15.1 Duration

Preparation: 3-5 days, depending on duration of sampling

Protocol: 2 days

## 16. Step 1 Preparation

### 16.1 Overview

Cast Phos-tag<sup>TM</sup> acrylamide gels, entrain cyanobacterial strains and collect samples

1.1. Phos-tag<sup>TM</sup> acrylamide SDS mini gels are prepared using a discontinuous buffer system (refer to materials section 3.1 for gel preparation, increasing resolving gel acrylamide concentration to 10% w/v). The resolving gel consists of 10% (w/v) 29:1 acrylamide: N, N'-methylene-bis-acrylamide containing Tris-HCl pH 8.8, 0.1% SDS, supplemented with 25  $\mu$ M of Phos-tag<sup>TM</sup> acrylamide reagent and 50  $\mu$ M Mn<sup>2+</sup>. Overlay the gel with a 2 mm layer of saturated butanol, or layer with isopropanol to exclude oxygen and ensure a flat interface between the resolving and stacking gels. After polymerization, rinse the isopropanol or butanol from the top of the gel with water, and drain the water by inverting the gel. Prepare the stacking gel using 4% (w/v) acrylamide:bis-acrylamide, Tris-HCl pH 6.8 and 0.1% SDS. Pre-chill acrylamide gels at 4°C prior to electrophoresis.

1.2. Entrain *S. elongatus* cells for two 12:12 LD cycles, move to constant light, and subsequently sample at chosen time points.

1.3. At each time point, harvest 10-15 mL of cells (grown to an optical density of  $A_{750}=0.1-0.75$ ) by centrifugation at  $6000 \times g$  for 5 min at 4°C. Carefully remove all residual growth medium from cell pellet. Quickly freeze each cell pellet and store at -80°C until ready to prepare lysates.

1.4. Resuspend the cell pellet in 30-80  $\mu$ L of lysis buffer (25 mM Tris pH 8.0, 0.5 mM EDTA, 1 mM DTT and bacterial protease inhibitor cocktail (Sigma)). Add glass beads (0.1-0.3 micron diameter) equivalent to one-third of the volume. Cell lysis is achieved with 2 cycles of 1 min in a homogenizer (BeadBeaterBioSpec) with 1 min cooling between cycles. Alternatively, cells may be lysed using a benchtop vortexer at maximum setting with ten cycles alternating 30 seconds vortexing/30 seconds cooling on ice.

1.5. Centrifuge cell extract at  $10\,000 \times g$  for 5 min at 4°C to pellet unbroken cells, cellular debris and beads. Transfer the blue-green supernatant to a new tube and use an



aliquot to quantify protein concentration using a Bradford assay. Samples may be used immediately for gel loading, or stored at  $-20^{\circ}\text{C}$ .

### 16.1 Tip

For proteins with heat-labile phospho-Asp or phospho-His residues, keep samples on ice after extraction and do not heat prior to gel loading. To obtain optimal levels of phosphorylated protein, proceed directly to electrophoresis and do not subject cell extracts to freeze-thaw cycles.

### 16.2 Tip

Samples must be prepared in buffer without phosphate. Do not substitute phosphate-buffered saline (PBS) for TBS during resuspension of cell pellets.

## 17. Step 2 Electrophoresis and Blotting

### 17.1 Overview

Load and run Phos-tag<sup>TM</sup> acrylamide gels and detect proteins by immunoblotting

- 2.1. Load 2-5  $\mu\text{g}$  of protein extract in SDS sample buffer per lane (for KaiC detection) and run at 90V  $4^{\circ}\text{C}$  for approximately 3 hours in a Tris/Glycine/SDS buffer.
- 2.2 Incubate gels twice for 5 min at RT in transfer buffer solution (Tris/Glycine/Methanol supplemented with 1 mM EDTA), then twice more in standard transfer solution without EDTA.
- 2.3. Transfer proteins from gel to PVDF membrane by wet electroblotting (tank transfer). Transfer at  $4^{\circ}\text{C}$  and 100V for at least 3 hours is recommended.
- 2.4. Block the membrane with 2.5% nonfat dry milk (NFDm)/Tris-buffered saline (TBS) + 0.1% Tween-20 for 1 hour at RT and then incubate with Anti-KaiC (1:2 000) overnight at  $4^{\circ}\text{C}$  on a rotary shaker. Then, rinse the membrane twice with TBS and incubate with HRP-linked secondary antibody (1: 10 000) in NFDm/TBS-T for 1 hour at RT.
- 2.5 Develop blot using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) according to the manufacturer's directions. For examples shown in Figure 3, chemiluminescence was detected using an Alpha Innotech FluorChem<sup>®</sup> HD2 Imaging System (Alpha Innotech, San Leandro, CA) (cf. Fig. 3B).
- 2.6. Perform image analysis to quantify band intensity using NIH ImageJ software. (cf. Fig. 3 B).

### 17.2 Tip

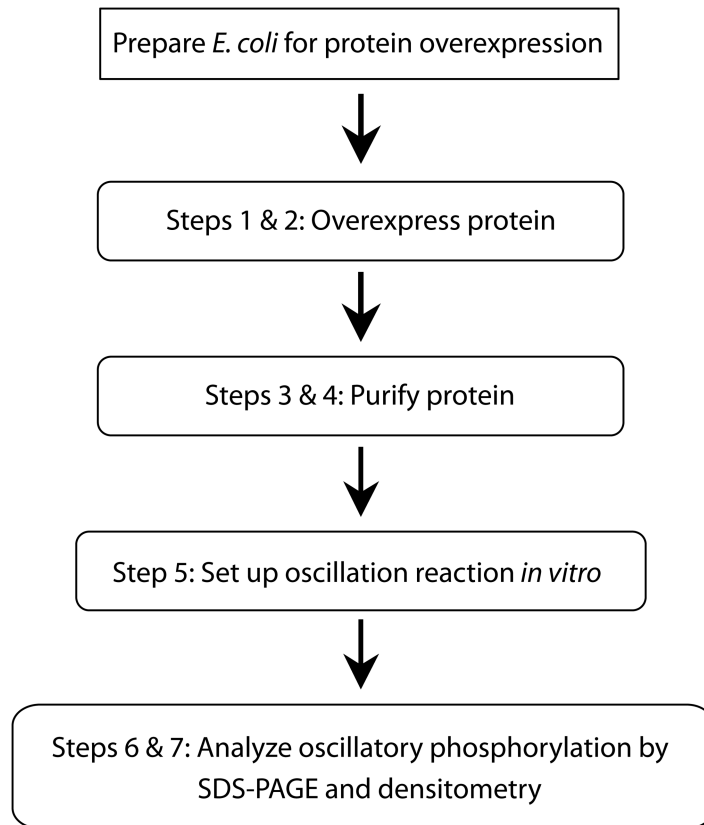
Resolution of multiple KaiC phospho-states may be obtained by running gels at lower current for an extended time and/or increasing concentration of Phos-tag<sup>TM</sup> acrylamide reagent.

## Acknowledgments

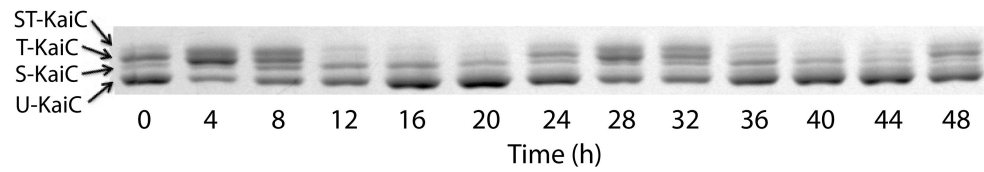
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## References

- Barbieri CM, Stock AM. Universally applicable methods for monitoring response regulator aspartate phosphorylation both in vitro and in vivo using Phos-tag-based reagents. *Analytical Biochemistry*. 2008; 376:73–82. [PubMed: 18328252]
- Gutu A, O'Shea E. Two Antagonistic Clock-Regulated Histidine Kinases Time the Activation of Circadian Gene Expression. *Molecular Cell*. 2013; 50:288–294. [PubMed: 23541768]
- Moronta-Barrios F, Espinosa J, Contreras A. In vivo features of signal transduction by the essential response regulator RpaB from *Synechococcus elongatus* PCC 7942. *Microbiology*. 2012; 158:1229–1237. [PubMed: 22322959]
- Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, et al. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science*. 2005; 308:414–415. [PubMed: 15831759]
- Pattanayek R, Wang J, Mori T, Xu Y, Johnson CH, Egli M. Visualizing a circadian clock protein: crystal structure of KaiC and functional insights. *Molecular Cell*. 2004; 15:375–388. [PubMed: 15304218]
- Tomita J, Nakajima M, Kondo T, Iwasaki H. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science*. 2005; 307:251–254. [PubMed: 15550625]
- Villarreal SA, Pattanayek R, Williams DR, Mori T, Qin X, Johnson CH, et al. CryoEM and Molecular Dynamics of the Circadian KaiB<sub>2</sub>KaiC Complex Indicates That KaiB Monomers Interact with KaiC and Block ATP Binding Clefs. *Journal of Molecular Biology*. 2013; 425:3311–3324. [PubMed: 23796516]
- Ye S, Vakonakis I, Ioerger TR, LiWang AC, Sacchettini JC. Crystal structure of circadian clock protein KaiA from *Synechococcus elongatus*. *Journal of Biological Chemistry*. 2004; 279:20511–20518.

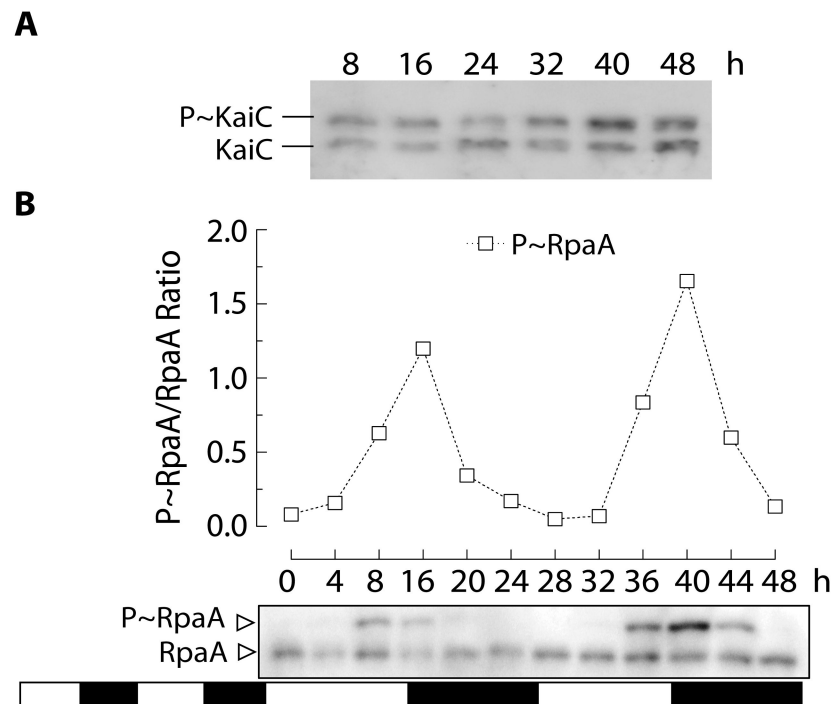


**Figure 1.**  
*In vitro* oscillation flowchart:



**Figure 2.**

Coomassie-stained SDS-PAGE image of KaiC phosphorylation cycles. The samples were taken every 4 h for 2 days. Four phosphoforms of KaiC can be separated with the method introduced here. ST-KaiC, S431 and T432 KaiC double phosphoform; T-KaiC, T432 KaiC phosphoform; S-KaiC, S431 KaiC phosphoform; and U-KaiC, unphosphorylated KaiC.



**Figure 3.** Detection of protein phosphorylation using Phos-tag acrylamide from whole-cell soluble protein extracts A) Immunoblot showing separation of phosphorylated and unphosphorylated KaiC after Phos-tag<sup>TM</sup> SDS-PAGE. Protein samples were collected every eight hours in constant light after entrainment to two 12-h light:dark (LD) cycles. B) Quantification of P~RpaA fractions from immunoblot. Specific detection of P~RpaA and RpaA with anti-RpaA polyclonal antibodies, respectively, is shown. The white and black boxes represent, respectively, light and dark periods. Boxes corresponding to first two LD cycles are not drawn to scale.