In vitro phosphorylation assay

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Abstract

This protocol can be used to analyze phosphate uptake by KaiC proteins over time.

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Guidelines

references:

Wiegard A, Dörrich AK, Deinzer HT, Beck C, Wilde A, Holtzendorff J, Axmann IM: Biochemical analysis of three putative KaiC clock proteins from *Synechocystis* sp. PCC 6803 suggests their functional divergence. *Microbiology 2013*, 159:948-958.

Axmann IM, Dühring U, Seeliger L, Arnold A, Vanselow JT, Kramer A, Wilde A: Biochemical evidence for a timing mechanism in *Prochlorococcus*. J Bacteriol 2009, 191:5342-5347

required chemicals/buffers:

chemical/buffer	comments
[y-32P]ATP (3000 Ci/mmol)	(3000 Ci/mmol), see materials tab
reaction buffer (RB)	depends on the protein of interest, see buffer table below
5 x SDS Gel loading buffer	see buffer table below

buffer compositions:

name	ingredients	reference
5 x SDS Gel loading buffer	250 mM Tris-HCl (pH 6,8) 40 % (v/v) glycerol 8 % (w/v) SDS 100 mM DTT 0.1 % (w/v) bromophenol blue	**Green MR and Sambrook J:** Molecular cloning: a laboratory manual - 4th ed. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2012*
reaction buffer for cyanobacterial KaiC proteins	20 mM Tris/HCl (pH 8) 150 mM NaCl 0.5 mM EDTA 5 mM MgCl2 1 mM ATP	**Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyama T, Kondo T:** Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science 2005*, 308:414-415.
reaction buffer for cyanobacterial KaiC from *Thermococcus litoralis*	50 mM HEPES (pH 7.2) 150 mM NaCl 5 mM MgCl2 1 mM ATP	
reaction buffer for cyanobacterial KaiC from *Pycrococcus horikoshii*	50 mM MES (pH 6) 150 mM NaCl 5 mM MgCl2 1 mM ATP	

required equipment:

Device
Heating block
Equipment for handling radioactive material
Gel dryer
radioactivity scanner (Fujifilm FLA-3000)

Before start

prepare gels for LowC SDS-PAGE

Materials

[y-32P]ATP (3000 Ci/mmol) SRP-301 by Hartmann Analytic GmbH

Protocol

Step 1.

Mix the purified recombinant KaiC protein in reaction buffer with [y-32P]ATP as described in the table below. If you want to determine the influence of KaiA, you will have to prepare samples with and without KaiA. You will need a master mix for all aliquots (timepoints) you want to analyze (e.g. 5) plus 1 additional aliquot (e.g. master mix for 6). Work on ice!

	- Kai A		**+ KaiA**	
	1 timepoint	**for 6 timepoints**	**1 timepoint**	**for 6 timepoints**
KaiC	variable (**2 µg**)	variable (**12 µg**)	variable (**2 µg**)	variable (**12 µg**)
KaiA	-	-	variable (**1 µg**)	variable (**6 µg**)
[y-32P]ATP	1,67 µCi	10 µCi (**1 µl**)	1,67 µCi	10 µCi (**1 µl**)
RB	**ad 10 µl**	**ad 60 µl**	**ad 10 µl**	**ad 60 µl**

Step 2.

Briefly spin down the reaction mixture using a bench-top centrifuge, take a 10 µl aliquot and mix with 2.5 µl 5 X SDS-PAGE buffer to stop the reaction (timepoint zero). Store the aliquot at -20 °C and immediatly proceed with the next step.

Step 3.

Incubate the remaining reaction mixture at the appropriate temperature (e.g. 30 °C for KaiC from *Synechococcus elongatus* PCC 7942).

Step 4.

Take samples at different timepoints (e.g. 0.75, 1.5, 3, 22 hours) as described in step 2.

Step 5.

Separate proteins and stain them with Coomassie brilliant blue as described in Protocol LowC SDS-PAGE.

Step 6.

Dry gels for 2-3 hours under vacuum at 60°C.

Step 7.

Expose gels to an autoradiography screen and analyze signals using the Fujifilm FLA-3000.

Step 8.

Scan dried gels to document Coomassie staining of the proteins.

Warnings

Make sure to be aware of the safety regulations associated with handling radioactive material!