Expression and purification of (GST-tagged) (Kai) proteins

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Abstract

This protocol can be used for:
(i) heterologous expression of GST-tagged proteins from pGEX-6P1 based expression vectors in *E. coli*.
(ii) purification of recombinant proteins via affinity chromatography using glutathione-agarose or glutathione-sepharose (GST tagged protein can be eluted with glutathione. Alternatively, the tag can be cleaved off by prescission protease)
(iii) further purification of the eluted protein via anion exchange chromatography

This protocol was modified from
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
Snijder J, Schuller JM, Wiegard A, Lössel, P, Schmelling NM, Axmann IM, Plitzko JM, Förster F, Heck AJR: Structures of the cyanobacterial circadian oscillator frozen in a fully assembled state. *Science* 2017, 355(6330):1181-1184

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Guidelines

Note that KaiC from *Synechococcus elongatus* PCC 7942 aggregates in the absence of ATP. Add 1 mM ATP and 5 mM $MgCl_2$ to all buffers for KaiC purification.

You can purify your recombinant protein with GST-tag or remove the GST-tag during elution.
If you want to retain the GST-tag, skip steps 17-22.
If you want to cleave off the GST-tag, skip steps 23-27.

Protocol

heterologous protein expression in E.coli

Step 1.

transformation:

- transform *E.coli* expression cells (e.g. *E.coli* BL21) with your pGEX-6P1 based expression plasmid

heterologous protein expression in E.coli

Step 2.

pre-culture:

- inoculate 100 ml terrific broth medium containing 100 µg ampicillin ml^{-1} with resulting transformants
- incubate over night at 37 °C and 200-250 r.p.m.

REAGENTS

Terrific-Broth-Medium X972.2 by Carl Roth

heterologous protein expression in E.coli

Step 3.

expression culture:

- inoculate 1 l terrific broth medium containing 100 µg ampicillin ml^{-1} with the pre-culture
- incubate at 37 °C and 200-250 r.p.m.

Note: use erlenmeyer flasks with a volume of at least 2 l to ensure sufficient aeration.

heterologous protein expression in E.coli

Step 4.

induction of protein expression:

For KaiC proteins:

- grow cells without induction at 37 °C for approx. 72 hours

Note: the promotor is leaky and will allow low protein expression and production of soluble protein, even without induction. Contrary to this, induction of kaiC expression leads to protein aggregation in inclusion bodies.

For KaiA and KaiB proteins:

- grow cells for 3 hours at 37 °C
- add IPTG to a final concentration of 1 mM and
- continue incubation at 37 °C and 200-250 r.p.m. over night

cell harvest

Step 5.

- spin down cells for 10 min at 4°C and 4000g
- discard supernatant and keep cells on ice.

cell disruption

Step 6.

enzymatic lysis by lysozyme:

- resuspend cells in 15 ml ice-cold extraction buffer [50 mM Tris/HCl (pH8), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT *(only for KaiC proteins: 5 mM $MgCl_2$, 1 mM ATP)*] using a paint brush.
- add a spatula tip's worth of lysozyme (or add lysozyme stock solution to a final concentration of 1mg lysozyme ml^{-1})

- add 125 U benzonase
- incubate on ice for 30 min.

READAGENTS

Benzonase 101654 by Merck Millipore

cell disruption

Step 7.

sonication:

- sonicate the cell suspension for 6 min on ice using e.g. a Bandelin sonopuls homogenizer and the following parameters:

tip	KE76
cycle	3 (0.3 sec active cycle, 0.7 sec passive cycle)
output	60 %

cell disruption

Step 8.

clarification of the lysate:

- centrifuge the resulting lysate for 20 min at 4 °C and 23000 g to remove insolubles
- Keep the resulting supernatant (= soluble proteins) on ice

Note: Many conical centrifugation tubes cannot withstand centrifugation of 23000g. If you want to use them, you can reduce centrifugal force, while increasing centrifugation time.

affinity purification

Step 9.

equilibration of glutathione resin:

- swirl stock solution of glutathione agarose 4B or glutathione sepharose 4B gently
- transfer 1 ml in a 50 ml conical centrifugation tube
- Add 5-10 ml extraction buffer
- centrifuge for 4 min at 1500 g and 4 °C using a swing out rotor
- discard supernatant carefully.

READAGENTS

Protino® Glutathione Agarose

4B 745500.10 by Macherey and Nagel

Glutathione sepharose 4B by Ge Life Sciences

affinity purification

Step 10.

protein binding:

- add soluble proteins from step 8 to glutathione resin
- rotate overhead for at least 20 min at RT (use e.g. an intelli mixer and program F1 at 5 r.p.m).

Note: incubation can be extended up to 3 hours.

affinity purification

Step 11.

- spin down glutathione resin for 4 min at 1500 g and 4 °C using a swing out rotor
- discard supernatant carefully.

affinity purification

Step 12.

washing step 1:

- add 45 ml ice cold extraction buffer
- mix thoroughly
- centrifuge for 4 min at 1500 g and 4 °C using a swing out rotor
- discard supernatant carefully.

affinity purification

Step 13.

washing step 2:

- add 45 ml ice cold extraction buffer
- mix thoroughly
- centrifuge for 4 min at 1500 g and 4 °C using a swing out rotor
- discard supernatant carefully.

affinity purification

Step 14.

washing step 3:

- add 45 ml ice cold extraction buffer
- mix thoroughly
- centrifuge for 4 min at 1500 g and 4 °C using a swing out rotor

- discard supernatant carefully.

affinity purification

Step 15.

washing step 4:

- add 45 ml ice cold extraction buffer
- mix thoroughly
- centrifuge for 4 min at 1500 g and 4 °C using a swing out rotor
- discard supernatant carefully.

affinity purification

Step 16.

- use 1 ml ice cold prescission buffer [50 mM Tris/HCl (pH8), 150 mM NaCl, 1 mM EDTA, 1 mM DTT *(only for KaiC proteins: 5 mM $MgCl_2$, 1 mM ATP)*] to transfer pelleted resin to a 2 ml reaction tube
- centrifuge for 4 min at 1500 g and 4 °C
- discard supernatant using a pipette
- repeat this step until all of glutathione resin is transferred to the reaction tube.

Note: if you want to elute the GST-fused protein later (without cleavage by prescission protease), you can use extraction buffer instead of prescission buffer.

option 1: elution of the untagged protein (part of affinity purification)

Step 17.

overnight cleavage:

- mix glutathione resin with 500 µl ice cold prescission buffer
- add 12.5 µl prescission protease
- incubate overnight at 4 °C under constant rotation of the tube (use e.g. an intelli mixer and program F4 at 5 r.p.m).

Note: This section describes elution of the untagged protein via cleavage by prescission protease. If you want to elute the recombinant GST-tagged protein (without removal of the tag), skip these steps and move on to step 23 instead.

REAGENTS

PreScission Protease 27084301 by Ge Life Sciences

option 1: elution of the untagged protein (part of affinity purification)

Step 18.

elution 1:

- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 1).

Note: This section describes elution of the untagged protein via cleavage by prescission protease. If you want to elute the recombinant GST-tagged protein (without removal of the tag), skip these steps and move on to step 23 instead.

option 1: elution of the untagged protein (part of affinity purification)

Step 19.

elution 2:

- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 2).

Note: This section describes elution of the untagged protein via cleavage by prescission protease. If you want to elute the recombinant GST-tagged protein (without removal of the tag), skip these steps and move on to step 23 instead.

option 1: elution of the untagged protein (part of affinity purification)

Step 20.

elution 3:

- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 3).

Note: This section describes elution of the untagged protein via cleavage by prescission protease. If you want to elute the recombinant GST-tagged protein (without removal of the tag), skip these steps and move on to step 24 instead.

option 1: elution of the untagged protein (part of affinity purification)

Step 21.

elution 4:

- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 4).

Note: This section describes elution of the untagged protein via cleavage by prescission protease. If you want to elute the recombinant GST-tagged protein (without removal of the tag), skip these steps and move on to step 23 instead.

option 1: elution of the untagged protein (part of affinity purification)

Step 22.

elution 5:

- spin down glutathione resin for 4 min at 1500 g and 4 °C

- transfer the supernatant to a fresh tube (=eluate 5).

Note: This section describes elution of the untagged protein via cleavage by prescission protease. If you want to elute the recombinant GST-tagged protein (without removal of the tag), skip these steps and move on to step 23 instead.

option 2: elution of the GST-tagged protein (part of affinity purification)

Step 23.

elution 1:

- add 1 ml glutathione solution [40 mM reduced L-glutathione, 50 mM Tris, pH8, *only for KaiC: 5 mM MgCl2, 1 mM ATP (!you have to adjust the pH after dissolving glutathione and Tris!)*]
- incubate for 5 min at RT under constant rotation of the tube (use e.g. an intelli mixer and program F4 at 5 r.p.m)
- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 1).

Note: This section describes the elution of the GST-tagged protein. If you removed the GST-tag by cleavage with prescission (steps 17-22), you have to skip these steps. Please move on to step 28 instead.

option 2: elution of the GST-tagged protein (part of affinity purification)

Step 24.

elution 2:

- Add 500 µl glutathione solution
- incubate for 5 min at RT under constant rotation of the tube (use e.g. an intelli mixer and program F4 at 5 r.p.m)
- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 2).

Note: This section describes the elution of the GST-tagged protein. If you removed the GST-tag by cleavage with prescission (steps 17-22), you have to skip these steps. Please move on to step 28 instead.

option 2: elution of the GST-tagged protein (part of affinity purification)

Step 25.

elution 3:

- add 500 µl glutathione solution and incubate for 5 min at RT under constant rotation of the tube (use e.g. an intelli mixer and program F4 at 5 r.p.m)
- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 3).

Note: This section describes the elution of the GST-tagged protein. If you removed the GST-tag by cleavage with prescission (steps 17-22), you have to skip these steps. Please move on to step 28 instead.

option 2: elution of the GST-tagged protein (part of affinity purification)

Step 26.

elution 4:

- add 500 µl glutathione solution and incubate for 5 min at RT under constant rotation of the tube (use e.g. an intelli mixer and program F4 at 5 r.p.m)
- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 4).

Note: This section describes the elution of the GST-tagged protein. If you removed the GST-tag by cleavage with prescission (steps 17-22), you have to skip these steps. Please move on to step 28 instead.

option 2: elution of the GST-tagged protein (part of affinity purification)

Step 27.

elution 5:

- add 500 µl glutathione solution and incubate for 5 min at RT under constant rotation of the tube (use e.g. an intelli mixer and program F4 at 5 r.p.m)
- spin down glutathione resin for 4 min at 1500 g and 4 °C
- transfer the supernatant to a fresh tube (=eluate 5).

Note: This section describes the elution of the GST-tagged protein. If you removed the GST-tag by cleavage with prescission (steps 17-22), you have to skip these steps. Please move on to step 28 instead.

affinity purification

Step 28.

qualitative analysis of elutate fractions:

- for each fraction, mix 80 µl of Bradford solution with 5-20 µl of your fraction in a well of a 96 well plate
- a Colour change to blue indicates sucessfull elution of proteins
- keep those fractions.

Note: You can further control quality of the protein by separation via SDS-PAGE.

affinity purification

Step 29.

buffer exchange:

- mix all eluate fractions of sufficient protein quality
- transfer them to a disposable centrifugal concentrator
- concentrate protein by centrifugation
- add your desired buffer and concentrate again
- repeat this step until the buffer is completely exchanged

Note: choose the molecular cut-off of the concentrator and centrifugal force according to the manufacturer's instructions.

For Kai proteins you can use the following buffers (depending on the desired application):

KaiA	20 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5 mM EDTA (*optional 5 mM $MgCl_2$, 1mM ATP*)
KaiB	20 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5 mM EDTA (*optional 5 mM $MgCl_2$, 1mM ATP*)
KaiC	20 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1mM ATP

Note: It is important to remove glutathione by sufficient buffer exchange. If you eluted the protein in prescission buffer and want to further purify it via anion exchange chromatography, buffer exchange is not necessary. You can control homogeneity of your protein by separation via SDS-PAGE. If it is sufficient for your application, you can stop the protocol here. If it is not sufficient for your application, you have to further purify your protein via anion exchange chromatography (steps 30-35)

anion exchange chromatography

Step 30.

set-up of your liquid chromatography system:

- connect 10 ml sample loop to your system
- connect a MonoQ column (1 ml) or ResourceQ column (1 ml) to your system
- exchange ethanol in capillaries and sample loop by water
- wash the column with at least 10 column volumes degassed MilliQ (*do not use higher flowrates than 1 min/ml*)
- connect the following buffers as eluant A and eluent B:

eluent	buffer composition
A	50 mM Tris/HCl (pH8), 1 mM EDTA, 1 mM DTT *only for KaiC proteins: 5 mM $MgCl_2$, 1 mM ATP*
B	50 mM Tris/HCl (pH8), 1 M NaCl, 1 mM EDTA, 1 mM DTT *only for KaiC proteins: 5 mM $MgCl_2$, 1 mM ATP*

anion exchange chromatography

Step 31.

equilibration of the column:

- equilibrate the column with at least 10 column volumes buffer A

Note: Do not use higher flowrates than 1 min/ml. It is better to equilibrate at lower flow rates overnight.

anion exchange chromatography

Step 32.

optional:

- if you want to test your set-up you can run the following program without injecting a sample
- monitor absorption at 280 nm

column volume	flowrate	eluent	action	fractions

10	1 ml/min	100 % A	autozero	no
2	1 ml/min	100 % A		no
20	1 ml/min	gradient from 100 % A to 100 % B		no
5	1 ml/min	100 % B		no
5	1 ml/min	100 A		no

Note: If your instrument can measure two wavelengths in parallel, monitor absorption at 280 and 220 nm

anion exchange chromatography

Step 33.

protein purification:

- dilute your protein in 10 ml eluent A and apply it to the sample loop (*injection valve must be set to load position*)
- run the following program
- monitor absorption at 280 nm
- collect fractions of 0.5 ml or 1 ml.

column volume	flowrate	eluent	action	fractions
10	1 ml/min	100 % A	autozero inject position	0.5-1ml
2	1 ml/min	100 % A	load position	0.5-1ml
20	1 ml/min	gradient from 100 % A to 100 % B		0.5-1ml
5	1 ml/min	100 % B		0.5-1ml
5	1 ml/min	100 % A		0.5-1ml

Note: If your instrument can measure two wavelengths in parallel, monitor absorption at 280 and 220 nm

anion exchange chromatography

Step 34.

qualitative analysis of eluate fractions:

- choose fractions of interest based on the absorption at 280 nm
- mix 5-20 µl of each fraction of interest with 80 µl of Bradford solution in a well of a 96 well plate
- a colour change to blue indicates that you successfully eluted proteins
- keep those fractions
- control homogeneity and size of your eluted protein by separation via SDS-PAGE.

anion exchange chromatography

Step 35.

buffer exchange:

- concentrate your protein by centrifugation
- add your desired buffer
- concentrate again
- repeat this step until buffer is completely exchanged

Note: choose the molecular cut-off of the concentrator and centrifugal force according to the manufacturer's instructions.

For Kai proteins you can use the following buffers (depending on your desired application):

KaiA	20 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5 mM EDTA (*optional 5 mM $MgCl_2$, 1mM ATP*)
KaiB	20 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5 mM EDTA (*optional 5 mM $MgCl_2$, 1mM ATP*)
KaiC	20 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1mM ATP