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ATPase activity assay

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ABSTRACT

This protocol can be used to analyse ATPase activity of KaiC proteins. Produced ADP is separated chromatographically using HPLC and monitored via its absorption at 260 nm.

Preparation of running buffer

1
- fill a 1l bottle with 963.4 ml MilliQ
- add 5.8 ml phosphoryc acid (17.2 M stock Nacalai tesque, CAS 121-44-8) [for pH adjustment] --> final concentration = 100 mM
- add 20.8 ml trimethylamine (7.15M stock, Nacalai tesque, CAS 7664-38-2) [counter-ion] --> final concentration = 150 mM
- add 10 ml acetonitrile (99.8 % stock, Sigma Aldrich, CAS 75-05-08) [mobile phase] --> final concentration = 1%
- degas shortly after mixing all samples (0.5-1.5 min) (*Note: longer degassing will lead to evaporation of acetonitrile)*

Preparation of MiliQ, MeOH (and ETOH)

2
- fill a 1l bottle with MiliQ and degas for 10 min
- fill a 1l bottle with 80 % MeOH and degas shortly
- optional: fill a 1l bottle with 20 % EtOH and degas for 10 min

Preparation of the HPLC instrument

3
- provide MilliQ for piston back flushing
- connect pump A, pump B and autosampler to MilliQ
- purge autosampler
- open pump valves, purge and close valves again
- clean all valves with at least 15 ml MilliQ (flowrate: 0.2-0.8 ml/min)

Note: do not exceed the maximal pressure, the column can withstand. If pressure is too high, reduce the flowrate

Equilibration of the column

4
- connect a Shim-Pack-VP-ODS column (SHIMADZU) (flow rate 0.4 ml/min)
- wash column with at least 36 ml MiliQ to remove EtOH or MeOH (0.2-0.4 ml/min)
- connect pump A to running buffer (leave pump and autosampler in MilliQ)
- open pump valves, purge and close valves again
- equilibrate column with at least 36 ml running buffer (0.2-0.8 ml/min)

Note: do not exceed the maximal pressure, the column can withstand. If pressure is too high, reduce the flowrate

buffer validation

5 - to test whether buffer still contains acetonitrile as mobile phase and to control quality of ATP
- mix 1 mM ADP, 1 mM ATP and 5 mM $MgCl_2$in reaction buffer (20 mM Tris/HCl (pH 8), 150 mM NaCl)

component	volume [µl]
Reaction buffer	97
0.1 M ATP	1
0.1 M ADP	1
0.5 M $MgCl_2$	1

- separate ADP and ATP using the following standard parameters

standard parameters:

parameter	Set to
pump	100 % A (running buffer)
flow rate	0.4 ml/min
run time	30 min
injection volume (autosampler)	2 µl
depth of the injection needle	52 nm (depends on your vials and instrument)
autosampler temperature	30 °C, if not stated otherwise
column oven temperature	30 °C, if not stated otherwise
PDA detector wavelength	190-800 nm (you will analyse absorption at 260 nm)
PDA detector sampling time	Every 0.64 sec

Separation of the sample

6 - mix 0.2 mg/ml KaiC with 1 mM ATP and 5 mM MgCl2 in reaction buffer
- optional: add 0.04 mg/ml KaiA and/or 0.04 mg/ml KaiB

Note: total volume can vary between 50-200 µl dependent on your incubation time and number of samples

- transfer mixture to reaction vial (make sure to eliminate all air bubbles)
- place reaction vial in autosampler of your HPLC instrument
- separate the sample using the above mentioned standard parameters
- for calculation you will have to substract the auto hydrolis of ATP (see step 9). For this purpose, separate samples containing only 1 mM ATP and 5 mM MgCl2 (but no protein) in reaction buffer. You can do this in paralell or use buffer values from an independent separation if you use the same column, buffer and ATP solution.
- To follow ADP production over time, program an autosampling shedule (e.g. 3 samples and 3 buffer controls measured every 3 hours as shown in the table below)
- run a final measurement without injecting a sample and water as running buffer (standard parameters, but 100 % B and 0 µl injection volume)

Time after starting first measurement [h]	Sample number	Sample name	Incubation time of the sample [h]	action
0	1	buffer-1	0	Insert sample vial, start shedule
0.5	2	buffer-2	0	Insert sample vial shortly before this timepoint

1	3	buffer-3	0	Insert sample vial shortly before this timepoint
1.5	4	KaiC-1	0	Insert sample vial shortly before this timepoint
2	5	KaiC-2	0	Insert sample vial shortly before this timepoint
2.5	6	KaiC-3	0	Insert sample vial shortly before this timepoint
3	1	buffer-1	3	
3.5	2	buffer-2	3	
4	3	buffer-3	3	
4.5	4	KaiC-1	3	
5	5	KaiC-2	3	
5.5	6	KaiC-3	3	
6	1	buffer-1	6	
6.5	2	buffer-2	6	
7	3	buffer-3	6	
7.5	4	KaiC-1	6	
8	5	KaiC-2	6	
8.5	6	KaiC-3	6	
9	1	buffer-1	9	
9.5	2	buffer-2	9	
10	3	buffer-3	9	
10.5	4	KaiC-1	9	
11	5	KaiC-2	9	
11.5	6	KaiC-3	9	
12	1	buffer-1	12	
12.5	2	buffer-2	12	
13	3	buffer-3	12	
13.5	4	KaiC-1	12	
14	5	KaiC-2	12	
14.5	6	KaiC-3	12	
15	1	buffer-1	15	
15.5	2	buffer-2	15	
16	3	buffer-3	15	
16.5	4	KaiC-1	15	
17	5	KaiC-2	15	
17.5	6	KaiC-3	15	
18	1	buffer-1	18	
18.5	2	buffer-2	18	
19	3	buffer-3	18	
19.5	4	KaiC-1	18	
20	5	KaiC-2	18	
20.5	6	KaiC-3	18	
21	1	buffer-1	21	
21.5	2	buffer-2	21	
22	3	buffer-3	21	
22.5	4	KaiC-1	21	
23	5	KaiC-2	21	
23.5	6	KaiC-3	21	

24	1	buffer-1	24	
24.5	2	buffer-2	24	
25	3	buffer-3	24	
25.5	4	KaiC-1	24	
26	5	KaiC-2	24	
26.5	6	KaiC-3	24	
27	-	MilliQ, waste	MilliQ, waste	

Separation of ADP standards (necessary for calibration curve)

7
- prepare standard solutions with defined amounts of ADP
- transfer to sample vials
- place in autosampler
- perform chromatography as described before

Cleaning and storage

8
- connect pump A to water
- purge autosampler
- open pump valves, purge and close pump valves again
- wash column with at least 36 ml MiliQ (0.4-0.8 ml/min)
- connect pump B and autosampler to 80 % MeOH
- purge autosampler
- open pump valves, purge and close pump valves again
- clean column with at least 36 ml 80 % MeOH (0.1-0.4 ml/min)
- detach column
- optional: if the capillaries you use, cannot withstand 80 % MeOH for a long time, or if you want to store your column in EtOH, perform the following steps to exchange it to EtOH:
- wash the column with at least 36 ml MiliQ (0.4-0.8 ml/min)
- connect pump B 20 % EtOH
- open pump valves, purge and close pump valves again
- wash the column with at least 36 ml 20 % MeOH (0.1-0.4 ml/min)

Data evaluation

9
- for each timepoint: determine the area of the ADP peak at 260 nm (width 60 sec, slope: 200 nU/min)

Note: retention time of ADP is ~14.5 min after separation at 30 °C, but varies with temperature.

- plot a calibration curve based on your standard measurements (step 7)
- samples: for each timepoint: substract ADP peak measured in the buffer sample from the ADP peak measured in the protein sample (ADP[protein]-ADP[buffer]=ADP[normalized])
- for each timepoint: calculate ADP molarity corresponding to ADP[normalized] from the calibration curve (e.g. (ADP[normalized]-10162)/4136.8=µM ADP)
- plot produced ADP over time, add a linear trend line and note it's slope (e.g. 1.32)
- divide slope by the molarity of your protein in the sample to obtain produced ADP per KaiC monomer (e.g. 1.32/3.45µM)
- multiply by 24 hours to obtain produced ADP per KaiC monomer and day