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# Native gel electrophoresis and Western Blot transfer of Kai-protein complexes

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ABSTRACT

This protocol could be used to

- analyze Kai-protein complexes in Clear-native PAGE
- perform Western Blot transfers of large native protein complexes with a semi dry blotting system
- perform the immunodetection of his-tagged proteins with a single monoclonal anti-his antibody conjugated to horseradish peroxidase (HRP) that catalyses the chemical reaction of the substrate ECL generating a chemiluminescence signal

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A chimeric KaiA-like regulator extends the nonstandard KaiB3-KaiC3 clock system in bacteria

KEYWORDS

Clear native PAGE, KaiC3 protein complexes, Western Blot, semi dry blotting, anti-his atibody, immunodetection



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MATERIALS TEXT

Reagents

ROTI®Nanoquant Bradford assay solution 5 x conc. **Carl Roth Catalog #K880.3**

SERVAGel™ N 4 - 16 10 Gele 12 lanes **Serva, Germany Catalog #43253.01**

Native Cathode Buffer for BN/CN (10x) **Serva, Germany Catalog #42536.01**

Native Anode Buffer for BN/CN (10x) **Serva, Germany Catalog #42535.01**

Sample Buffer for Clear Native (2x) **Serva, Germany Catalog #42534.01**

SERVA Native Marker Liquid Mix for BN/CN **Serva, Germany Catalog #39219.01**

ROTI®Blue quick **Carl Roth Catalog #4829.2**

ROTI®Blot A 10x conc. **Carl Roth Catalog #L510.1**

ROTI®Blot K 10 x conc. **Carl Roth Catalog #L511.1**

6x-His Tag Monoclonal Antibody (HIS.H8) HRP **Thermo Scientific Catalog #MA121315HRP**

Pierce™ ECL Western Blotting Substrate **Thermo Scientific Catalog #32109**

Buffers

**Phosphorylation assay buffer**
20 mM Tris-HCl, pH 8.0
150 mM NaCl
0.5 mM EDTA, pH 8.0
5 mM $MgCl_2$
1 mM ATP

**10 x TEN Buffer**
0.5 M Tris-HCl (pH7.4)
0.05 M EDTA
1.5 M NaCl in $H_2O$

Other material and equipment

- electrophoresis system suitable for Serva native mini gels (e.g. Hoefer SE260 Mighty Small vertical electrophoresis system)
- PVDF Transfermembran (ROTI®PVDF 0.2)
- semi-dry blotter (Trans-Blot®SD Semi-Dry Transfer Cell, BioRad)
- blotting filter paper (Whatman, 3 MM)
- platform shaker
- Gel Doc imager (BioRad)

SAFETY WARNINGS



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SAFETY WARNINGS

CAUTION: Methanol is toxic!! If ingested or inhaled it can cause a wide range of harmful effects, from sickness, heart and liver damage to reproductive harm, blindess or death. Always ware neoprene gloves and work under the hood. Avoid breathing in the vapour. Collect waste in a specific solvent container for disposal.

## formation of Kai protein complexes

1
- The protocol was developed for the analysis of protein complexes of the alternativ KaiC3 clock system of *Synechocystis* spc. PCC 6803, but might work for other KaiC clock systems as well
- Strep-KaiC3 purified from *E. coli* Rosetta gami B (DE3) cells is phosphorylated (Strep-KaiC3-P)
- in order to get dephosphorylated KaiC3 (Strep-KaiC3-NP) incubate Strep-KaiC3 (minimum concentration 1 mg/ml) in phosphorylation-assay buffer (20 mM Tris/HCl, pH 8, 150 mM NaCl, 0.5 mM EDTA, 5 mM MgCl2, 1 mM ATP) for 18 h at 30 °C
- check the solution for protein precipitation and remove precipitate by centrifugation (1' at 13000 rpm); take the supernatant and measure the protein concentration with Bradford assay
- prepare protein stock-solutions of 1 mg/ml for each protein in phosphorylation-assay buffer
- prepare 10 µl samples containing 2 µg Strep-KaiC3 and 1 µg of other Kai proteins (KaiA3-His6, KaiB3, KaiA) following the table below
- prepare bigger pools in case you want to take more samples at different time points or for different analyzes
- prepare at least two aliquots for each sample (for running two gels, one for staining with Coomassie, one for blotting)
- incubate Kai-proteins for 16 h at 30°C
- stop the reaction by adding 10 µl of 2 x CN sample buffer, mix and store at -20°C for future analysis or proceed to next section

| A | Strep-KaiC3 | KaiA3-His6 | Strep-KaiC3 + KaiA3-His6 |
|---|---|---|---|
| Strep-KaiC3 (1mg/ml) | 2 µl | - | 2 µl |
| KaiA3-His6 (1 mg/ml) | - | 1 µl | 1 µl |
| buffer | 8 µl | 9 µl | 7 µl |
| total volume | 10 µl | 10 µl | 10 µl |

## Clear native PAGE

2
- keep samples on ice
- thaw marker (Serva native marker liquid mix) on ice
- meanwhile assemble the Hoefer SE260 Mighty Small vertical electrophoresis system by inserting the upper buffer chamber in the lower buffer chamber
- unwrap a precast Serva 4-16% native gradient gel and fix it to the upper chamber with the clamps, comb to the back
- put a well locating decal to the plate to mark the wells
- fill the upper chamber with native 1 x cathode buffer and the lower chamber with 1 x anode buffer
- carefully remove the comb and rinse the wells intensively with a syringe (e.g. Hamilton syringe)
- add 5 µl of marker and your samples (20 µl) to the wells; fill empty wells with 1 x sample buffer to avoid smiley effects in the gel run
- put the gel system on ice and perform electrophoresis with 8 mA/gel (~ 90V) for 10 min, followed by 15 mA/gel (~ 200 V) until the colour-front reaches the end of the gel (~ 3 h)
- disassemble the gel according to manufacturer's instructions
- carefully remove the upper gel part; the KaiC3/KaiA3/KaiB3 complex is > 720 (838.2 kDa) and runs close to the upper edge of the separating gel and might be removed accidentally by cutting of the stacking gel
- rinse the gel shortly with dionized water
- stain one gel with Coomassie (RotiBlue Quick stain), proceed with blotting with the second gel

NOTE: CN-PAGE works only for proteins that have a negative net charge at neutral pH! Neutrally or positively charged proteins will not migrate into the gel; try Blue native PAGE for such kind of proteins (BN-PAGE did not work for the Kai-Proteins)

## Western Blot



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3 The following section describes the transfer of high molecular weight proteins from a clear-native polyacrylamide gradient gel (4-16% CN-PAGE) onto an PVDF transfer membrane (0.2 µm pore size) using a semi-dry transfer system; it is specific for semi-dry transfer devices with the anode plate serving as a base

Prepare the following buffer:

Anode buffer: 20 ml 10 x Roti®-Blot A (includes 0.6% SDS), 180 ml a. dest
→ 200 mL 1 x buffer + 0.06% SDS

Cathode buffer: 20 ml 10 x Roti®-Blot K (includes 0.6% SDS), 180 ml a. dest
→ 200 ml 1 x buffer + 0.06% SDS

NOTE: do not use Methanol for transfer of large proteins, include SDS for better transfer

Materials needed to assemble the transfer stack:
- gel containing the resolved proteins
- semi-dry blotter
- Six sheets of blotting filter paper (Whatman, 3 MM), cut to the same dimensions as the gel (9.0 x 8.0 cm2)
- One sheet of PVDF membrane, cut to the same dimensions as the gel
- Deionised water
- 100% methanol
- Pasteur pipettes

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Set up:
- Use 10 $cm^2$ plate-trays
- Cut off one edge of the gel; immerse the gel in cathode buffer for at least 30'
- Soak three pieces of filter paper in anode buffer for at least 30''
- Soak three pieces of filter paper in cathode buffer for at least 30''

Prepare the membrane:
- Wash the membrane in methanol for 10-15''; the membrane should uniformly change from opaque to semitransparent
- Wash the membrane in deionized water for 10-20''
- Equilibrate the membrane in anode buffer for at least 5'

Assembling the Transfer Stack for a Semi-Dry System (Single Transfer)
- Assemble in the cold room
- wet the graphite anode plate with anode buffer using a Pasteur pipette
- Place the three sheets of filter paper soaked in anode buffer solution in the center of the graphite anode electrode plate
- Place the membrane on the filter paper, use foreceps
- Place the gel on top of the membrane
- Place three sheets of filter paper soaked in cathode buffer on top
- Wet the cathode plate with cathode buffer

CAUTION: To ensure an even transfer, remove air bubbles by carefully rolling a pipette over the surface of each layer of the stack. Applying excessive pressure may damage the membrane and gel

- Place the cathode plate cover on top of the assembled transfer stack; do not bump the cathode plate cover since it could disturb the alignment of the transfer stack and cause inaccurate results
- Insert the black cathode lead (-) into the cathode plate jack and the red anode lead (+) into the anode plate jack
- Connect the anode lead and cathode lead to their corresponding power supply outputs.
- Let it run at 4.0 mA/$cm^2$ for 3 h at 4°C, set appropriate current according to the size of the stack, e.g. 8.0 x 9.0 $cm^2$



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x 4.0 mA/$cm^2$ = 288 mA

Removing the Blot from the Semi-Dry System

- Turn off the power supply and disconnect the system leads
- Remove the cover; peel off and discard the upper filter papers
- Cut the membrane to the exact size of the gel including the notched corner
- Peel off the gel and transfer it to a 10 x 10 cm2 plate; rinse the gel shortly with deionized water and stain it afterwards with Coomassie Blue to check the transfer
- Peel off the blotted membrane with a pair of forceps, transfer it to a 10 x 10 cm plate
- Rinse the membrane shortly with deionized water and proceed to immunodetection

single antibody immunodetection of his-fusion proteins

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- Block non-specific binding sites by immersing the membrane in 1xTEN + 1% Tween20 for 2h 4°C o/n on a platform shaker
- Antibody: Dilute anti-his conjugate 1:2000 (10 µl /20 ml) in 1xTEN + 1% Tween; allow sufficient antibody solution to cover the membrane (at least 0.25 ml/$cm^2$)
- incubate the membrane in diluted conjugate for 2h at RT on a platform shaker (60 rpm)
- Discard the antibody conjugate and wash the membrane in 1xTEN + 0.1% Tween for 6 x 5' (= 30') with fresh changes of buffer at RT with gentle shaking

NOTE: in case you have antibodies that require a second antibody conjugated to HRP, repeat the previous three steps with the 2nd antibody

- Mix the two ECL-substrate solutions, ratio 1:1 (1.5 ml each in a 5 ml Eppendorf tube)
- Cut a transparent disposal bag (Roth), place the membrane on the lower sheet
- Cover the membrane completely with development substrate mix
- Incubate for 1'
- lift up the sheet and remove the substrate solution with a paper towel
- cover the membrane with the upper sheet; squeeze out remaining liquid, remove air bubbles
- place the membrane in the in the Gel Doc Imager, image area 13.4 x 10.0 cm, and make a picture of the membrane in program "protein, Coomassie Blue" to visualize the dimensions of the membrane (Upper marker bands and intensive chemiluminescence bands are visible even in this mode)
- expose the membrane, program "blot, Chemi", image area 13.4 x 10.0 cm; signals appear after 20''
- make an image of the Coomassie stained gels from before and after the transfer using same image area
- use all images to assign the signals to the samples; gels might swell in the staining/destaining process, keep this in mind when assigning the signals from the membrane



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