Immunoblot Analysis of Histone H4 Acetylation and Histone H2A Phosphorylation in *Candida albicans*

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[Abstract] Posttranslational modifications of histones are required for different processes including transcription, replication and DNA damage repair. This protocol describes the preparation of a whole-cell extracts for the fungal pathogen *Candida albicans*. Furthermore, the extract is used to detect lysine acetylation of histone H4 as well as serine 129 phosphorylation of histone H2A by immunoblot analysis.

Materials and Reagents

1. *Candida albicans*
2. TCA (trichloroacetic acid) (Merck KGaA, catalog number: 641730)
3. Recombinant histone H4 (New England Biolabs, catalog number: M2504S)
4. Calf histones (Sigma-Aldrich, catalog number: H9250)
5. 0.033% sodium azide (Merck KGaA, catalog number: 8223350250)
6. Nitrocellulose membrane (Millipore, catalog number: Protran BA79)
7. Whatman filter paper 3 MM Chr (Whatman, catalog number: 3030-917)
8. BSA (PAA Laboratories GmbH, catalog number: K41-001)
9. Sodium azide (Merck KGaA, catalog number: 822335)
10. Rabbit polyclonal antibody to histone H4 acetyl K5 (Abcam, catalog number: ab51997) (dilution: 1:5,000)
11. Rabbit polyclonal antibody to histone H4 acetyl K8 (Active Motif, catalog number: 39172) (dilution: 1:3,000)
12. Rabbit polyclonal antibody to histone H4 acetyl K12 (Millipore, catalog number: 07-959) (dilution: 1:3,000)
13. Rabbit polyclonal antibody to histone H4 C-terminus (Abcam, catalog number: ab10158) (dilution: 1:1,000)
14. Rabbit polyclonal antibody to histone H2A phospho-serine 129 (Active Motif, catalog number: 39271) (dilution: 1:2,000)

15. Rabbit polyclonal antibody to histone H2A (Active Motif, catalog number: 39236) (dilution: 1:5,000)
16. IRDye 800CW goat anti-rabbit IgG (H + L) (LI-COR, catalog number: 926-32211)
17. IRDye 680RD goat anti-rabbit IgG (H + L) (LI-COR, catalog number: 926-68071)
18. 4.5% stacking gel
19. 20% running gel
20. Bacto Yeast Extract (Becton, Dickinson and Company, catalog number: 212720)
21. Bacto Peptone (Becton, Dickinson and Company, catalog number: 211820)
22. Glucose (Merck KGaA, catalog number: 346351)
23. β-Mercaptoethanol (Sigma-Aldrich, catalog number: M3148-100ML)
24. Urea (Sigma-Aldrich, catalog number: U5378-1KG)
25. SDS (AppliChem GmbH, catalog number: A1112,1000)
26. EDTA (Sigma-Aldrich, catalog number: E5134-1KG)
27. Bromphenolblue
28. YPD medium (see Recipes)
29. Yex lysis buffer (see Recipes)
30. Protein sample buffer (see Recipes)
31. SDS-PAGE running buffer (see Recipes)
32. Running buffer (see Recipes)
33. Transfer buffer (see Recipes)
34. TBS and TBS-T (see Recipes)
35. PBS (see Recipes)

Equipment

1. Mini-Protean gel electrophoresis system (Bio-Rad Laboratories, model: 165-8000)
2. Mini Trans-Blot cell (Bio-Rad Laboratories, model: 170-3930)
3. 1.5 ml microcentrifuge tubes
4. 15 ml Falcon tubes
5. 14 ml Snap-cap tubes
6. Eppendorf Thermomixer comfort (Eppendorf, model: 5355 000.011)
7. Shaking incubator
8. Orbital shaker
9. LI-COR Odyssey CLx Infrared scanner (LI-COR, model: P/N 9140-WP)
10. CASY Cell Counter Model TT (Roche, catalog number: 05651735001)

Software

1. LI-COR Odyssey CLx imaging system

Procedure

A. Whole-cell extract preparation
   1. Inoculate a single colony in 5 ml of YPD medium in a 14 ml Snap-cap tube and grow overnight at 30 °C shaking with 220 rpm.
   2. Dilute overnight culture to an OD_{600} of 0.1 in 5 ml YPD medium in a 14 ml Snap-cap tube and grow cells to OD_{600} of 1 at 30 °C shaking with 220 rpm.
      Note: For strains/conditions with different cell morphologies determine cell number by CASY measurement according to the CASY operator manual and use a total number of 5 x 10^7 cells. Flasks can also be used instead of Snap-cap tubes.
   3. Harvest cells by centrifugation in 15 ml Falcon tubes for 3 min at 1,500 *x g.*
   4. Resuspend pellet in 1 ml ice-cold H_2O.
      Note: Work on ice from now on.
   5. Transfer suspension to 1.5 ml tube.
   6. Add 150 µl ice-cold Yex lysis buffer, vortex thoroughly and incubate on ice for 10 min.
   7. Add 150 µl ice-cold 50% (w/v) TCA and incubate on ice for 10 min to precipitate proteins.
   8. Spin for 5 min at 10,000 *x g* 4 °C and discard supernatant with a pipette.
   9. Spin for 1 min at 10,000 *x g* 4 °C and remove rest of the supernatant.
   10. Resuspend pellet in 100 µl protein sample buffer.
       Note: Resuspend by pipetting; if pH turns acidic (protein sample buffer turns yellow), add 1 M Tris base to increase pH (until sample buffer turns blue again).
   11. Incubate at 37 °C for 15 min shaking at 900 rpm.
   12. Spin down cell debris at 10,000 *x g* for 5 min and use 10 µl (0.5 OD_{600} units) of the supernatant for SDS-PAGE.

B. SDS-PAGE and western blotting
   1. Load samples on a polyacrylamid gel (4.5% stacking gel and 20% running gel).
   2. Load 0.5 µg recombinant histone H4 in protein sample buffer as negative control and 2 µg calf histones in protein sample buffer as positive control for acetylation analysis.
      Note: Gel cast as described previously (Sambrook and Russell, 2001); Bio-Rad Mini-Protean gel electrophoresis system was used.
   3. Run gel at 150 V.

4. Transfer proteins to nitrocellulose membrane by electroblotting using the Bio-Rad Mini Trans-Blot cell.
5. Assemble blotting cassette in transfer buffer according to the Mini Trans-Blot instruction manual and run at 200 mA for 1 h at room temperature.
6. Block membrane for 1 h at room temperature on an orbital shaker using 5% (w/v) BSA in TBS.
7. Incubate with primary antibody diluted in TBS-T overnight at 4 °C on an orbital shaker.
8. Pour off primary antibody solution and wash 3 x 10 min in TBS-T at room temperature.
   Note: Primary antibody solution can be reused several times (depending on the antibody); if solution is reused, add 0.033% sodium azide as preservative.
9. Incubate with secondary antibody diluted 1:10,000 in TBS-T for 45 min at room temperature on an orbital shaker.
   Note: IRDye 800CW or IRDye 680RD secondary antibodies can be used.
10. Pour off secondary antibody solution and wash 3 x 10 min in TBS-T at room temperature.
11. Rinse blot briefly with PBS and scan using the LI-COR Odyssey CLx Infrared scanner.
    Note: Scanner settings: Intensity: 5; Resolution: 168 µm; Quality: medium.

Recipes

1. YPD medium
   10 g/L Bacto Yeast Extract
   20 g/L Bacto Peptone
   20 g/L Glucose (add after autoclaving as 10x stock)
2. Yex lysis buffer
   1.85 M NaOH
   7.5% (v/v) β-Mercaptoethanol (freshly added)
3. Protein sample buffer
   40 mM Tris-HCl, pH 6.8
   8 M Urea
   5% (w/v) SDS
   0.1 mM EDTA
   1% (v/v) β-Mercaptoethanol (freshly added)
   0.1 g/L Bromphenolblue
4. Running buffer
   25 mM Tris
   192 mM Glycine
   0.1% (w/v) SDS

5. Transfer buffer
   25 mM Tris
   192 mM Glycine
   20% (v/v) Methanol
6. TBS
   25 mM Tris
   140 mM NaCl
   2.5 mM KCl
   pH 7.4
7. TBS-T
   TBS with 0.1% (v/v) Tween 20
8. PBS
   140 mM NaCl
   2.5 mM KCl
   8.1 mM Na_2HPO_4
   1.5 mM KH_2PO_4
   pH 7.3

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