

Western Analysis of Histone Modifications (*Aspergillus nidulans*)

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[Abstract] Western blotting allows for the specific detection of proteins and/or modifications of proteins by an antibody of interest. This protocol utilizes a crude nuclei extraction protocol for *Aspergillus nidulans* to enrich for histones and other nuclear proteins prior to gel electrophoresis. Post translational modifications of histones may then be easily detected. After electrophoresis, the selected antibodies are used to detect and quantify levels of the modifications of interest.

Materials and Reagents

1. D-glucose
2. NaNO₃
3. KCl
4. MgSO₄·7H₂O
5. KH₂PO₄
6. ZnSO₄·7H₂O
7. H₃BO₃
8. MnCl₂·4H₂O
9. FeSO₄·7H₂O
10. CoCl₂·5H₂O
11. CuSO₄·5H₂O
12. (NH₄)₆Mo₇O₂₄·4H₂O
13. EDTA
14. Spermine
15. Spermidine
16. PMSF
17. Sorbitol
18. Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, catalog number: PI-78437)
19. NaCl

20. KCl
21. Tris base
22. Glycine
23. Methanol
24. Bio-Rad protein assay (Bio-Rad Laboratories, catalog number: 500-0006)
25. Miracloth (Calbiochem, catalog number: 475855)
26. Rabbit polyclonal antibody to histone H4 acetyl K5 (1:2,500) (Abcam, catalog number: ab51997)
27. Rabbit polyclonal antibody to histone H4 acetyl K8 (1:2,500) (Abcam, catalog number: ab15823)
28. Rabbit polyclonal antibody to human C-terminus histone H4 antibody (1:2,500) (Abcam, catalog number: ab10158)
29. Mouse monoclonal antibody to TATA Binding Protein (TBP) (1:1,000) (Abcam, catalog number: ab61411)
30. Mouse monoclonal antibody to pan acetyl H4 (1:1,000) (Active Motif, catalog number: 3992)
31. Goat anti-mouse alkaline phosphatase conjugated antibody (1:5,000) (Life Technologies, Gibco[®], catalog number: 13864-012)
32. Goat anti-rabbit alkaline phosphatase conjugated antibody (1:5,000) (Pierce Antibodies, catalog number: 31342)
33. 1-Step NBT/BCIP (Thermo Fisher Scientific, catalog number: 34042)
34. Glucose Minimal Medium (GMM) (see Recipes)
35. 2x Laemmli buffer (see Recipes)
36. Nuclear Isolation buffer (see Recipes)
37. Resuspension buffer (see Recipes)
38. ST buffer (see Recipes)
39. TBS-T (see Recipes)
40. Western transfer buffer (see Recipes)
41. Media Recipes (see Recipes)

Equipment

1. Mortar and pestle
2. Bio-Rad Mini-Protean gel electrophoresis system (Bio-Rad Laboratories, catalog number: 170-3940)
3. Semi-Dry Blotter (Bio-Rad Laboratories, catalog number: 170-3940)
4. Orbital shaker

5. 15 ml Falcon tubes
6. 50 ml Falcon tubes
7. 30 ml centrifuge tubes
8. Microfuge tubes

Procedure

1. Nuclear extract:

Note: All nuclear isolation steps and solutions should be kept on ice.

- a. Inoculate 250 ml of liquid glucose minimal medium (GMM) to a final concentration of 1×10^6 spores/ml and incubate at 37 °C, 250 rpm for 48 h.
 - b. Collect mycelia by vacuum filtration through two layers of miracloth. Remove excess moisture by squeezing in between paper towels.
 - c. Place mycelia into a 15 ml Falcon tube and flash freeze in liquid nitrogen. After freezing, grind mycelia to a fine powder with a mortar and pestle under liquid nitrogen.
 - d. Transfer ~ 5 g ground mycelia to 50 ml Falcon tubes.
 - e. Add 40 ml of ice cold Nuclear Isolation buffer and vortex to mix.
 - f. Centrifuge for 10 min at 1,000 x g, 4 °C. Filter supernatant through a double layer of miracloth into 30 ml centrifuge tubes.
 - g. Centrifuge for 10,000 x g for 15 min, 4 °C.
 - h. Discard supernatant and resuspend pellet in 10 ml of ice cold Resuspension buffer.
 - i. Centrifuge for 10,000 x g for 15 min, 4 °C.
 - j. Discard supernatant and resuspend pellet in 1 ml of ice-cold ST buffer.
 - k. Transfer suspension into 1.5 ml centrifuge tube and pellet debris by centrifugation at 4,000 x g for 30 sec, room temperature.
 - l. Transfer supernatant into a sterile 1.5 ml centrifuge tube. Store at -20 °C if desired.
 - m. Quantify protein levels using Bio-Rad Protein Assay or equivalent.
- ### 2. Western blotting:
- a. Load standardized amount of protein per sample (e.g. 50 µg) onto an SDS-PAGE gel with appropriate sample loading buffer (such as Laemmli 2x sample buffer).
I used 10% Tricine-SDS-PAGE, cast according to Schägger (2006), and the Bio-Rad Mini Protean gel electrophoresis system.
 - b. Transfer to nitrocellulose membrane by electroblotting using the Bio-Rad Semi-Dry Blotter (can be done at room temperature).
 - i. Soak your gel in western transfer buffer for 30 min and soak the nitrocellulose membrane in western transfer buffer for 10 min before blotting.

- ii. Soak extra thick blotting paper in western transfer buffer – construct the “sandwich” according to the semi-dry transfer manual. From the bottom (+ pole) working to the top (- pole): Extra thick blotting paper, nitrocellulose membrane, acrylamide gel, extra thick blotting paper.
- iii. Assemble apparatus and run at 15 V for 1 h.
- c. Block membranes for 1 h at room temperature on an orbital shaker using 5% nonfat dry milk in TBS-T.
- d. Incubate with primary antibodies for 1 h in TBS-T. 10 ml is sufficient for a small blot. Incubation may also be performed at 4 °C, especially if planning to reuse primary antibody solutions.
- e. Pour off the primary antibody solutions. Primary antibody solutions may be resused up to 5 times, although antibody concentration will decrease with each use (*note: These solutions contain sodium azide as preservative*).
- f. Wash 3 x 10 min in TBS-T. Apply secondary antibodies in TBS-T for 1 h at room temperature. 10 ml is sufficient for a small blot.
- g. Pour off secondary antibody solution (no need to retain). Wash blots 3 x 10 min in TBS-T at room temperature on the orbital shaker.
- h. Add One-step NBP/BCIP developing solution and agitate gently. Keep an eye on the signal intensity, it will only be a matter of minutes to develop and development time will be different for each antibody. When reaches desired intensity (or just before), pour off the developing solution and rinse in several changes of distilled water. Incubate in ddH₂O for ~20 min.
(Note: NBP/BCIP developer results precipitation of a colored substance on the membrane, thus, membranes cannot be stripped and reprobred. If intending to reprobe the membrane, alternative secondary antibodies (e.g. HRP or fluorescent conjugated) should be used.)
- i. Blots can be dried on bench to preserve. Signal will fade upon direct exposure to light.

Recipes

- | | |
|---------------------------|------------------------------------|
| 1. 2x Laemmli buffer | 1 ml |
| 4% SDS | 400 µl of 10% SDS |
| 10% 2-mercaptoethanol | 100 µl |
| 20% glycerol | 200 µl |
| 0.004% bromophenol blue | 40 µl of saturated solution (0.1%) |
| 0.125 M Tris HCl | 125 µl of 1 M Tris HCl (pH 6.8) |
| Bring to 1 ml with water. | |

2. Nuclear Isolation buffer 1 L
 - 1 M Sorbitol 182.2 g
 - 10 mM Tris-HCl (pH 7.5) 10 ml of 1 M Tris-HCl
 - 10 mM EDTA 20 ml of 0.5 M EDTA

Autoclave, then add the following:

 - 0.15 mM Spermine 52.2 mg
 - 0.5 mM Spermidine 127 mg

Right before use, add:

 - 2.5 mM PMSF 435 mg

Note: PMSF has a short half-life in aqueous solutions, 30-100 min.
3. Resuspension buffer 1 L 500 ml
 - 1 M Sorbitol 182.2 g 91.1 g
 - 10 mM Tris-HCl pH 7.5 10 ml of 1M Tris-HCl 5 ml
 - 1 mM EDTA 2 ml of 0.5 M EDTA 1 ml

Autoclave, then add the following:

 - 0.15 mM Spermine 52.2 mg 26.1 mg
 - 0.5 mM Spermidine 127 mg 63.5 mg

Right before use, add (optional):

 - 2.5 mM PMSF 435 mg 218 mg
4. ST buffer 100 ml
 - 1 M Sorbitol 18.2 g
 - 10 mM Tris-HCl (pH 7.5) 1 ml of 1 M Tris-HCl

Autoclave

Add Protease Inhibitor Cocktail right before use at 10 µl per 1 ml.
5. TBS-T 1 L
 - NaCl 8 g
 - KCl 0.2 g
 - Tris base 3 g

Mix in ~ 800 ml dH₂O, adjust pH to 7.4 with HCl, then adjust volume to 1 L.

Autoclave

After cooling, add 1 ml Tween 20.
6. Western transfer buffer 1 L 9 L
 - Tris base 3.03 g 27.3 g
 - Glycine 14.4 g 129.6 g
 - Methanol 200 ml 1.8 L

Adjust volume to correct volume with dH₂O

Store at 4 °C for up to 1 month

7. Media Recipes

Glucose Minimal Medium (GMM)

20x sodium nitrate salts *	50 ml/L
Trace elements (shake before using)	1 ml/L
D-glucose	10 g/L

Adjust pH to 6.5

Add ddH₂O up to one liter

Autoclave 15 min, 121 °C

*20x sodium nitrate salts solution

NaNO ₃	120 g
KCl	10.4 g
MgSO ₄ ·7H ₂ O	10.4 g
KH ₂ PO ₄	30.4 g

Add ddH₂O up to 1 L

Autoclave and store at room temperature.

**Trace elements

ZnSO ₄ ·7H ₂ O	2.2 g
H ₃ BO ₃	1.1 g
MnCl ₂ ·4H ₂ O	0.5 g
FeSO ₄ ·7H ₂ O	0.5 g
CoCl ₂ ·5H ₂ O	0.16 g
CuSO ₄ ·5H ₂ O	0.16 g
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.11g
Na ₄ EDTA	5.0g

Add the solids in order to 80 ml of H₂O, dissolving each completely before adding the next. Heat the solution to boiling, cool to 60 °C. Adjust the pH to 6.5-6.8 with KOH pellets.

Cool to room temperature and adjust volume to 100 ml with ddH₂O.

References

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