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Histone extraction for mass spectrometry-based analysis of post-translational modifications in the fungal genus *Aspergillus*

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ABSTRACT

Mass spectrometry is a powerful technique to uncover histone post-translational modifications in an unbiased and quantitative manner (Noberini,*et al.*,The FEBS Journal, 2021). Yet, such analyses require good quality protein samples, a challenge when working with fungi. Here, we present a protocol based on the work by Noberini and colleagues (Noberini, *et al.*, Methods, 2020) to extract histone proteins from filamentous fungi for applications like mass spectrometry-based analyses of post-translational modifications. Several modifications were made: 1) additional cell disruption and homogenization steps using freeze-drying and TissueLyser II; 2) increased Triton X-100 concentration in the lysis buffer; and 3) prolonged incubation time in lysis buffer. This protocol is reproducible, and the proteins extracted from three *Aspergillus* species (*A. niger*, *A. fumigatus*, and *A. nidulans*) were of good quality for mass spectrometry.

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GUIDELINES

1. Germinated spores from overnight cultures are the ideal starting material.

2. Disruption by TissueLyser II and sonication are necessary steps because fungal cells are protected by the cell wall which prevents efficient lysis.

3. 100 mg ground starting material per sample of *Aspergillus* ends up with enough yield (around 20 µg) for Bradford Protein Assay, SDS-PAGE visualization, and mass spectrometry-based analysis (which ideally requires 3-4 µg of histones). Different fungal species may vary.

4. Different medium are used to culture fungal strains, e.g. *A. niger* and *A. fumigatus* grow on MEA (Malt Extract Agar) medium at 30 °C and 25 °C, respectively; *A. nidulans* grows on YAG (Yeast Agar Glucose) medium at 37 °C.

MATERIALS TEXT

Solutions and reagents:

- ACES buffer (1.822 g/L N-(2-Acetamido)-2-aminoethanesulfonic acid, 0.2 mL/L Tween 80)
- Solid medium and liquid medium depending on the strain (*A. niger* and *A. fumigatus* are cultured in MEA (Malt Extract Agar Oxoid 50 g/L) medium; *A. nidulans* is cultured in YAG medium (5 g/L yeast extract, 15 g/L agar, 20 g/L D-glucose, 1mL/L trace elements (Solution A: Dissolve 10 g EDTA and 1 g $FeSO_4 \times 7H_2O$ in 80 mL water and adjust pH to 5.5; Solution B: Dissolve 4.4 g $ZnSO_4 \times 7H_2O$, 1 g $MnCl_2 \times 4H_2O$, 0.32 g $CuSO_4 \times 6H_2O$, and 0.22 g $(NH_4)_6Mo_7O_{24} \times 4H_2O$ in 80mL water; Combine Solution A and B, adjust pH to 6.5)
- PBS (Phosphate Buffered Saline) buffer (8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L $Na_2HPO_4 \times 2\ H_2O$, 0.2 g/L KH_2PO_4, 1.32 g/L $CaCL_2 \times 2H_2O$, and 0.5 mM $MgCl_2 \times 6H_2O$)
- Lysis buffer (1× PBS, 0.5 mM PMSF, 5 µM Leupeptin, 5 µM Aprotinin, 5 mM Na-butyrate, and 1% or 10% Triton X-100)
- Aprotinin bovine (Sigma, A6103)
- Leupeptin (Sigma, L2884)
- Sodium butyrate (Sigma, B5887)
- Phenylmethylsulfonyl fluoride (PMSF; Fluka, 78830)
- Triton X-100 (Sigma, T8787)
- Acetone (Biosolve,1046021)
- Coomassie protein assay reagent (Thermo, 1856209)

Equipment:

- Centrifuge (Eppendorf, 5810R)
- Centrifuge (Eppendorf, 5430R)
- Hemacytometer (Marienfeld, 0.0025mm^2)
- Freeze dryer (Christ, Alpha 1-4 LSCbasic)
- TissueLyser II (QIAGEN, C.0659)
- Orbital shaker (Grant-bio, PS-3D)
- Incubator (Yourtech, C.0663)
- Vortex (IKA, S000)
- Ultrasonic Cleaner (Branson 2510)

SAFETY WARNINGS

Acetone has strong flammability, always operate it inside a fume hood.

BEFORE STARTING

1. PBS buffer should be kept on ice.

2. Set centrifuges at 4 °C beforehand.

3. Prepare lysis buffers with different Triton X-100 concentration fresh and add PMSF last. PMSF stock solution in isopropanol can be stored at -20 °C for several months, but it can be inactivated within 35 minutes once it is added in aqueous solution.

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Sample collection and disruption 2d

1 Harvest fungal spores from 4- or 5 -day old sporulating plates with a spreader in 12.5 mL ice-cold ACES buffer 5m

2 Centrifuge the spore suspension at 2,500 xg at 4 °C for 5 minutes. 5m

3 Discard supernatant and resuspend spores in 25 mL ice-cold ACES buffer. 1m

4 Centrifuge the spore suspension at 2,500 xg at 4 °C for 5 minutes, and discard supernatant. 5m

5 Resuspend spores in 10 mL ice-cold ACES buffer, and count spore concentration with an hemacytometer or spore counter. Ideally, the spore concentration is around 1×10^7 spores/ mL. 1m

6 Inoculate 50 mL of liquid medium with $1 \sim 10 \times 10^7$ spores in a 250 mL flask, and incubate at 30 °C, 200 rpm, for 16 hours. 16h

7 Collect germinated spores by centrifugation at 4,000 xg for 10 minutes and discard supernatant. 10m

8 Resuspend germinated spore pellet in 25 mL 0.8 M NaCl. 1m

9 Centrifuge at 4,000 xg for 10 minutes and discard supernatant to get rid of liquid medium. 10m

10 Freeze germinated spores in liquid nitrogen. 1m

11 Freeze dry overnight. 1d

12 Grind freeze-dried samples with a mortar and pestle, and then with TissueLyser II using two ø5 mm metal beads at 30 Hz for 30 seconds, which is repeated twice. 1h

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Histone protein extraction 1d

13 Resuspend 100 mg ground sample in 1 mL lysis buffer with high Triton X-100 concentration (1×PBS, 0.5 mM PMSF, 5 μM Leupeptin, 5 μM Aprotinin, 5mM Na-butyrate, and 10% Triton X-100). 1m

14 Incubate on an orbital shaker for 30 minutes at 4 °C. 30m

15 Add 10 ml lysis buffer with low Triton X-100 concentration (1×PBS, 0.5 mM PMSF, 5 μM Leupeptin, 5 μM Aprotinin, 5mM Na-butyrate, and 1% Triton X-100), mix by vortexing, and incubate on the orbital shaker at 4 °C for another 30 minutes. 30m

16 Centrifuge at 3,250 xg for 30 minutes at 4 °C, and discard supernatant. 30m

17 Resuspend in 1 mL ice-cold PBS buffer. 1m

18 Sonicate for 30 seconds; this process is repeated 10 times with 30 seconds break in between. 10m

19 Add 9 mL ice-cold PBS buffer and mix by vortexing. 1m

20 Centrifuge at 3,250 xg for 20 minutes at 4 °C, and discard supernatant. 20m

21 Resuspend pellet in 2 mL 0.4 N H_2SO_4 and incubate at 4 °C for 4 hours on an orbital shaker. 4h

22 Centrifuge at 16,000 xg for 10 minutes at 4 °C. The supernatant contains soluble core histone proteins. 10m

23 Add 4 volumes of acetone to the sample, and keep at -20 °C overnight to precipitate histone proteins. 16h

24 Centrifuge at 6,000 xg for 25 minutes at 4 °C to pellet proteins, then dissolve in 100 μL water. 25m

Protein quantity and quality measurement 6h 20m

25 Measure protein concentration with the Bradford Protein Assay (He, F., Bio-protocol, 2011). 20m

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26 Check protein quality via 16% SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel (Simpson, R. J., Cold Spring Harbor Protocols, 2006). 6h

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