

**Catalog No.:** 40026

**Name:** Histone Purification Mini Kit

**Format:** 20 rxns

The following guideline is designed to be used in combination with Active Motif's Histone Purification Mini Kit (Catalog No. 40026) and consists of recommendations for the preparation and labeling of purified core histones for analysis in liquid chromatography-mass spectrometry (LC/MS). The protocol provided has been demonstrated to improve the recovery of histones, including low abundance histone marks, phosphorylated and hydrophilic histone peptides.

Active Motif's Histone Purification Mini Kit enables you to purify the core histone proteins while preserving their post-translational modifications (e.g. acetylation, methylation, and phosphorylation). This purification method is an improvement over acid precipitation methods and utilizes a convenient spin column and a proprietary buffer system to purify the core histones from cells and tissue samples. The following is an optimized protocol for acylation, trypsin cleavage and chemical labeling of histones for use in LC/MS.

The optimized chemical labeling protocol improves the representation of hydrophilic peptides containing H3K4me2/me3, phosphopeptides and low abundance acetylation events such as H3K27ac and H3K36ac as compared to standard labeling methods. The method is simple: first, an extract is made and applied to the purification column, then histones are eluted, enabling the purification of core histones. Histones are then subjected to an acylation agent which blocks the highly abundant lysine residues in the histone tails from tryptic cleavage. During digestion, cleavage occurs at the carboxylic side of arginine residues resulting in longer peptide length for better analysis. Finally, histones samples are subjected to chemical labeling and analyzed by a mass spectrometer, which measures the mass-to-charge ratio (m/z).

The protocol provided has been validated for use with the Orbitrap Elite™ hybrid ion trap-orbitrap mass spectrometer or equivalent capillary LC-MS/MS instrument. Active Motif is not responsible for the analysis of data generated using this guideline. We recommend checking with your mass spectrometry provider to determine any special considerations required for your instrument or analysis prior to starting the protocol.

## Additional materials required:

- Dounce homogenizer with a small clearance pestle (Catalog No.40401); the large pestle supplied can be used for initial sample reduction, while the small pestle should be used to process the final homogenate).
- 1.7 ml microcentrifuge tubes (centrifugation of spin columns)
- For precipitation of histones: Perchloric acid, 70% (Acros Organics, part no. 424030010)
- For washing of histones:
  - 4% perchloric acid (Aldrich brand)
  - 0.2% HCl in acetone\*
  - Cold 100% acetone (VWR, part no. BDH1101)

\* Add 0.5 ml of HCl stock (36.8%, Sigma, part no. H-7020) and adjust the volume with acetone until 92 ml. Store in a glass bottle at 4°C.

- Bicarbonate Buffer
  - 1 M triethylammonium bicarbonate (Sigma brand)
- Acylation Agent
  - Propionic anhydride (Fluka brand)
- Quenching Buffer
  - 80 mM Hydroxylamine (Aldrich brand)
- Sequencing grade modified Trypsin (Promega, Catalog No. V511A)
- Phenyl isocyanate (PIC), which is also available in deuterated ( $d_6$ ) and carbon-13 ( $^{13}C_6$ ) labeled forms (Sigma-Aldrich)
- C18-stage-tips for desalting (Thermo Scientific, Catalog No. SP201)
- 1% and 0.1% Trifluoroacetic acid (TFA)
- 60% Acetonitrile/0.1% TFA
- HPLC-grade water ( $ddH_2O$ )

- Solvent A: 0.1% v/v formic acid, 2% v/v acetonitrile in HPLC grade water
- Solvent B: 0.1% v/v formic acid, 98% v/v acetonitrile in HPLC grade water
- Orbitrap Elite™ hybrid ion trap-orbitrap mass spectrometer (Thermo Scientific) or equivalent capillary LC-MS/MS instrument
- Analysis software

## Section A thru Section D:

Follow the instructions provided in the Histone Purification Mini Kit (Catalog No. 40026) from Section A thru Section D for the preparation, purification and precipitation of histone proteins. Resuspend the histones in HPLC-grade water (ddH<sub>2</sub>O). Flick the bottom of the tube gently with a finger. Let the pellet resuspend 10 to 20 minutes at room temperature, then gently vortex and centrifuge briefly. Check to make sure that pellet is completely resuspended.

## Section E: Histone Protein Resuspension and Quantification

1. Total core histone proteins can be quantified by measuring the absorbance at 230 nm. An OD of 0.42 from a sample diluted 1:10 indicates a protein concentration of 1 mg/ml.

Undiluted samples may be outside the linear range of your spectrophotometer. Therefore, we recommend preparing a 1/10 dilution of your samples before quantifying.

$$\frac{\text{OD 0.42}}{1 \text{ mg/ml}} = \frac{\text{Your OD}}{x \text{ mg/ml}}$$

OD of 0.42 = 1 mg / ml

Solve for x = Concentration of diluted stock

Multiply by 10 = Actual histone concentration

2. Dilute the purified core histones to a final protein concentration of 1 mg/ml in ddH<sub>2</sub>O. Prepare 1 to 5 µg aliquots of the histones. Flash freeze and store at -80°C.

## Section F: Acylation of Purified Histones

1. Prepare samples by diluting between 1 to 5 µg of purified core histones with ddH<sub>2</sub>O to a final volume of 9 µl.
2. Add 1 µl Bicarbonate Buffer to each reaction.
3. Working in a fume hood, prepare a 1:100 dilution of Acylation Agent in ddH<sub>2</sub>O. Immediately add 1 µl of diluted solution to each reaction. Vortex and incubate for 2 minutes at room temperature. Discard any unused dilution.
 

**Note:** As the bicarbonate buffer neutralizes the protons created during acylation, CO<sub>2</sub> may buildup in the tubes. Open and close the tubes several times during the incubation to release any CO<sub>2</sub> buildup and prevent tube explosion.
4. Add 1 µl Quenching Buffer and incubate 20 minutes at room temperature.

## Section G: Tryptic Digestion and Chemical Labeling

1. Resuspend trypsin to a final concentration of 0.1 mg/ml with the appropriate resuspension buffer. We recommend using Promega Sequencing grade modified Trypsin (Catalog No. V511A). It is important to limit the number of freeze-thaw cycles to five or dispense into single-use aliquots after resuspending.
2. Add 1 µl of the trypsin solution to each sample. Incubate 4 hours or overnight at 37°C.
3. Add 3 µl of a freshly prepared 30 mM phenyl isocyanate (PIC) solution in acetonitrile (1 % v/v solution). Incubate for 1 hour at 37°C.
 

**Note:** We suggest checking the efficiency of the PIC-labeling using MALDI-TOF.

4. Acidify samples by adding 8 µl 1% TFA to each sample.
5. Desalt histone samples using C18-stage-tips. Tips are wetted with 60% acetonitrile/0.1% TFA and then equilibrated with 20 µl of 0.1% TFA. Acidified samples are then diluted in 60 µl ddH<sub>2</sub>O and loaded into the C18-stage-tips. Tips are washed twice with 20 µl 0.1%TFA and eluted with 3 µl of 60% acetonitrile/0.1% TFA and used immediately or dried in a vacuum centrifuge and stored at -20°C.
6. Follow the recommendation for resuspension of the stage-tip desalted histone and mass spectrometry analysis provided for the LC/MS being used. Below are the conditions utilized on the Orbitrap Elite hybrid linear ion trap/orbitrap mass spectrometer:
  - a. Resuspend in Solvent A (0.1% v/v formic acid, 2% v/v acetonitrile in HPLC-grade water) to the recommended concentration for the LC/MS.
  - b. Load peptides onto a C18 column (Waters BEH-C18; 100 µm i.d. x 10 cm; 1.7 µm particles, 130Å pores) for 10 minutes at 1.5 µl/min in 2% solvent B (0.1% v/v formic acid, 98% v/v acetonitrile) and separated at 1 µl per minute by a linear gradient from 2% solvent B to 25% solvent B over 60 minutes followed by a ramp to 40% solvent B in 15 minutes, then to 90% solvent B and re-equilibration at 2% solvent B for a 90 minute total run time.
  - c. Full mass range spectra were collected at 60,000 resolution (defined as M/dM at m/z 400) and product ions were collected in a top 15 data dependent scan cycle at unit resolution in the ion trap mass analyzer (resonance collision-induced dissociation) or at 15,000 resolution in the orbitrap (higher energy collisional dissociation, HCD), respectively.
  - d. AUC values for peptide peaks were analyzed using an in-house program.

#### References:

**Mass Spectrometric Quantification of Histone Post-translational Modifications by a Hybrid Chemical Labeling Method** by Maile, T. M. *et al.* (2015) *Molecular & Cellular Proteomics* 14 (4): 1148-1158.