

# Protocol for Decoding the Protein Composition of Whole Nucleosomes with Nuc-MS: Sample Preparation, Data Acquisition and Analysis

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## Method Article

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# Abstract

The disassembly and digestion of nucleosome particles in current proteomics approaches forfeits correlations among histones and blurs nucleosome-level information. We developed Nuc-MS which analyzes whole nucleosomes and displays their histone modifications and variants in a single mass spectrum. In this protocol, we provide step-by-step instructions for preparation of mononucleosomes for mass spectrometry (MS) analysis, and parameter sets for Nuc-MS data acquisition and data analysis.

## Introduction

## Reagents

## Equipment

## Procedure

### Sample Preparation

#### Mononucleosome Extraction from Cells and Purification

1. With 2.5 mL cell pellet, resuspend cell pellet with 2.5X PCV of Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 340 mM sucrose (12% w/v), 10% glycerol (v/v), 0.5 mM DTT, and 1X Roche cOmplete EDTA-free protease inhibitor cocktail).
2. Lyse cells by adding equal volume of Buffer A supplemented with 2% Triton X-100
  - a. Note: Final concentration of Triton X-100 in cell suspension is 1%.
3. Incubate on ice for 10 mins with occasional mixing.
4. Pellet nuclei by centrifuging at 1,300 xg for 5 minutes at 4C.
5. Resuspend nuclei pellet with 6X PCV of Buffer A.
6. Transfer nuclei suspension onto 35 mL sucrose cushion (10 mM HEPES pH 7.9, 30% (w/v) sucrose, 1.5 mM MgCl<sub>2</sub>).
7. Pellet nuclei by centrifuging at 1,300 xg for 12 minutes at 4C to separate nuclei from cell debris.
8. Resuspend nuclei pellet with 2X PCV of Buffer A.
9. Incubate at room temperature for 5 mins.

10. Supplement nuclei suspension with 1 mM CaCl<sub>2</sub>.
11. Supplement nuclei suspension with 2  $\mu$ L of NEB micrococcal nuclease per 1 mL of cell suspension.
12. Incubate at 37C for 15 minutes with occasional mixing.
13. To quench micrococcal nuclease digestion, incubate on ice and supplement nuclei suspension with 2 mM EGTA and 1 mM EDTA.
14. Supplement nuclei suspension with final concentration of 100 mM KCl and 0.05% Triton X-100.
15. Incubate 15 minutes on ice with occasional mixing.
16. Centrifuge at 20,000 xg for 20 mins at 4C and collect resulting supernatant containing nucleosomes.
17. Mononucleosomes are concentrated and buffer exchanged into Buffer A supplemented with 650 mM NaCl using 30 kDa MWCO spin filter (*Millipore-Sigma*).
18. Concentrated nucleosomes are purified using HiPrep™ 26/60 Sephacryl S-300 HR column (Size Exclusion Chromatography) equilibrated with Buffer A supplemented with 650 mM NaCl using AKTA Prime Plus FPLC (*GE Lifescience*).
19. Fractions containing mononucleosomes are validated by purifying 10 ug of material using Qiagen DNA clean up kit and resolving it on 2% agarose gel in 0.5X TBE.
  - a. Note: Fractions showing ~150 DNA bp are pooled together for Nuc-MS analysis.

### *Sample Desalting for Nuc-MS*

1. Purified mononucleosomes and synthetic nucleosomes (Nucs; e.g. *EpiCypher* designer Nucs) must be concentrated and desalted into 150 mM ammonium acetate solution. Best results are achieved using the 0.5 mL 30 kDa MWCO spin filter (*Millipore-Sigma*). A concentration >2  $\mu$ M in a volume of 50  $\mu$ L yields adequate signal intensity for MS<sup>1-3</sup>.
2. Filter is first conditioned by spinning 500  $\mu$ L of 150 mM ammonium acetate for 3 mins at 13,000 xg. Upon completion of spin, empty the filter of any remaining liquid.
3. Add up to 500  $\mu$ L of Nuc sample into the filter – topping off with ammonium acetate – and spin for 5 mins at 13,000 xg or until sample is concentrated at or below 100  $\mu$ L.
4. For sample desalting, add ammonium acetate up to the 500  $\mu$ L mark and spin sample for 5 mins at 13,000 xg. Repeat desalting process 10-15 times for best results.

5. For final spin, centrifuge for 10 minutes to concentrate sample  $>2 \mu\text{M}$  in  $<50 \mu\text{L}$ .

## Nuc-MS

### *Data Acquisition*

1. Instrumentation: Given the high mass of Nuc particles, the Q Exactive HF mass spectrometer with Extended Mass Range (QE-EMR) and Q Exactive HF Ultra-High Mass Range (QE-UHMR) instrumentation are best for Nuc-MS analysis. Other instruments with up to 8000 m/z transmission and detection range may also be suited for Nuc-MS.
2. XCalibur QualBrowser 4.0.27.10 (*Thermo Fisher Scientific*) is used for MS data acquisition.
3. Native electrospray ionization (nESI) can be achieved with commercial Nanospray and Nanospray Flex Ion Sources with a static NSI probe (*Thermo Fisher Scientific*) as well as a capillary-based ion source as described previously [1].
4. Once stable electrospray is achieved, the  $\text{MS}^1$  (intact mass of Nuc particle) can be collected using the following parameters in positive ion mode (QE-EMR ; QE-UHMR):
  - a. Injection time: Fixed at 50 ms ; Fixed at 200 ms
  - b. Number of microscans: 10 ; 10
  - c. Resolution ( $\text{MS}^1$ ): 15,000 at 400 m/z ; 17,500 at 200 m/z
  - d. Ultra-High Vacuum (UHV) Pressure: 4 V:  $1.65\text{E-}9$  mBar ; 2-4 V:  $7.7\text{E-}11$  to  $1.55\text{E-}10$  mBar
  - e. Source Induced Dissociation (SID): 25 V ; 5 V
  - f. In-Source Trapping (IST): Not available ; 25 to 50 V
  - g. HCD trapping energy: 10 V ; 0 V
  - h. Capillary temperature: 330 °C ; 300 °C
5. Ejection of histones ( $\text{MS}^2$ ) is achieved with high-energy collision dissociation (HCD) activation of an isolated nucleosome charge state for synthetic Nucs or isolation of the entire charge state distribution for

endogenous mononucleosomes. The isolation window using the quadrupole mass filter should be optimized based on heterogeneity of the sample and signal intensity output. The following parameters are recommended once the desired species/population is isolated (QE-EMR ; QE-UHMR):

- a. Injection time: Fixed at 500 ms ; Fixed at 500 ms
- b. Number of microscans: 10-20 ; 10-20
- c. Resolution (MS<sup>2</sup>): 120,000 at 400 m/z ; 100,000 at 200 m/z
- d. Ultra-High Vacuum (UHV) Pressure: 2 V: 7.11E-10 mBar ; 2 V: 7.7E-11 mBar
- e. Source Induced Dissociation (SID): 5 V ; 0 V
- f. In-Source Trapping (IST): Not available ; 25 to 50 V
- g. HCD trapping energy: 120 V ; 5-30 V (Normalized Collision Energy, NCE)

6. For histone ejection at the source in preparation for pseudo-MS<sup>3</sup>, the following parameters are recommended (QE-EMR ; QE-UHMR):

- a. Injection time: Fixed at 100-300 ms ; Fixed at 100-300 ms
- b. Number of microscans: 2 ; 2
- c. Resolution (MS<sup>2</sup>): 120,000 at 400 m/z ; 100,000 at 200 m/z
- d. Ultra-High Vacuum (UHV) Pressure: 2 V: 7.11E-10 mBar ; 1-2 V: 4.67E-11 to 7.7E-11 mBar
- e. Source Induced Dissociation (SID): 50-150 V \* ; 0 V
- f. In-Source Trapping (IST): Not available ; 150 to 200 V
- g. HCD trapping energy: 0 V ; 0 V (Normalized Collision Energy, NCE)

h. \*Note that histone ejection at the source using SID on the QE-EMR is not as efficient as IST on the QE-UHMR. A 50-150 V SID range is provided given that some DNA fragmentation occurs which must be balanced with histone ejection.

7. For fragmentation of source-ejected histones ( $MS^3$  / pseudo- $MS^3$ ), the desired histone proteoform charge state is manually isolated using the quadrupole mass filter with an isolation window of 5-10  $m/z$ . The following parameters are recommended once stable signal is achieved for the isolated species (QE-EMR ; QE-UHMR):

- a. Injection time: Fixed at 1000-2000 ms ; Fixed at 1000-2000 ms
- b. Number of microscans: 10 ; 10
- c. Resolution ( $MS^2$ ): 120,000 at 400  $m/z$  ; 100,000 at 200  $m/z$
- d. Ultra-High Vacuum (UHV) Pressure: 2 V: 7.11E-10 mBar ; 2 V: 7.7E-11 mBar
- e. Source Induced Dissociation (SID): 50 V ; 0 V
- f. In-Source Trapping (IST): Not available ; 150 to 200 V
- g. HCD trapping energy: 120 V ; 30-40 V (Normalized Collision Energy, NCE)

### *Data Analysis*

8.  $MS^1$  and  $MS^2$  data at medium-resolution (not isotopic) can be deconvoluted and visualized using MagTran 1.03 [2] (mass range: 15-300 kDa; max no. of species: 10-15; S/N threshold: 1; mass accuracy: 0.05 Da; charge determined by: charge envelop only) and UniDec 3.2.0 [3] (Charge Range: 20 – 50, Mass Range: 15 – 300 kDa, Sample mass every 0.5 Da).

9. Isotopically resolved  $MS^2$  data can be analyzed using Xtract (Signal-to-Noise threshold ranging from 1-30, *Thermo Fisher Scientific*) and mMass 5.5.0 ([www.mmass.org](http://www.mmass.org)).

10.  $MS^3$  data can be processed using Xtract (Signal-to-Noise threshold ranging from 1-30, *Thermo Fisher Scientific*), which provides the monoisotopic masses for the detected fragment ions. mMass 5.5.0 ([www.mmass.org](http://www.mmass.org)), ProSight Lite 1.4 [4] (precursor mass type: average; fragmentation method: HCD; fragmentation tolerance: 10-15 ppm), and TDValidator 1.0 [5] (max ppm tolerance: 25 ppm; cluster tolerance: 0.35; charge range: 1-10; minimum score: 0.5; S/N cutoff: 3; Mercury7 Limit: 0.0001; minimum size: 2) are used to assign recorded fragment ions to the primary sequence of the subunits.

11. For careful fragmentation analysis, ProSight Lite and TDValidator are used to analyze spectra in medium throughput to assign and validate *b* and *y* fragment ions to the histone sequences, and for

generating a p-score. mMass generates *in silico* a list of theoretical fragment ions for a target proteoform and is thus used to interrogate individual fragment ions within a spectrum not identified by TDValidator or ProSight Lite.

12. Unexplained mass shifts ( $\Delta m$ ) observed at the MS<sup>1</sup>, MS<sup>2</sup>, and MS<sup>3</sup> levels were manually interrogated using the UNIMOD database ([http://www.unimod.org/modifications\\_list.php](http://www.unimod.org/modifications_list.php)) as a reference for candidate modifications.

## Troubleshooting

## Time Taken

## Anticipated Results

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