A mass spectrometry-based workflow to analyze histone post-translational modifications and the associated acid extractome

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Method Article

Keywords: Histone post-translational modification, LC-MS/MS, protein, acid extractome, peptide, relative abundance, proteomics, epigenetics, human embryonic stem cells

Posted Date: May 31st, 2024

DOI: https://doi.org/10.21203/rs.3.pex-1909/v1

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Abstract

Worldwide, there is an increasing interest in epigenetics because of its involvement in several diseases. An important epigenetic mechanism is the histone post-translational modifications and the histone code they generate. Mass spectrometry (MS) offers the opportunity to comprehensively characterize the histone code in one single experiment. We developed a workflow that allows starting from a cell pellet to: 1) extract the histones, 2) analyze them via bottom-up proteomics using data-dependent acquisition (DDA) mass spectrometry, and 3) identify and quantify them via our data analysis pipeline. The time required to complete the entire workflow is highly dependent on the number of samples to be analyzed and should be considered as at least 1 week of sample preparation prior to the acquisition, approximately 90 minutes of acquisition time per sample and thereafter one to several weeks of data analysis depending on the complexity of the data.

Introduction

For a long time, the focus within epigenetics was largely on DNA methylations and non-coding RNAs. The increasing interest in histones and the associated post-translational modifications (hPTMs) in recent years has led to many new insights. In addition to the well-known hPTMs such as acetylation, methylation, phosphorylation, and ubiquitination, new hPTMs are being discovered with recently lactylations, dopaminylation, and serotonylation. Until today, sequencing-based approaches such as ChIP-seq have been mainly used. Much of the information we currently have on hPTMs is therefore obtained using antibodies that target specific hPTMs providing the benefit of localizing the hPTM within the genome. However, in this way, no genome-wide picture or info can be obtained on combinations of hPTMs, which can lead to misconceptions about histone biology.

Our workflow allows us to get a broader picture of the underlying dynamics of the histone code by simultaneously mapping different hPTMs in an untargeted way. The workflow is in principle applicable to any cell type but was optimized mainly for the purpose of using stem cells.

Reagents

- Phosphate Buffered Saline (Thermo Fisher Scientific)
- Tris-HCl
- Potassium Chloride
- Magnesium chloride
- Dithiothreitol
- Halt Protease and Phosphatase Inhibitor Cocktail 100x (78440)
- Phosphatase inhibitor cocktails II and III (P5726 and P0044, Sigma-Aldrich)
- Hydrogen chloride (Chem-Lab NV)
- Trichloroacetic acid (Chem-Lab NV)
- Acetone (Sigma Aldrich)
- MilliQ water (Sartorius)
- Laemmli-buffer (Bio-Rad Laboratories)
- β-mercaptoethanol (Bio-Rad Laboratories)
- β-galactosidase (Sciex)
- Sodium dodecyl sulfate
- Glycine
- Bovine histone standard (Promega)
- Acetic acid
- Methanol
- Sypro® Ruby protein gel stain (IntivtrogenTM Molecular ProbesTM)
- Triethylammonium bicarbonate (Sigma Aldric)
- Isopropylalcohol (Biosolve Chimie SARL)
- Propionic anhydride (Sigma Aldric)
- Acetonitrile (Merck)
- Calciumchloride (Thermo Fisher Scientific)
- Sequencing Grade Modified Trypsin (Promega)
- Hydroxylamine (Sigma Aldrich)
Ammonium hydroxide (Sigma Aldrich)
Formic acid (Chem-Lab NV)
8–16% TGX gel (Biorad)

**Equipment**

**Equipment:**
- Eppendorf Thermomixer Comfort (Eppendorf)
- VersaDoc, Imaging System Model 3000 (Bio-Rad Laboratories)
- NanoLC 425 system (Eksigent)
- TripleTOF 6600+ (Sciex)
- Centrifuge (Sigma Aldrich)
- Speedvac concentrator SPD 111V (Thermo Fisher Scientific)

**Software:**
- Progenesis QIP 4.2. (Nonlinear Dynamics, Waters)
- Mascot Daemon v2.6.0 (Matrix Science)
- MSqRob v0.7.6 (https://github.com/statOmics/MSqRob )

**Procedure**

**Protocol 1: Histone extraction (HLB)**

1. Resuspend the cells in cold Phosphate Buffered Saline (PBS): 1*10^6 cells/50µl.
2. Distribute in 1.5 mL Eppendorfs: 200µl = 4*10^6 cells
3. Spin down for 10minutes at 4°C and 300g
4. Remove the supernatant
5. Add 800 µL Hypotonic Lysis Buffer (HLB) to the cell pellet (200 µL for 1*10^6 cells)
6. Prepare HLB buffer: 10 mM Tris-HCl pH 8.0, 1 mM KCl, 1.5 mM MgCl2 supplemented with 1 mM DTT, Halt Protease and Phosphatase Inhibitor Cocktail 100x (78440) and phosphatase inhibitor cocktails
II and III (P5726 and P0044, Sigma-Aldrich, 1 mL of cocktail for 100 mL of buffer) (See Table 1 for quantities).

7. Rotate for 30 minutes at 4°C to promote lysis of cell membrane (mechanical shear)

8. Pellet the nuclei in centrifuge for 10 minutes at 4°C and 10 000 g

9. Discard supernatant

10. Resuspend the pellet in 125 µL (for 1*10^6 cells) 0.4N HCl by soft pipetting until no clumps left in solution (if necessary: vortex)

11. Incubate 30 minutes in acid on rotator at 4°C to promote lysis of nuclei and solubilization of histones

12. Spin down for 10 minutes at 4°C and 16000 g.

13. Transfer supernatant to new Eppendorf (histones are present in the acid since they are alkaline proteins)

14. Add drop by drop, TCA until a final concentration of 33% is reached to promote precipitation of histones and invert the tube several times (results in a milky solution)

15. Incubate on ice for 30 minutes

16. Spin for 10 minutes at 4°C and 16000 g to pellet the histones

17. Remove the supernatant (be careful: the pellet is not always visible)

18. Add ice-cold acetone (do not resuspend the pellet) to remove TCA, make sure the pellet is fully covered with acetone

19. Spin for 5 minutes at 4°C and 16000 g

20. Remove the supernatant

21. Add cold acetone again (do not resuspend the pellet) to remove TCA.

22. Spin for 5 minutes at 4°C and 16000 g

23. Remove the supernatant

24. Dry at room temperature for 30 minutes (until no acetone left)

25. Resuspend in MilliQ water (50 µl for 1*10^6 cells)
26. Transfer 400,000 cells (20 µl) to a new Eppendorf tube for gel-electrophoresis (optionally) (If there are still clumps left: Spin for 10 minutes at 4°C and 16000 g and transfer the supernatant in a fresh Eppendorf)

27. Vacuum dry the samples (centrivap)

Protocol 2: Gel-electrophoresis

Sample preparation

1. Dry sample (equal to 400,000 cells)
2. Resuspend samples in 10 µl laemmlí-buffer
3. Add 1 µl β-mercaptoethanol in a fume hood to each sample
4. Vortex and spin down
5. Incubate for 7 minutes at 95°C in a thermoshaker
6. Spin down

Prepare Criterion Cell

7. Place the criterion cell on ice in a fume hood
8. Remove the sticker from the bottom of the gel cassette and check the gel for cracks
9. Put the gel cassette in the criterion cell
10. Fill the reservoir with running buffer (25 mM Tris, 0.1% SDS, and 192 mM glycine in MilliQ water) and take out the comb

Running of the samples

11. Load the samples and standards (2 µg of bovine histones) on the gel (3 standards per gel: lane 1, lane 9 and lane 18)
12. Put the cover on the criterion cell
13. Start running the gel on 200V
14. Stop running when the frontline is almost gone

Visualization
15. Take out the cassette

16. Incubate in fixation-solution (7% acetic acid, 10% methanol in MilliQ water) for 10 minutes on a shaker

17. Wash the gel 3 times for 5 minutes in MilliQ water on a shaker

18. Incubate in SyproRuby overnight

19. Wash the gel 3x for 10 minutes in MilliQ water on a shaker

20. Visualize the gel by using a Versadoc

**Protocol 3: Propionylation and tryptic digestion**

**Propionylation before**

1. Vacuum dry the samples (20 µg/sample)

2. Add 20 µL TEAB (1M)

3. Add 20 µL Prop-reagent (Isopropylalcohol:propionic anhydride (79:1))

4. Spin down & Incubate at room temperature for 30 minutes

5. Add 20 µL H₂O

6. Spin down & Incubate at 37°C for 30 minutes

7. Vacuum dry samples

**Trypsin digest (Final Volume: 50 µl)**

8. Add 500 mM TEAB

9. Add CaCl₂ and ACN

   a. Final conc CaCl₂: 1 mM

   b. Final conc ACN: 5%

10. Resuspend trypsin in 500 mM TEAB

11. Add trypsin at a 1:20 ratio (w/w) => 1 µg trypsin/20 µg histones

12. Spin down & incubate overnight at 37°C
13. Vacuum dry samples

**Propionylation after**

14. Vacuum dried samples (20 µg/sample)

15. Add 20 µL TEAB (1M)

16. Add 20 µL Prop-reagent (Isopropylalcohol:propionic anhydride (79:1))

17. Spin down & Incubate at room temperature for 30minutes

18. Add 20 µL H₂O

19. Spin down & Incubate at 37°C for 30minutes

20. Vacuum dry samples

**Reversing overpropionylation hydroxylamine mediated**

21. Vacuum dried samples (20 µg/sample)

22. Add 50 µL 0.5 M NH₂OH

23. Add 15 µL NH₄OH at pH 12

24. Spin down & Incubate at room temperature for 20minutes

25. Adjust pH with formic acid: 30 µl 100% FA

26. Vacuum dry samples

**Data-dependent acquisition mass-spectrometry**

1. The propionylated samples, complemented with a Beta-Galactosidase (ß-gal) (Sciex) are resuspended in 0,1% FA resulting in 1.5 µg histones and 50 fmol ß-gal in a 9 µL injection.

2. A quality control (QC) mixture is created by combining 2 µL of each sample.

3. The samples are acquired on a mass spectrometer according to the parameters in **Parameter file 1**.

**Data-analysis of hPTMs**
1. For all runs, raw data is imported in Progenesis QIP 4.2. (Nonlinear Dynamics, Waters) followed by alignment, feature detection, and normalization.

2. Next, an *.mgf file is created based on the twenty MS/MS spectra closest to the elution apex and exported for searches using Mascot (Matrix Science).

3. First, a standard search is performed on the exported *.mgf file to identify non-propionylated standards (ß-gal and MPDS) and to verify underpropionylation (Parameter file 2).

4. Second, to identify the proteins present in the sample and to detect unexpected histone post-translational modifications, an error-tolerant search with fixed K- and N-term propionylation without biological modifications is carried out against a complete Human Swissprot database (downloaded from Uniprot and supplemented with contaminants from the cRAP database (https://www.thegpm.org/crap/)) (Parameter file 3).

5. Subsequently, a FASTA database is created based on the results of the error-tolerant search.

6. Next, the three MS/MS spectra closest to the elution apex per feature are merged into a single *.mgf file and exported for a Mascot-search including the following parameters: 1) a mass error tolerance of 10 ppm and 50 ppm for the precursor ions and the fragment ions respectively; 2) Arg-C enzyme specificity, allowing for up to one missed cleavage site; 3) variable modifications included acetylation, butyrylation, crotonylation, and trimethylation on K, methylation on R, dimethylation on both K and R, deamidation on asparagine (N), glutamine (Q) and R (the latter representing citrullination), phosphorylation on serine (S) and threonine (T), and oxidation of methionine (M); and 4) fixed modifications included N-terminal propionylation and propionylation on K (Parameter file 4).

7. The search is performed against the above-mentioned custom-made FASTA database.

8. This Mascot result file (*.xml-format) is imported into Progenesis QIP 4.2 for annotation.

9. To resolve isobaric near-coelution, features that are identified as histone-peptidoforms are manually validated and curated.

10. To correct for variations in sample loading, samples are normalized against all histone peptides.

11. Outlier detection and removal are done based on the principal component analysis (PCA).

12. Finally, the deconvoluted peptide ion data of all histones can be exported from Progenesis QIP 4.2 for further analysis.

A fully detailed description of the use of Progenesis QIP for histone analysis can be found in Provez et al. (https://www.biorxiv.org/content/10.1101/2022.05.05.490796v1)
Acid extractome analysis after histone extraction

Next to histones, other alkaline proteins remain in the HCl during acid extraction. For this purpose, we export the protein data from Progenesis QIP and use the MSqRob software for statistical analysis. Herein, relative protein quantification is done by implementing the peptide-level robust ridge regression method.

1. First, the deconvoluted peptide ion data of all identified peptides is exported from Progenesis QIP 4.2.

2. MSqRob requires an annotation file that contains the name of the runs included in the experiment and as well as the condition to which each run belongs (i.e. naïve or primed).

3. Log transformation and quantile normalization of the data are performed by the software.

4. Finally, pairwise comparisons (e.g. naive versus primed) are carried out and the result files can be exported for further use.

Troubleshooting

Time Taken

Anticipated Results

References


Acknowledgements

We want to thank Elisabeth Govaert and Paulien Meert for all their efforts in optimizing the different steps of the protocol.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ParameterFile1.pdf
- ParameterFile2.pdf
- ParameterFile3.pdf
- ParameterFile4.pdf
- Table1HLBBuffer.pdf