Quantitative Studies of Cancer Epigenetics Using Label-Free Top Down and Bottom Up to Interrogate Histone Modifications

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Overview

→ nLC QqQ assay developed for studying multiple methylation (Lysine 9, 27, 36, and 79) states of histone H3.

→ Technical triplicates and biological duplicates have CVs of 1 to 15 %, depending on signal magnitude; percent standard deviations between 0.25 and 2.00 %, dependent on the limit-of-quantitation.

Introduction

Changes in histone modifications (methylation, acetylation, phosphorylation, *etc.*) may be characterized by mass spectrometry.[1, 2] This includes all histones (H1, H2A, H2B, H3, and H4) and their combinatorial modification patterns, which may be described as a the "histone code".[3] Here, we report the optimization and application of nano-liquid chromatography triple quadrupole mass spectrometry (nLC QqQ MS) to measure methylation (unmodified, mono-, di-, and tri-) and acetylation changes on Histone H3 lysines 4, 9, 14, 27 ,36, and 79 – along with acetylation of K9 and K14. The assay is applied to histones extracted from many different types of cancer, such as glioma and leukemia. The result is a relative quantitation assay to complement methylation profiling via top down MS.

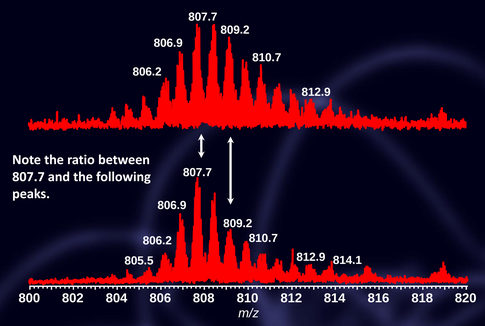
[1]Garcia B.A., et al., NatureMethods, 4, 487-489, 2007.
[2]Plazas-Mayorca, M.D., et al., JPR, 8, 5367-5374, 2009.
[3]Jenuwein T., et al., Science, 293, 1074-1080, 2001.

Methods

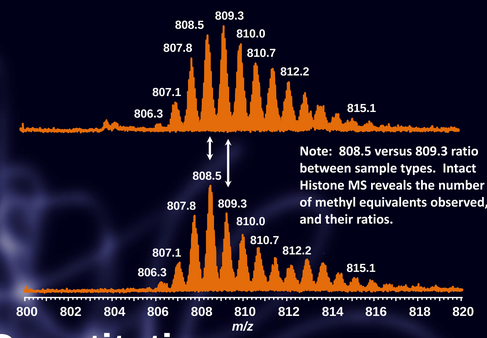
Histones were extracted with a reported acid extraction protocol.[4] A small portion of extract from some samples were profiled by on-line top down proteomics with a 12 tesla linear ion trap FT ICR mass spectrometer to estimate the number of methyl-equivalents. The rest of the sample was processed through a standard propionylation/trypsin digestion protocol.[5] LC-ready samples (~1ug/uL on column) were analyzed with a TSQ Quantum Ultra triple quadrupole mass spectrometer in technical triplicate. Optimization of MRM transitions for the histone peptides of interest was performed with a combination of standard synthetic peptides, Skyline,[6] and Pinpoint software. Final results for relative quantitation were generated by custom algorithms and scripts.

[4] Martinez-Garcia, E., et al., Blood, 117, 211-220, 2010.
[5] Garcia, B.A., et al., NatureProtocols, 2, 933-938, 2007.
[6] MacLean, B., et al., Bioinformatics, 26, 966-968, 2010.

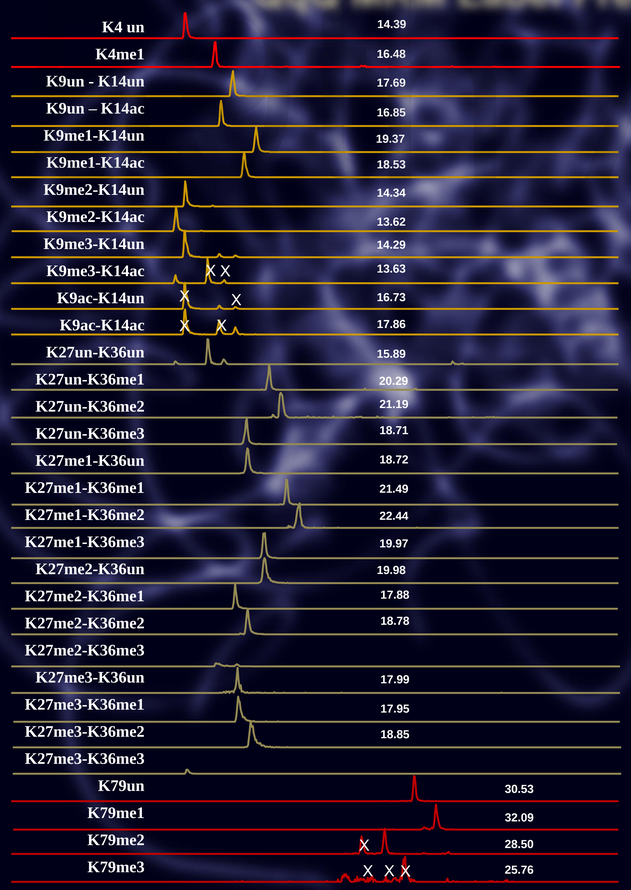
On-line (nanoLC) Histone H3 Profiles



To the left, H3.2 (red), to the right H3.1 (orange). The top spectra are histone extracted from glioblastoma; the bottom spectra are histone extracted from HeLa S3 cells. The two forms were separated (~5 ug of total protein from acid extract) on PLRP-S material. The exact mass of the resulting de-isotoped and deconvoluted spectra revealed methylation (mass difference of 14 Da). There is an apparent shift in the amount of methylation for glioblastoma and HeLa derived H3.2 and H3.3.



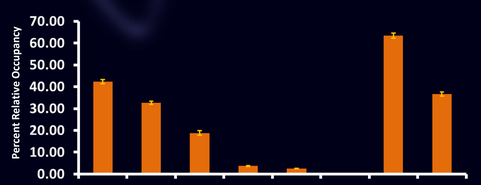
QqQ MRM Label Free Relative Quantitation



Percent Relative Occupancy for K9 K14 Peptide

Total Protein Amount (µg)	K9un K14un	K9un K14Ac	K9me 1K14un	K9me1 K14Ac	K9me2 K14un	K9me2 K14Ac	K9me3 K14un	K9me3 K14Ac	K9Ac K14un	K9Ac K14Ac
1.00	26.58	14.51	18.71	12.59	13.59	7.49	3.73	0.28	0.93	1.61
1.00	30.17	16.82	20.03	12.02	9.30	5.65	2.90	0.22	1.14	1.76
2.00	26.34	14.68	18.80	12.94	12.95	8.04	3.51	0.29	0.99	1.46
3.00	26.76	14.00	20.89	13.17	11.62	7.46	3.35	0.30	0.98	1.47
4.00	25.28	14.08	19.55	12.30	15.23	6.77	4.08	0.28	0.94	1.49
0.50	28.34	13.27	18.20	12.81	11.65	9.39	3.71	0.33	0.92	1.38
0.50	30.73	13.52	19.11	15.08	10.35	5.51	3.00	0.20	1.02	1.47
1.00	27.34	13.06	18.83	13.12	12.79	8.46	3.66	0.29	0.91	1.52
1.00	29.52	13.91	19.40	14.48	10.68	5.84	3.37	0.20	1.00	1.60
1.50	27.94	13.80	18.12	12.54	12.57	8.62	3.64	0.31	0.97	1.48
1.50	28.53	14.80	20.49	15.78	9.35	5.29	2.86	0.25	1.03	1.61
2.00	27.42	13.95	18.51	12.53	12.79	8.50	3.59	0.35	0.92	1.44
2.00	26.86	15.43	19.41	13.60	11.25	7.49	3.19	0.32	1.02	1.43
0.33	27.34	13.71	21.51	12.08	11.82	7.20	3.50	0.23	1.00	1.61
0.33	32.09	13.05	19.28	12.33	10.60	7.37	2.78	0.23	1.00	1.28
0.67	28.71	14.02	19.53	12.76	11.25	7.59	3.39	0.28	0.98	1.49
1.00	28.93	15.76	19.74	15.26	9.07	5.51	2.87	0.27	0.98	1.61
1.33	29.59	16.18	19.39	13.47	10.15	5.06	3.21	0.24	1.13	1.58
0.25	27.43	14.41	19.40	14.06	11.19	7.16	3.31	0.24	1.09	1.70
0.50	26.51	13.50	18.12	13.14	13.40	8.54	3.97	0.30	0.91	1.62
0.75	28.24	13.01	18.72	13.40	12.45	7.93	3.61	0.31	0.90	1.43
1.00	27.39	14.03	20.03	13.53	11.39	7.55	3.43	0.30	0.87	1.49
AVG	28.09	14.25	19.35	13.32	11.61	7.20	3.39	0.27	0.98	1.52
STD	1.61	1.02	0.87	1.04	1.53	1.23	0.36	0.04	0.07	0.11
	K9un	K9me1	K9me2	K9me3	K9ac				K14un	K14ac
Sum	42.34	32.67	18.81	3.67	2.51			Sum	63.43	36.57
Error	1.91	1.36	1.97	0.36	0.13			Error	2.42	1.91
CV%	4.50	4.16	10.47	9.79	5.23			CV%	3.81	5.24

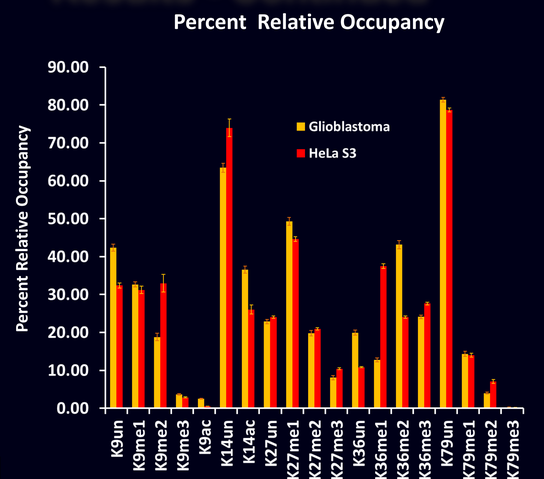
Percent Relative Occupancy K9 and K14



To the left, the percent relative occupancy of K9/K14 from 22 injections of a single sample over one week. The sample was diluted to 4 different concentrations prior to 4 different injection volumes. The final estimated amount of protein is found in the left column. The percent relative occupancy is calculated by adding all of the peak areas for the signal associated with peptides that contain K9/K14, then dividing each individual peak area by the total sum. Remember, trypsin will not likely cleave propionylated or hyper methylated lysine. At different concentrations of total protein loaded, the % relative standard deviation for each peptide is typically less than 2.0%. Further, the coefficient of variance is less than 10 %.

To the left, the percent relative occupancy plotted for the above presented final averages and standard deviations. The system is stable over time and repeated injections. Note, the propionylation of lysine will change the ionization and fragmentation efficiencies.

Results - Continued



K9un	K9me1	K9me2	K9me3	K9ac	K14un	K14ac	K27un	K27me1	K27me2	K27me3	K36un	K36me1	K36me2	K36me3	K79un	K79me1	K79me2	K79me3
9.96	1.46	-14.17	0.82	1.93	-10.52	10.52	-1.12	4.64	-1.18	-2.34	9.12	-24.78	19.16	-3.50	2.66	0.34	-3.06	0.06

The above plot presents the methylation status of K9/K27/K36/K79 (and acetylation status for K9/K14) for histone extracted from glioblastoma and HeLa S3 cell lines. Top down analysis revealed a mixed mode of differences for methylation equivalents. The QqQ analysis of peptides further illustrates the mixture of differences between the two sample types. The above table is percent relative occupancy for glioblastoma subtracted from the hela derived samples (for each mark). Thus, the K9 site reveals less methylation for glioblastoma.

Coefficient of Variance	K9un	K9me1	K9me2	K9me3	K9ac	K14un	K14ac	K27un	K27me1	K27me2	K27me3	K36un	K36me1	K36me2	K36me3	K79un	K79me1	K79me2	K79me3
(%) CV Glio IV	4.50	4.16	10.47	9.79	5.23	3.81	5.24	4.99	4.21	7.10	13.49	6.86	8.52	5.06	4.05	1.54	9.44	12.56	64.36
(%) CV HeLa	4.11	6.44	14.25	9.82	8.34	6.34	9.41	2.61	2.68	3.03	5.59	2.65	3.26	2.40	2.91	1.27	7.17	15.56	45.82

The percent coefficient of variance (above table) for both samples is presented above. Note, the % CV increases with a peptide of low relative abundance due to the lower limits-of-detection and lower limits-of-quantitation for methylation marks that are of low biological abundance.

Conclusions

A robust nLC-QqQ assay has been developed for the analysis of histone H3.(1,2,3) methylation and acetylation site specific status. Further, all transitions and retention times were verified with synthetic peptides or by accurate mass measurement with a LTQ-FT. Finally, the assay is stable over different concentrations of total protein loaded and multiple injections. We are currently applying the assay to understand the epigenetic causes of cancer.

Acknowledgements

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