A Label-Free Screening Approach to Epigenetic Drug Discovery: Using Mass Spectrometry to Monitor Protein and Nucleic Acid Modification Events

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Introduction

Epigenetics, the study of changes in gene expression caused by mechanisms other than deviations in DNA sequence, is rapidly emerging as a field with tremendous drug development potential across a broad range of therapeutic areas. Epigenetic discovery via traditional screening technologies is complicated by the ability of these enzymes to impart multiple modification events onto singular substrates resulting in complicated and dynamic enzyme kinetic environments. In this study, we investigated the use of mass spectrometry as an almost universal platform for the detection and quantification of multiple protein and nucleic acid modification events (i.e. histone acetylation status, histone methylation status, DNA methylation status). The use of mass spectrometry facilitates direct detection of native enzyme substrate and product pairs, and allows for rapid assay development of a wide array of chemical analytes (peptides, oligonucleotides, whole proteins, etc...). The label-free methodology mass spectrometry provides can enable targeted screening across a large collection of epigenetic processes and represents a ubiquitous approach to this complex research area.

Experimental

All assays were investigated via solid phase extraction electrospray mass spectrometry using the Agilent RapidFire High-throughput MS System (RapidFire/MS) which facilitates cycle times of < 10 seconds per sample.

Assay Specific Conditions

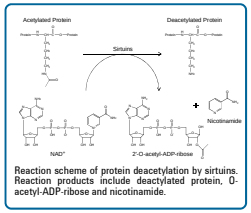
Analyte Class	RF Cartridge	Wash Buffer	Elute Buffer	MS System
Peptides	C4	ddH_2O 0.09% formic acid 0.01%TFA	100% MeCN 0.09% formic acid 0.01%TFA	QQQ (6460)
Co-factors	Graphitic Carbon	ddH_2O	25% MeCN 25% acetone 5 mM NH_4-acetate	QQQ (6460)
Whole Proteins	C4	ddH_2O 0.09% formic acid 0.01%TFA	100% MeCN 0.09% formic acid 0.01%TFA	Q-TOF (6530)
DNA Oligos	Cyano	ddH_2O 6 mM octylammonium acetate	80% MeCN 6 mM octylammonium acetate	QQQ (6460)

Mass spectrometry parameters were independently developed for each specific analyte of interest on the appropriate MS platform.

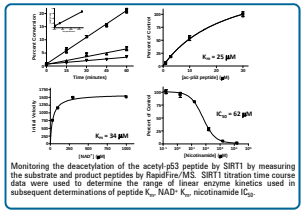
Histone Protein Acetylation Status

SIRT-1 Protein Deacetylation

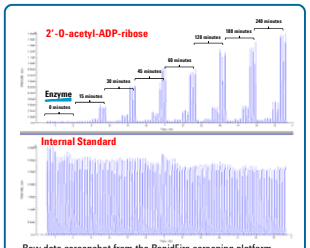
The sirtuin enzymes, a class of NAD+-dependent histone deacetylases, are a focal point of epigenetic research because of their roles in regulating gene expression and cellular differentiation by deacetylating histones and a host of transcription factors, including p53. SPE-MS/MS technology offers two independent label-free screening methodologies to study sirtuin activity; 1) peptide specific quantitation and 2) O-acetyl-ADP-ribose co-product quantitation.



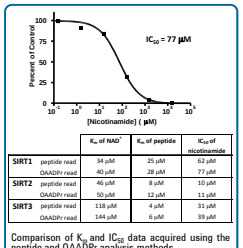
Reaction scheme of protein deacetylation by sirtuins. Reaction products include deactylated protein, O-acetyl-ADP-ribose and nicotinamide.



Monitoring the deacetylation of the acetyl-p53 peptide by SIRT1 by measuring the substrate and product peptides by RapidFire/MS. SIRT1 titration time course data were used to determine the range of linear enzyme kinetics used in subsequent determinations of peptide K_m, NAD+ K_m, nicotinamide IC_{50}.

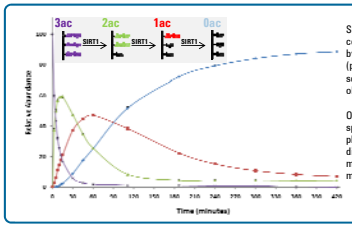


Raw data screenshot from the RapidFire screening platform.



		K_m of NAD+	K_m of peptide	IC_{50} of nicotinamide
SIRT1	peptide read	34 µM	25 µM	62 µM
	OAADPr read	40 µM	28 µM	77 µM
SIRT2	peptide read	46 µM	8 µM	10 µM
	OAADPr read	50 µM	12 µM	11 µM
SIRT3	peptide read	118 µM	4 µM	31 µM
	OAADPr read	144 µM	6 µM	39 µM

Comparison of K_m and IC_{50} data acquired using the peptide and OAADPr analysis methods.

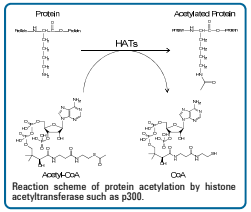


Successive deacetylation of a p53 peptide containing three acetylated (ac) lysine residues by SIRT 1. Conversion over time of 3ac peptides (purple X's) to 2ac (green triangles), 1ac (red squares), and 0ac (blue diamonds) species is observed.

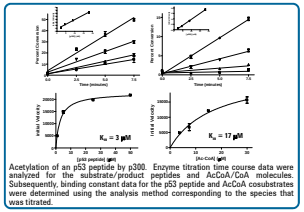
One of the distinct advantages of mass spectrometry over traditional optical screening platforms is the ability to simultaneously distinguish and independently quantitate multiple modfication events on single substrate molecules.

p300 Protein Acetylation

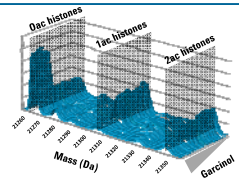
Histone acetyltransferases (HATs) catalyze the transfer of an acetyl group from an acetyl-coenzyme A donor molecule to specific lysine residues within proteins. The acetylation state of proteins, particularly histones, is known to modulate their intermolecular binding properties and control various cellular processes, most notably transcriptional activation. In addition, deregulation of HAT activity has been linked to the development of a number of cancers; therefore, compounds that affect these enzymes have strong potential as therapeutic agents. SPE/MS/MS technology offers three distinct label-free HAT screening approaches; 1) ACoA/CoA pair of cofactors, 2) direct peptide measurement and 3) whole protein measurement.

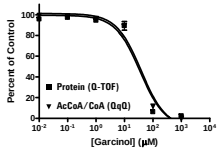


Reaction scheme of protein acetylation by histone acetyltransferase such as p300.



Acetylation of an p53 peptide by p300. Enzyme titration time course data were analyzed for the substrate/product peptides and AcCoA/CoA molecules. Subsequently, binding constant data for the p53 peptide and AcCoA cosubstrates were determined using the analysis method corresponding to the species that was titrated.





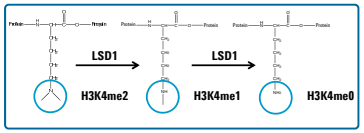
Inhibition of p300 by garcinol when using whole histone proteins as a substrate. Deconvoluted Q-TOF-MS data for 18 reactions (endpoint IC_{50}, 6 concentration in triplicate) showing increasing acetylation (going towards back) with decreasing inhibitor concentration. Comparison of inhibition data analyzed from Q-TOF-MS on whole histone proteins and traditional QQQ on AcCoA/CoA is included.

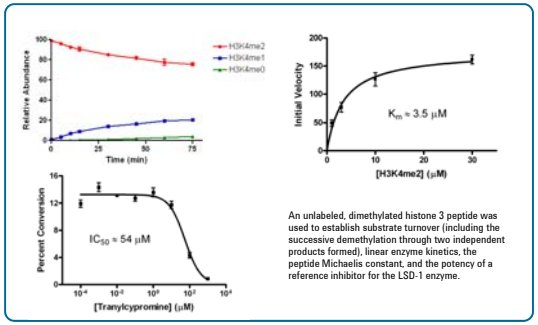
Incorporation of a high-resolution (Q-TOF) mass spectrometer into the RapidFire workflow allows the substitution of whole proteins for representative peptide based sequences in a high-throughput screening compatible mode.

Histone Protein Metylation Status

LSD-1 Histone Demethylation

Histone methylation status can be a major determinate in gene expression by altering histone protein structure thereby regulating the access to specific DNA sequences. The ability to regulate the transcription rate of a multitude of genes has been linked to a variety of disease states, making enzymes that control histone methylation attractive drug discovery targets. SPE/MS/MS technology presents the unique ability to uniquely identify and quantitate independent methylation events on single lysine residues within histone proteins.



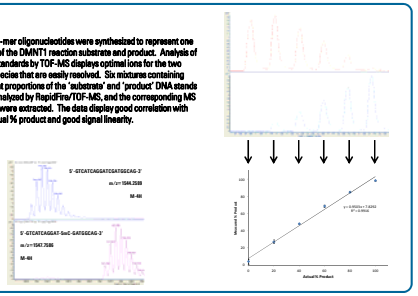


An unlabeled, dimethylated histone 3 peptide was used to establish substrate turnover (including the successive demethylation through two independent products formed), linear enzyme kinetics, the peptide Michaelis constant, and the potency of a reference inhibitor for the LSD-1 enzyme.

Nucleic Acid Methylation Status

Over the past couple years, the application of the RapidFire system to studying epigenetic modifications has grown significantly. Primarily, however, the target substrates have been peptide-based. To help establish the applicability of the platform for investigating epigenetic changes to DNA, preliminary experiments using DNA standards were conducted.

Two 20-mer oligonucleotides were synthesized to represent one strand of the DNMT1 reaction substrate and product. Analysis of these standards by TOF-MS displays optimal ions for the two DNA species that are easily resolved. Six mixtures containing different proportions of the 'substrate' and 'product' DNA stands were analyzed by RapidFire/TOF-MS, and the corresponding MS signals were extracted. The data display good correlation with the actual % product and good signal linearity.



Conclusions

We have demonstrated the utility of solid phase extraction electrospray mass spectrometry in multiple epigenetic screening applications. In addition to methylation and acetylation changes; histone tails can be modified by a diverse range of mechanisms including phosphorylation, deimination, ubiquitination, sumoylation, ADP-ribosylation, etc...; all of which may be monitored by mass spectrometry. With cycle times of < 10 seconds per sample, the RapidFire High-throughput MS System could be used to establish a range of peptide-based screening assays in primary epigenetic discovery programs.