Development of High-Throughput Assays to Study Methylases, Demethylases and Deacetylases Targeting Histone H3K4, H3K27 and H3K36 Residues

Mathieu Arcand, Mireille Caron, Julie Blouin, Claire Normand, Anne Labonté, Hendrick Plante, Lucille Beaudet & Jaime Padrós

PerkinElmer, 1744 William St., Montreal, QC H3J 1R4, Canada

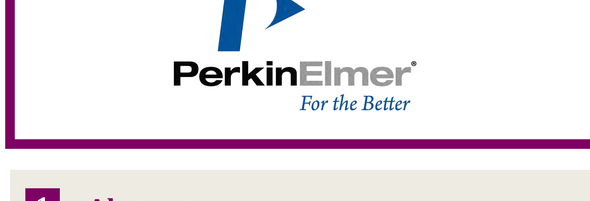
1 Abstract

Several assay methods have been developed for quantifying the activity of histone deacetylases (HDACs and sirtuins), histone methyltransferases (HMTs), and histone demethylases (HDMs). These include radioactive assays, enzyme-linked immunoassays (ELISA), mass spectrometry, and enzyme-coupled detection of fluorescent peptides or reaction co-products (e.g. S-adenosylhomocysteine, formaldehyde, hydrogen peroxide). These assays suffer from various drawbacks such as low throughput, lack of sensitivity, generation of hazardous waste, requirement for expensive equipment, or artifacts associated with the use of non-physiological fluorescent moieties or enzyme-coupled assays (generation of false positives/negatives).

In this study, we describe the development and optimization of homogeneous antibody-based assays for measuring the catalytic activity of a series of epigenetic lysine-modifying enzymes acting on histone H3 Lys4 (SIRT1 deacetylase and LSD1 demethylase), Lys27 (HDAC1 deacetylase, EZH2 methyltransferase and JMJD3 demethylase) and Lys36 (JMJD2A demethylase). Two different non-radioactive, no-wash technologies were used for detection of the enzymatic reaction products: amplified luminescent proximity homogeneous (AlphaLISA®) assay and time-resolved Förster energy transfer (LANCE® *Ultra*) assay.

Results demonstrated that all assays were sensitive, rapid and robust (Z′ factors ≥ 0.69), requiring only nanomolar concentrations of enzyme and peptide. Furthermore, profiling of known inhibitors for each epigenetic enzyme showed the expected potency with either technology. These assays will therefore be ideal for the identification of selective small molecule inhibitors. The approach described here is broadly suitable for measuring the catalytic activity of other histone-modifying enzymes by combining the appropriate biotinylated histone-derived peptides and mark-selective antibodies.

2 Assay Principles



In AlphaLISA and LANCE *Ultra* epigenetics proximity assays, biotinylated histone H3-derived peptide substrates are incubated in enzymatic reactions in the presence of the required cofactors (see Methods). Detection of reaction products occurs *via* mark-specific antibodies coupled to Acceptor beads (AlphaLISA) or labeled with europium chelate (LANCE *Ultra*). The biotin moiety of the histone H3-derived peptide substrates is captured by streptavidin coupled to a Donor bead (AlphaLISA) or labeled with the U*Light*™ acceptor dye (LANCE *Ultra*). In both assay technologies, irradiation of the captured reaction products triggers an energy transfer leading to light emission proportional to the enzyme activity.

3 Materials

Enzymes and biotinylated peptide substrates. Recombinant enzymes LSD1, SIRT1, JMJD2A, JMJD3 and the EZH2/EED/SUZ12/RbAp48/AEBP2 protein complex were from BPS Bioscience. HDAC1 was obtained from Cayman Chemical. Biotinylated peptides were from AnaSpec.

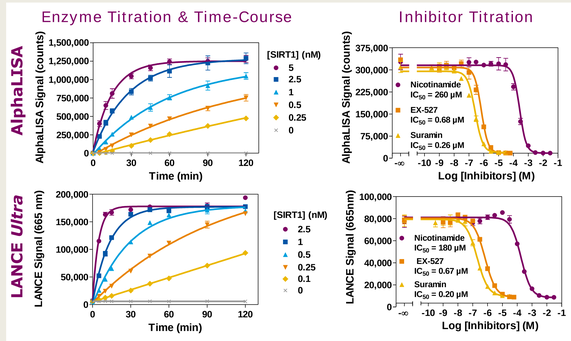
Reagents and inhibitors. S-(5′-adenosyl)-L-methionine chloride (SAM), α-ketoglutaric acid potassium salt (2OG), (+) sodium L-ascorbate, ammonium iron(II) sulfate hexahydrate (Fe(II)), nicotinamide adenine dinucleotide (NAD^+), trans-2-phenylcyclopropylamine (tranylcypromine), trichostatin A, sinefungin, 2,4-pyridinedicarboxylic acid (2,4-PDCA) and nicotinamide were from Sigma-Aldrich. Ethylenediaminetetraacetic acid (EDTA) was obtained from Invitrogen, suberoylanilide hydroxamic acid (SAHA) from Cayman Chemical, and suramin from EMD Chemicals.

Detection reagents, consumables and instrument. The anti-mark AlphaLISA Acceptor beads, Alpha Streptavidin Donor beads, AlphaLISA 5X Epigenetics Buffer 1 kit, LANCE *Ultra* europium-labeled anti-mark antibodies, U*Light*-Streptavidin, 10X LANCE Detection Buffer, white opaque 384-well OptiPlate™ microtiter plates, TopSeal™-A film and EnVision® Multilabel Plate Reader were all from PerkinElmer.

4 Methods

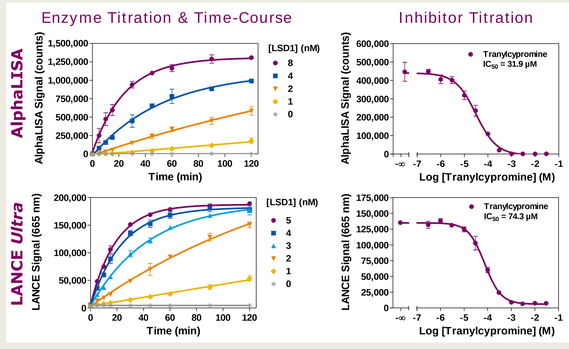
	Enzyme		Substrate		Cofactor		Reaction time	Detection reagent
AlphaLISA	SIRT1	1 nM	H3K4ac	200 nM	NAD^+	800 µM	30 min	Anti-H3K4 (unmodified)
	LSD1	2 nM	H3K4me1	80 nM	N/A		60 min	Anti-H3K4 (unmodified)
	HDAC1	1 nM	H3K27ac	3 nM	N/A		30 min	Anti-H3K27ac
	EZH2	150 ng/well	H3 (21-44)	100 nM	SAM	3 µM	120 min	Anti-H3K27me2-1
	JMJD3	1 nM	H3K27me3	50 nM	2OG	1 µM	45 min	Anti-H3K27me2-1
	JMJD2A	0.5 nM	H3K36me3	100 nM	2OG	2 µM	30 min	Anti-H3K36me2
LANCE *Ultra*	SIRT1	0.5 nM	H3K4ac	300 nM	NAD^+	500 µM	30 min	Anti-H3K4 (unmodified)
	LSD1	2 nM	H3K4me1	200 nM	N/A		60 min	Anti-H3K4 (unmodified)
	HDAC1	1 nM	H3K27ac	3 nM	N/A		45 min	Anti-H3K27ac
	EZH2	150 ng/well	H3 (21-44)	500 nM	SAM	3 µM	180 min	Anti-H3K27me2-1
	JMJD3	5 nM	H3K27me3	200 nM	2OG	0.5 µM	120 min	Anti-H3K27me2-1
	JMJD2A	1 nM	H3K36me3	250 nM	2OG	5 µM	30 min	Anti-H3K36me2

5 H3K4ac Deacetylation by SIRT1



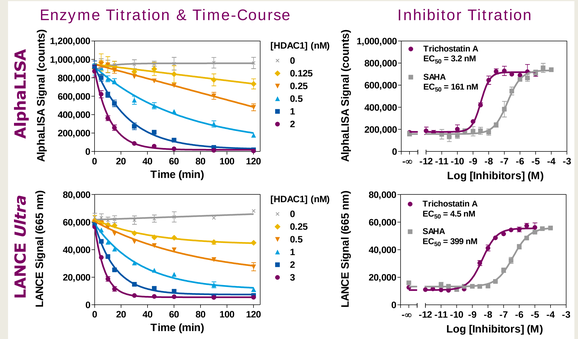
SIRT1 Assay Buffer: *50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT, 0.01% Tween-20 and 0.01% BSA.*

6 H3K4me1 Demethylation by LSD1



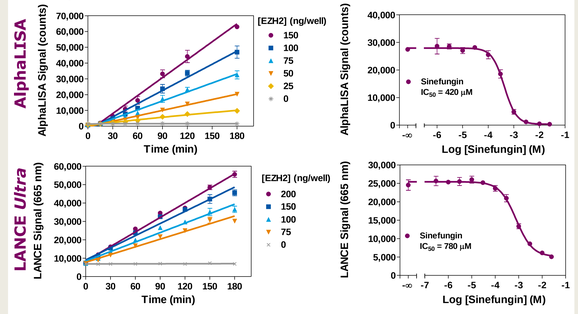
LSD1 Assay Buffer: *50 mM Tris-HCl pH 9.0, 50 mM NaCl, 1 mM DTT and 0.01% Tween-20.*

7 H3K27ac Deacetylation by HDAC1



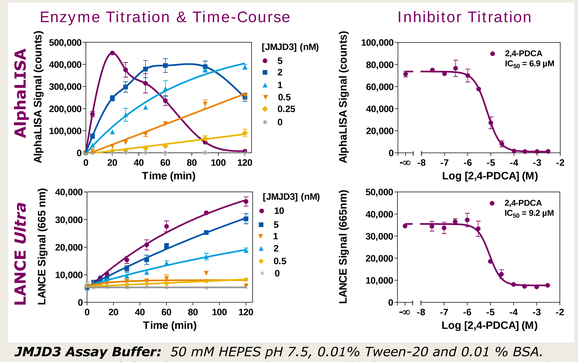
HDAC1 Assay Buffer: *50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 0.01% Tween-20 and 0.01% BSA.*

8 H3 (21-44) Methylation by EZH2



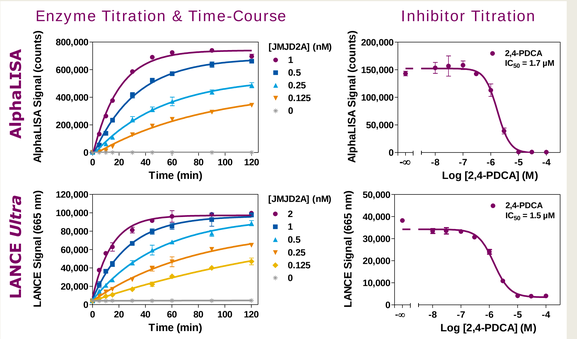
EZH2 Assay Buffer: *50 mM Tris-HCl pH 9.0, 50 mM NaCl, 1 mM DTT, 0.01% Tween-20 and 0.01% BSA.*

9 H3K27me3 Demethylation by JMJD3



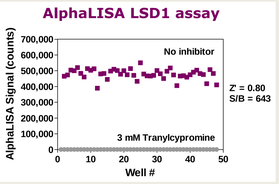
JMJD3 Assay Buffer: *50 mM HEPES pH 7.5, 0.01% Tween-20 and 0.01 % BSA.*
Note: *5 µM Fe(II) and 100 µM ascorbate were added to enzymatic reactions along with the biotinylated histone H3K27me3 peptide substrate.*

10 H3K36me3 Demethylation by JMJD2A



JMJD2A Assay Buffer: *50 mM HEPES pH 7.5, 0.01% Tween-20 and 0.1 % BSA.*
Note: *5 µM Fe(II) and 100 µM ascorbate were added to enzymatic reactions along with the biotinylated Histone H3K36me3 peptide substrate.*

11 Assay Robustness



Enzyme	AlphaLISA S/B	AlphaLISA Z′	LANCE *Ultra* S/B	LANCE *Ultra* Z′
SIRT1	384	0.77	15	0.75
LSD1	643	0.80	20	0.88
HDAC1	2.9	0.69	3.6	0.80
EZH2	52	0.71	4.6	0.80
JMJD3	69	0.70	7.4	0.75
JMJD2A	144	0.85	7.3	0.74

Z′-factor values and signal to background ratios (S/B) were determined for each AlphaLISA and LANCE Ultra optimized epigenetic assay by analyzing 48 assay wells for both total and inhibited signals. Calculated Z′-factor values were ≥ 0.69 and remained stable after overnight incubation (not shown).

12 Summary

- AlphaLISA Acceptor beads and LANCE *Ultra* europium-labeled anti-mark antibodies were used for the successful optimization of robust and sensitive epigenetic assays using histone H3-derived peptides as substrates.
- Signal increase assays were developed for three demethylases (LSD1, JMJD2A and JMJD3) and the SIRT1 deacetylase taking advantage of antibody specificity for unmodified (H3K4) or di-methylated (H3K27me2 and H3K36me2) residues.
- The AlphaLISA and LANCE *Ultra* HDAC1 signal decrease assays showed a robust Z′-factor value (0.69 for AlphaLISA and 0.80 for LANCE *Ultra*), despite of S/B ratios <4.
- IC_{50} values for known inhibitors and rank order of potency were as expected from the literature with either detection technology.
- IC_{50} and Z′-factor values remained stable after overnight incubation, allowing both online and offline HTS plate reading.
- A comprehensive description of these assays and their optimization is available on our website at www.perkinelmer.com/epigenetics.