Development of Homogeneous Non-radioactive Assays for Studying Histone H3 Methyltransferases and Demethylases

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1 Abstract

Post-translational modifications of histones are among the epigenetic mechanisms that can affect chromatin structure and function. Disruption of epigenetic processes can lead to altered gene expression and malignant cellular transformation. Epigenetic changes including DNA methylation, histone acetylation and histone methylation are now considered to play important roles in the initiation and progression of cancer.

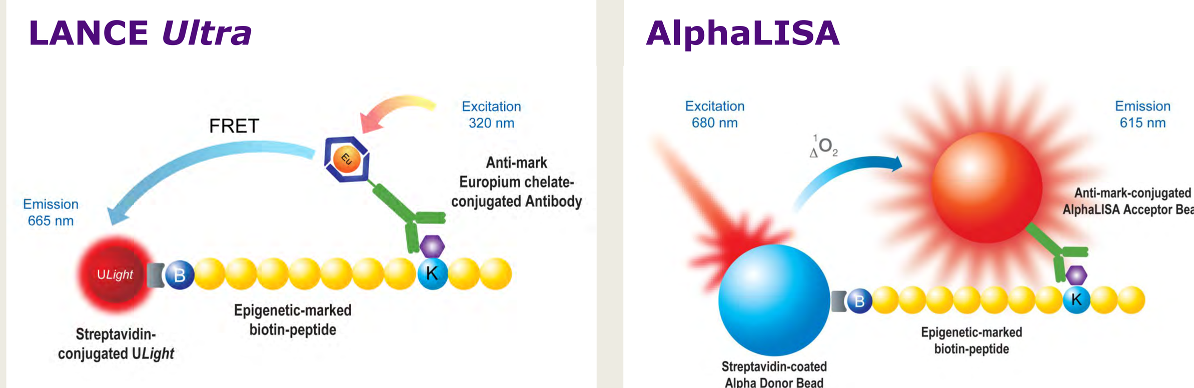
Histone methylation and demethylation are enzymatically dynamic processes controlled respectively by histone methyltransferases (HMTs) and histone demethylases (HDMs). Several assay methods have been developed for quantifying the activity of HMTs and HDMs. These include radioactive assays, enzyme-linked immunoassays (ELISA), mass spectrometry, and enzyme-coupled detection of reaction co-products (e.g. S-adenosylhomocysteine, formaldehyde, hydrogen peroxide). These assays suffer from various drawbacks such as generation of hazardous waste, low throughput, lack of sensitivity, requirement for expensive equipment, or artifacts associated with the use of enzyme-coupled assays (generation of false positives/negatives).

In this study, we describe homogeneous (mix-and-read) assays for measuring the catalytic activity of both HMTs and HDMs acting on histone H3 using two different non-radioactive technologies: amplified luminescent proximity homogeneous assay (AlphaLISA®) and time-resolved fluorescence energy transfer (LANCE®). The EZH2 complex (which dimethylates histone H3 on lysine 27), LSD1, JMJD3 and JMJD2A (which demethylate mono-methyl lysine 4, tri-methyl lysine 27 and tri-methyl lysine 36, respectively, on histone H3) were selected as model enzymes due to their association with various human diseases.

Assays were developed in 384-well format and used as substrates two synthetic biotinylated peptides derived from the N-terminus of histone H3 (amino acids 1 to 21 for LSD1 and 21 to 44 for EZH2, JMJD3 and JMJD2A). All assays were designed as signal-increase homogeneous assays, where direct detection of product formation was conducted using methyl-state selective antibodies conjugated to either AlphaLISA Acceptor beads or LANCE europium chelate.

Results demonstrated that all assays were sensitive and robust, requiring only nanomolar concentrations of enzyme. 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. Although these studies focused on LSD1, EZH2, JMJD3 and JMJD2A enzymes, the approach described here is broadly suitable for measuring the catalytic activity of other HMTs and HDMs by combining the appropriate biotinylated histone-derived peptides and methyl-state selective antibodies.

2 Assay Principles

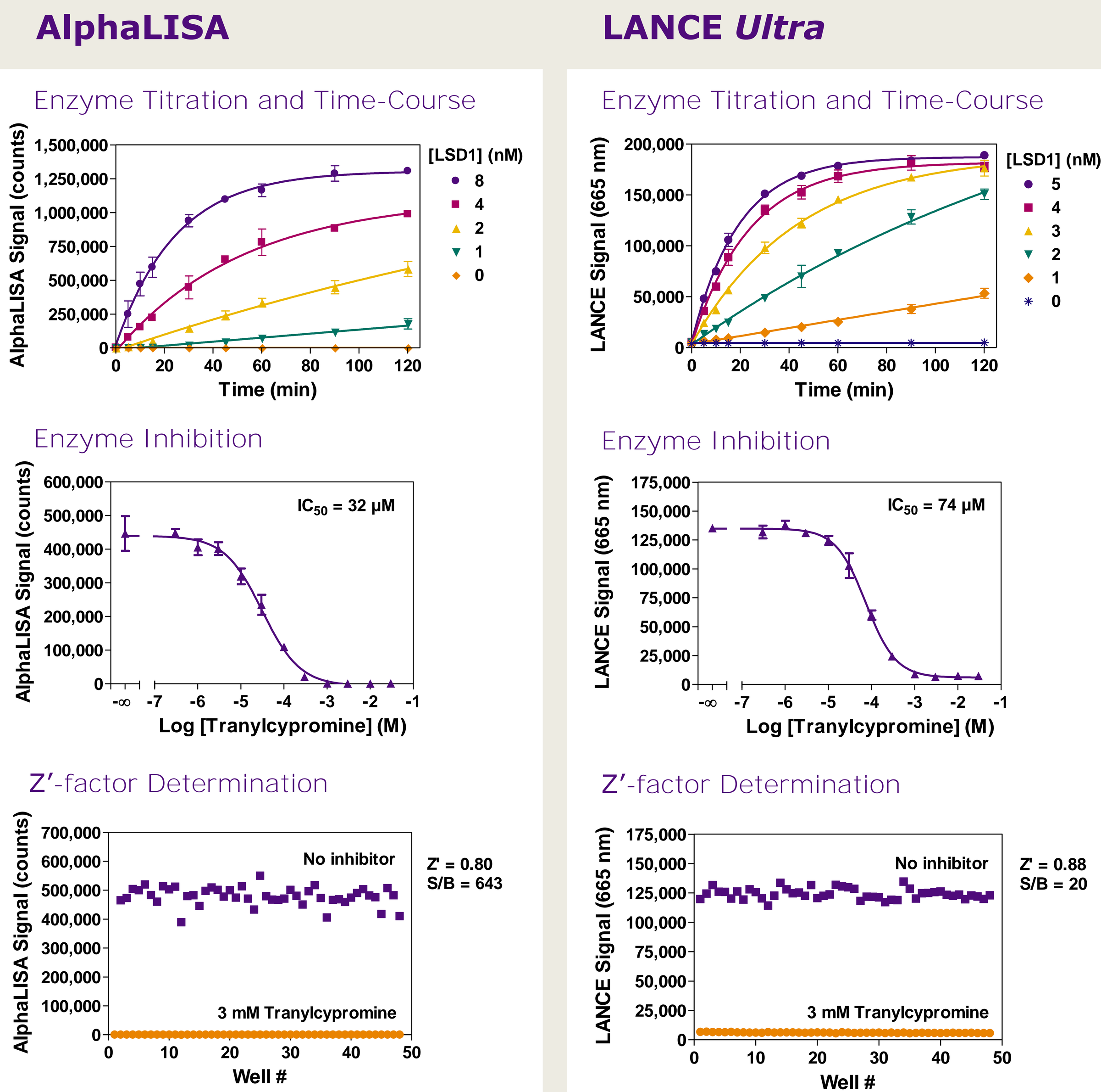


Each epigenetic enzymatic immunoassay was developed using a biotinylated histone H3-derived peptide as substrate. Two different non-radioactive, no-wash technologies were used for detection of the modified substrate:

- LANCE *Ultra* time-resolved fluorescence resonance energy transfer (TR-FRET) assays use a proprietary europium chelate donor dye, W1024 (Eu), together with U*Light*™, a small molecular weight acceptor dye with a red-shifted fluorescent emission. The modified peptide is captured by the Eu-labeled anti-mark antibody (Eu-Ab) and U*Light*-Streptavidin (SA), which bring the Eu donor and U*Light* acceptor dye molecules into close proximity. Upon irradiation at 320 or 340 nm, the energy from the Eu donor is transferred to the U*Light* acceptor dye which, in turn, generates light at 665 nm.
- AlphaLISA assays use Alpha Donor and AlphaLISA Acceptor beads. The modified peptide is captured by the Streptavidin (SA) Alpha Donor beads and AlphaLISA Acceptor beads conjugated to an anti-mark antibody (Ab), which brings the Donor and Acceptor beads into proximity. Upon laser irradiation of the beads-target complexes at 680 nm, short-lived singlet oxygen molecules produced by the Donor beads can reach the Acceptor beads to generate an amplified chemiluminescent signal at 615 nm.

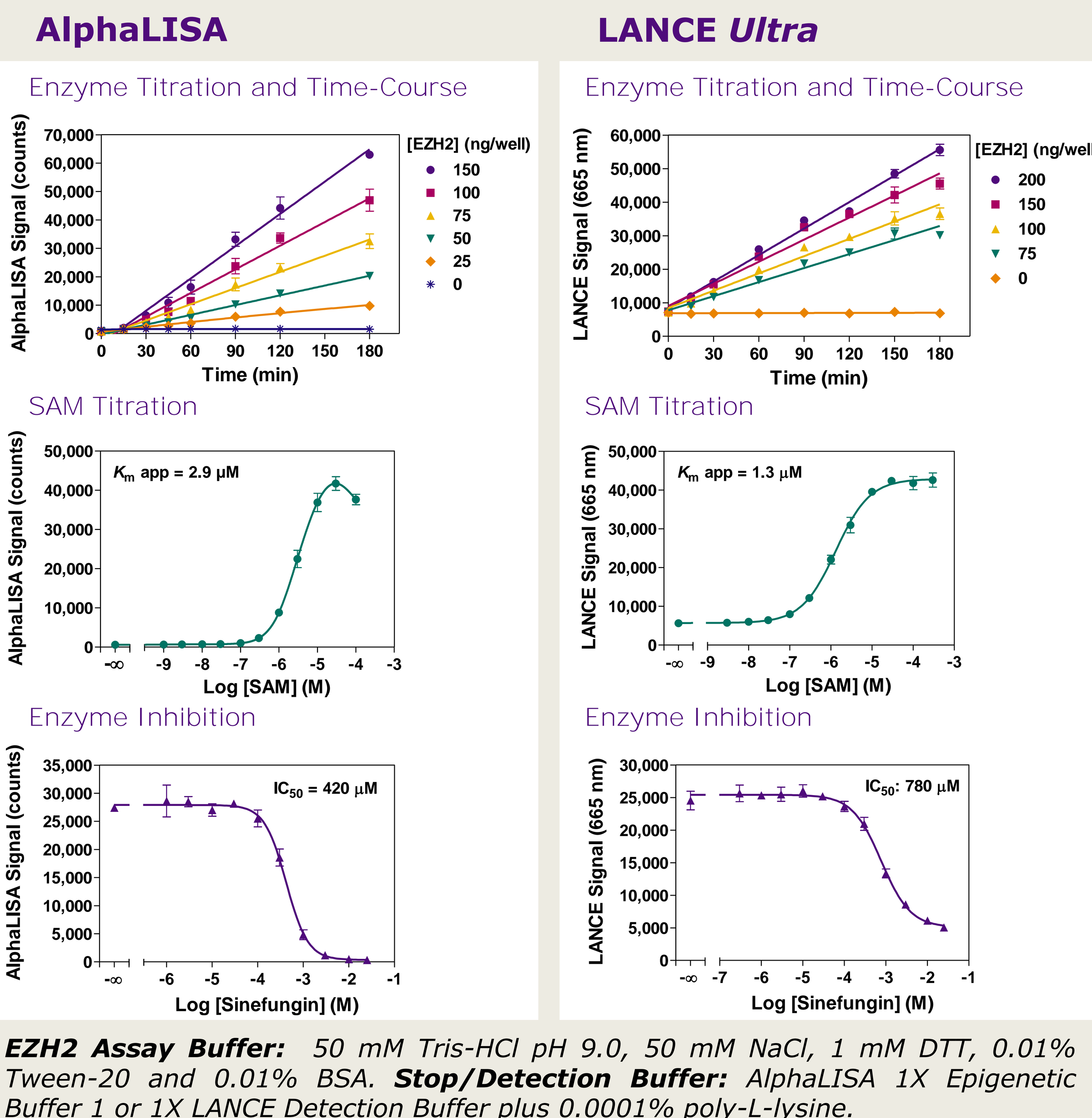
In both assay technologies, the intensity of the light emission is proportional to the level of biotinylated substrate modification.

3 H3K4me1 Demethylation by LSD1



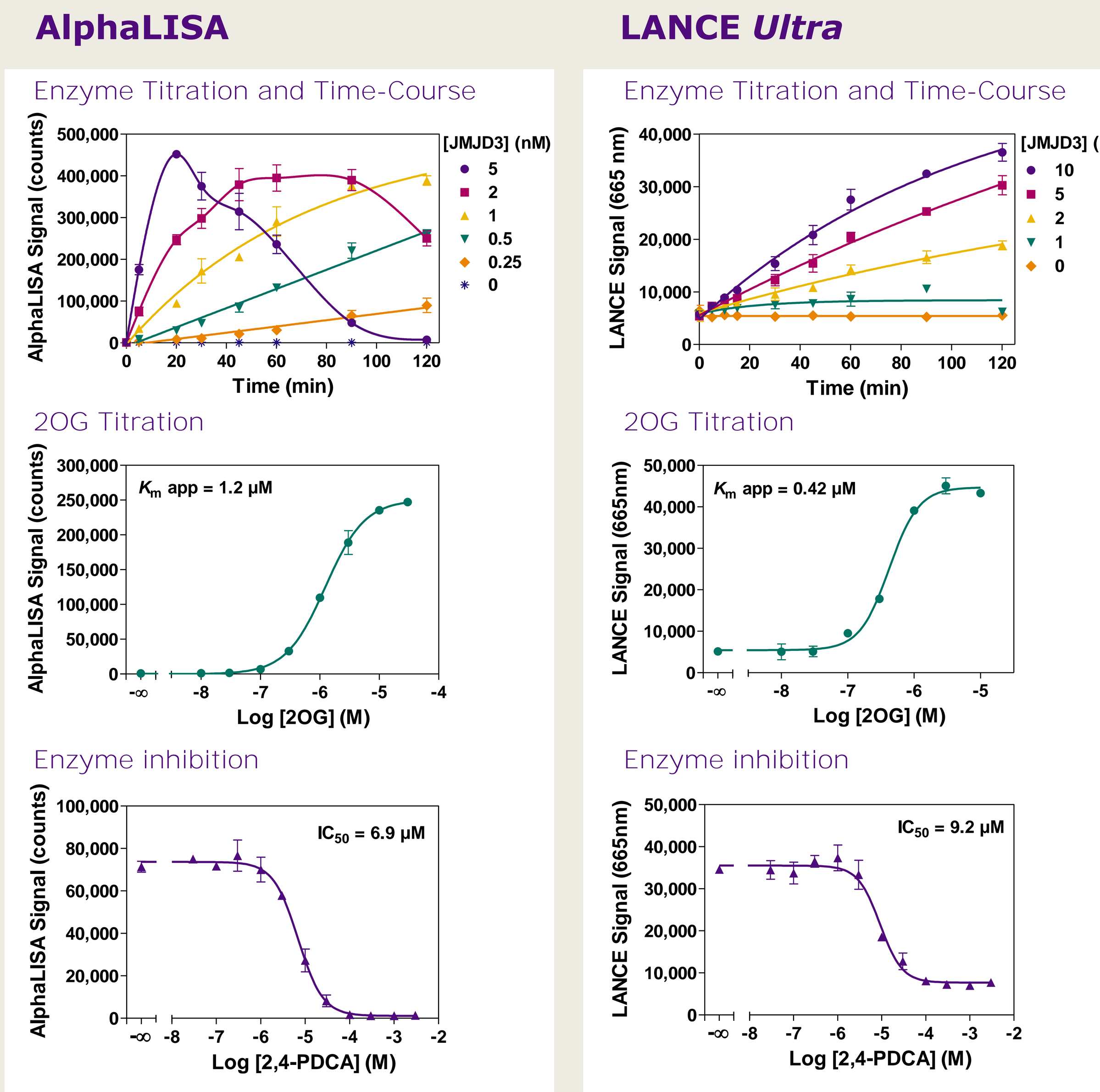
LSD1 Assay Buffer: *50 mM Tris-HCl pH 9.0, 50 mM NaCl, 1 mM DTT and 0.01% Tween-20.* ***Stop/Detection Buffer:*** *AlphaLISA 1X Epigenetic Buffer 1 or 1X LANCE Detection Buffer plus 300 µM tranylcypromine.*

4 H3 (21-44) Methylation by EZH2



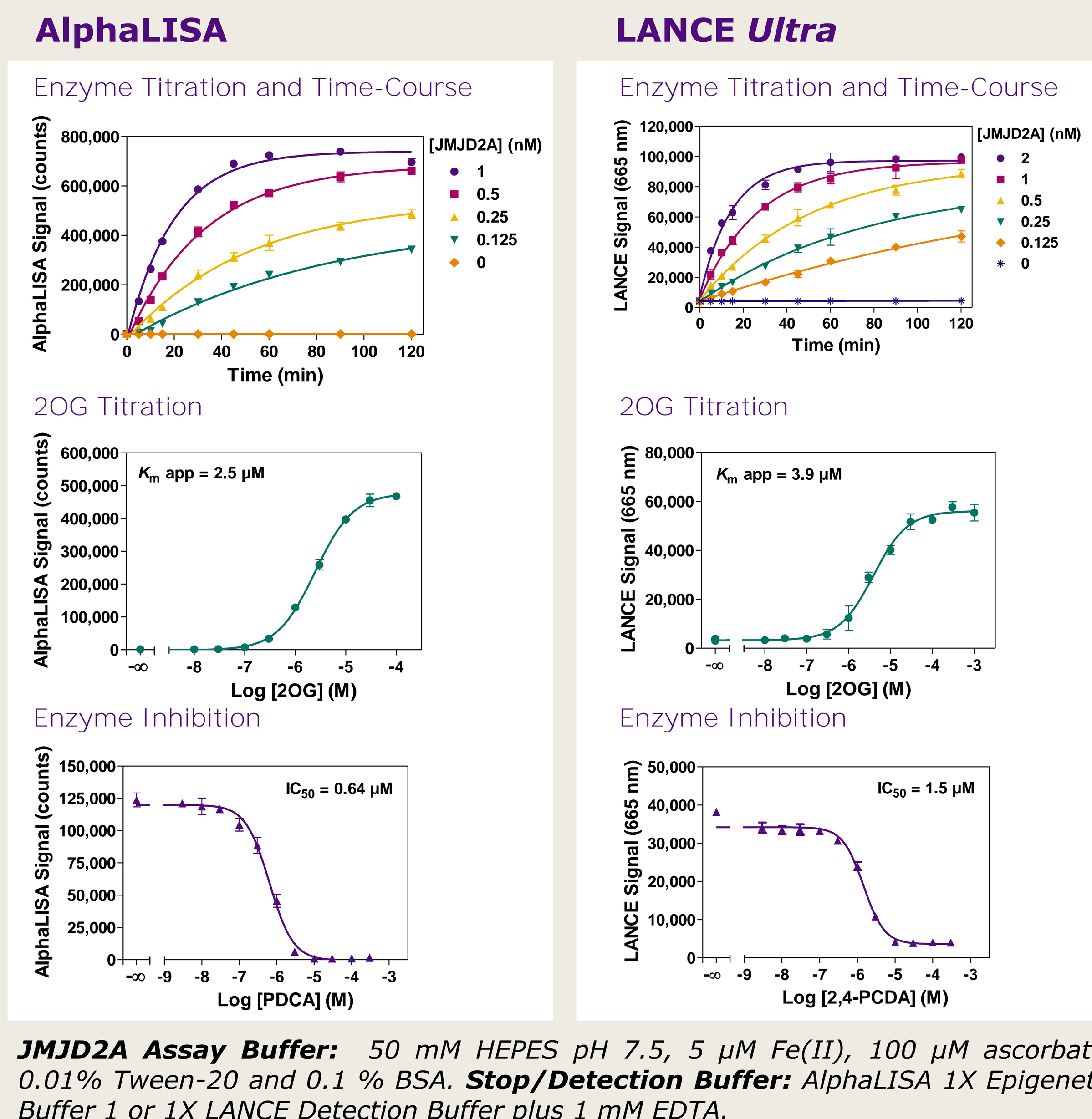
EZH2 Assay Buffer: *50 mM Tris-HCl pH 9.0, 50 mM NaCl, 1 mM DTT, 0.01% Tween-20 and 0.01% BSA.* ***Stop/Detection Buffer:*** *AlphaLISA 1X Epigenetic Buffer 1 or 1X LANCE Detection Buffer plus 0.0001% poly-L-lysine.*

5 H3K27me3 Demethylation by JMJD3



JMJD3 Assay Buffer: *50 mM HEPES pH 7.5, 5 µM Fe(II), 100 µM ascorbate, 0.01% Tween-20 and 0.01 % BSA.* ***Stop/Detection Buffer:*** *AlphaLISA 1X Epigenetic Buffer 1 or 1X LANCE Detection Buffer plus 1 mM EDTA.*

6 H3K36me3 Demethylation by JMJD2A



JMJD2A Assay Buffer: *50 mM HEPES pH 7.5, 5 µM Fe(II), 100 µM ascorbate, 0.01% Tween-20 and 0.1 % BSA.* ***Stop/Detection Buffer:*** *AlphaLISA 1X Epigenetic Buffer 1 or 1X LANCE Detection Buffer plus 1 mM EDTA.*

7 Assay Robustness

Enzyme	AlphaLISA		LANCE *Ultra*	
	S/B	Z'	S/B	Z'
LSD1	643	0.80	20	0.88
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 ≥ 0.7 and remained stable after overnight incubation (not shown).

8 Materials

- **Enzymes and substrates** Recombinant active enzyme LSD1, EZH2/EED/SUZ12/RbAp48/AEBP2 protein complex, JMJD2A and JMJD3 were purchased from BPS Biosciences. All 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)), 2,4-pyridinedicarboxylic acid (2,4-PDCA), trans-2-phenylcyclopropylamine (tranylcypromine) and sinefungin were from Sigma-Aldrich. Ethylenediaminetetraacetic acid (EDTA) was from Invitrogen.
- **Detection reagents and materials** AlphaLISA Acceptor beads, Alpha Streptavidin Donor beads, AlphaLISA 5X Epigenetics Buffer 1 kit, europium-labeled antibodies, U*Light*-Streptavidin, 10X LANCE Detection Buffer, white opaque 384-well OptiPlates™, TopSeal-A™, EnVision® Multilabel Plate Reader were all from PerkinElmer.

9 Methods

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

10 Summary

- Anti-mark antibodies coupled to AlphaLISA Acceptor beads or labeled with LANCE *Ultra* europium chelate 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 histone demethylases (LSD1, JMJD2A and JMJD3) and one histone methyltransferase (EZH2) taking advantage of antibody specificity for unmodified (H3K4) or di-methylated (H3K27me2 and H3K36me2) residues.
- IC_{50} values for known inhibitors and rank order of potency were as expected from the literature with either technology.
- IC_{50} and **Z'**-factor values remained stable after overnight incubation (data not shown), 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.